THE ACTIVITY OF DIETARY BETA-CAROTENE AGAINST CARCINOGEN-INDUCED URINARY BLADDER CANCER IN THE RAT

VOLUME 1

A thesis presented by

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ABSTRACT

The activity of dietary beta-carotene (BC) has been evaluated against urinary bladder cancer in rats treated with the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN). This experiment was designed to demonstrate whether any anti-cancer activity shown by BC was mediated through conversion to vitamin A, or through some other mechanism. Weanling female F344 rats were randomised into four dietary groups. Group 2 received a basic vitamin A-deficient diet which was supplemented with vitamin A. Group 1 also received the vitamin A-deficient diet supplemented with vitamin A until week 7, when 3 mM/kg BC was added. Groups 3 and 4 were fed the basic diet (without vitamin A supplementation) to week 7, at which time plasma vitamin A levels had fallen to approximately 10% of control values. Group 3 was then given 3 mM/kg BC; Group 4 received a low level of vitamin A supplementation in the drinking water, to maintain a healthy but vitamin A-deficient condition. At week 15, each group was divided into two; Groups 1-4 were dosed with BBN (a total of 635 mg/rat in 5 weekly aliquots), and Groups 5-8 received carcinogen vehicle. The animals were killed 42 weeks later. Urinary bladders were weighed, and total tumour volume/bladder calculated prior to histological examination. There was no statistically significant evidence that BC reduced the incidence of carcinomas, or relative bladder weights or tumour volumes in BBN-treated rats.

Before this investigation could be undertaken, several other experiments were necessary. Three trials evaluated commercially-available vitamin A-deficient diets for use in the main experiment. Other studies were conducted to determine: 1) a method of maintaining vitamin A-deficient rats for long-term experimentation, 2) that the absorption of appreciable amounts of unconverted BC by rats did occur, and 3) the survival of vitamin A-deficient rats following carcinogen treatment.

To date, the present investigation into the activity of BC against carcinogen-induced urinary bladder cancer is the only one to be carried out in rats. Furthermore, it is the only experiment to have investigated the effect of BC against bladder cancer in vitamin A-normal and vitamin A-deficient animals. If the negative results reported here for BC against bladder cancer are repeated in other experimental systems, this may indicate that BC is not an effective chemopreventative agent for bladder cancer.
<table>
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<tr>
<td>2-AAF</td>
<td>2-acetylaminofluorene</td>
</tr>
<tr>
<td>4-HPR</td>
<td>N-(4-hydroxyphenyl)retinamide (retinide)</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous-polyposiscoli</td>
</tr>
<tr>
<td>APL</td>
<td>acute promyelocytic leukaemia</td>
</tr>
<tr>
<td>BBN</td>
<td>N-butyl-N-(4-hydroxybutyl)nitrosamine</td>
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<tr>
<td>BC</td>
<td>beta-carotene</td>
</tr>
<tr>
<td>BL6D2F₁</td>
<td>C57BL/6 x DBA/2 F₁ hybrid mice</td>
</tr>
<tr>
<td>BP</td>
<td>benzo(a)pyrene</td>
</tr>
<tr>
<td>BPV</td>
<td>bovine papillomavirus</td>
</tr>
<tr>
<td>c-onc</td>
<td>cellular oncogene</td>
</tr>
<tr>
<td>cis</td>
<td>carcinoma in situ</td>
</tr>
<tr>
<td>DBN</td>
<td>dibutylnitrosamine</td>
</tr>
<tr>
<td>DCC</td>
<td>deleted-in colorectal-cancer</td>
</tr>
<tr>
<td>DEN</td>
<td>diethylnitrosamine</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>DMH</td>
<td>dimethylhydrazine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>F344</td>
<td>Fischer 344 rat</td>
</tr>
<tr>
<td>FANFT</td>
<td>N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide</td>
</tr>
<tr>
<td>FFQ</td>
<td>food frequency questionnaire</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GTPase</td>
<td>guanine triphosphatase</td>
</tr>
<tr>
<td>Gy</td>
<td>gray (1 gray is a unit of radiation absorption equivalent to 100 rads)</td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Harvey-ras</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>iu</td>
<td>international unit</td>
</tr>
<tr>
<td>kg</td>
<td>kilograms</td>
</tr>
<tr>
<td>Ki-ras</td>
<td>Kirsten-ras</td>
</tr>
<tr>
<td>MCA</td>
<td>methylocholanthrene</td>
</tr>
<tr>
<td>Mesna</td>
<td>2-mercapto-ethane sodium sulphonate</td>
</tr>
<tr>
<td>mg</td>
<td>milligrams</td>
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\[\begin{array}{ll}
\mu gRE & \text{micrograms retinol equivalent} \\
mM & \text{millimoles} \\
MNNG & \text{N-methyl-N'-nitro-N-nitrosoguanidine} \\
MNU & \text{N-methyl-N-nitrosourea} \\
MOCA & \text{4,4-methylene-bis(2-chloroaniline)} \\
N-ras & \text{Normal-ras} \\
NCI/NTP & \text{National Cancer Institute/National Toxicology Program} \\
nm & \text{nanometre} \\
NSAID & \text{non-steroidal anti-inflammatory drug} \\
p53 & \text{p53 gene or the protein product of that gene} \\
PCR & \text{polymerase chain reaction} \\
PDGF & \text{platelet derived growth factor} \\
PhIP & \text{2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine} \\
RA & \text{all-trans-retinoic acid} \\
RAR & \text{retinoic acid receptor families} \\
\text{RA} & \text{RAR & RXR} \\
Rb & \text{retinoblastoma gene} \\
RBP & \text{retinol-binding protein} \\
RDA & \text{recommended daily amount (of nutrients)} \\
RNA & \text{ribonucleic acid} \\
SCC & \text{squamous cell carcinoma} \\
SSCP & \text{single-strand conformation polymorphism} \\
SV40 & \text{simian virus-40} \\
TCC & \text{transitional cell carcinoma} \\
TMMP & \text{all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl} \\
& \text{-2,4,6,8-nonatetraenoate} \\
TPA & \text{12-O-tetradecanoylphorbol-13-acetate} \\
tRNA & \text{transfer ribonucleic acid} \\
\text{Trypt-P-2} & \text{3-amino-1-methyl-5H-pyridol(4,3-b)indol} \\
UV & \text{ultraviolet light} \\
UVmax & \text{maximum ultraviolet absorbance} \\
\text{v-onc} & \text{viral oncogene} \\
\text{WT-1} & \text{Wilms' tumour gene}
\end{array}\]
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- $\alpha$-tocopherol  
- BC  

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- Retinol  
- $\alpha$-tocopherol  
- BC  

c Group 3: Female Wistar rats  

- Retinol  
- $\alpha$-tocopherol  
- BC  

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- Retinol  
- $\alpha$-tocopherol  
- BC

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c Female Wistar rats
d Female Sprague-Dawley rats

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CHAPTER 1

URINARY BLADDER CARCINOGENESIS

1 EPIDEMIOLOGY OF URINARY BLADDER CANCER

After coronary heart disease, cancer is the second highest cause of death throughout the western world. Cancer of the urinary bladder is the sixth most common malignancy worldwide (Cordon-Cardo et al. 1994), accounting for 4.4% of all cancers (Doll 1992). It is the most common neoplasm of the urinary tract, accounting for about 75% of urinary system cancers in men and about 50% in women (Ruddon 1987). In the USA, it was estimated that the disease would afflict 51,200 people in 1994, and would result in roughly 10,600 deaths (Kroft and Oyasu 1994).

Mortality rates from urinary bladder cancer vary in different countries, but are increasing for males and, to a lesser extent, for females also (Bouffioux 1984). The highest mortality rates are observed in Denmark, the UK, Belgium and Italy, the lowest rates in Japan, Singapore and Venezuela (Paneau et al. 1992). In countries with a high mortality such as Belgium, the death rate increases exponentially after the age of 40, peaking between 65 and 75 years (Bouffioux 1984). In general, the number of deaths from bladder cancer falls in people older than 85 years, but this is probably related to the limited numbers surviving to the end of the eighth decade (Clayson and Cooper 1970). Mortality is particularly high in the first years after diagnosis, but subsequently falls to almost negligible levels in patients with a history of the disease for at least 15 years (Malmstrom et al. 1993). The five-year relative survival rates are 41% for males and 35% for females (Paneau et al. 1992). In the USA, the five-year survival rates for bladder cancer have improved significantly since 1974; nevertheless, approximately 10,000 deaths in the USA per year are still attributed to the disease (Reznikoff et al. 1993).

International comparisons of bladder cancer mortality and morbidity statistics suffer from those inaccuracies common to other types of cancer (Clayson and Cooper 1970). Thus, all comparisons are affected by differences in the standard and availability of medical care in different countries, by differences in the accuracy of the diagnosis and treatment, combined with variations in the frequency of autopsy examinations, and practice of medical recording and retrieval. In addition, bladder cancer statistics are further confused by lack of agreement on what constitutes a papilloma and because patients with low-grade bladder tumours may have very long survival times so that
death from other causes supervenes, leading to higher morbidity rates than those of mortality (Dom 1962; Clayson and Cooper 1970). Therefore morbidity, rather than mortality, is a more valid criterion for bladder cancer epidemiology. For example, in the classical epidemiological studies of bladder cancer in the British chemical industry (Case et al. 1954), there were 127 death certificates mentioning the disease (out of a total population of 4622 men exposed to aromatic amines), but a retrospective attempt to assess the actual number of instances of carcinoma or papilloma in this population discovered 262 examples.

The incidence of bladder cancer increases with age and is particularly high after the age of 60. In fact, bladder cancer before the age of 40 is extremely uncommon (Bouffouix 1984; Cohen and Johansson 1992). Most tumours in younger patients are low-grade, papillary, non-invasive transitional cell carcinomas (TCC), which differ from the usual distribution of tumours in older patients (Cohen and Johansson 1992). In addition, younger patients developing these low-grade tumours may have considerably less chance of recurrence than older patients with the same lesions (Fitzpatrick and Reda 1986).

With the exception of Japan, the worldwide incidence of bladder cancer is increasing in males and, to a lesser extent, in females also (Bouffouix 1984; Mellemgaard et al. 1993). Since 1973, the incidence of bladder cancer in the USA has been reported to be increasing at a rate of 1% per year for white males and 2% per year for black males, an increase which has been partially attributed to a rise in the diagnosis of localised bladder cancer (Cordon-Cardo et al. 1994). Wide differences are observed in incidence rates from country to country and even within the same country (Bouffouix 1984). International studies reveal age-standardised incidence rates of from 2.8 to 28.7 per 100,000 in males and from 0.2 to 7.0 per 100,000 in females (King 1982). The highest incidences are found in Europe, the USA and in some countries of Africa, particularly in the north (Egypt) and in the east (Tanzania) of the continent (Paneau et al. 1992; Doll 1992). In some of these same areas, the incidence rates are higher for males than for females and are increasing with time (Paneau et al. 1992). Indeed, throughout most parts of the world, urinary bladder cancer is markedly more common in males than in females, being two to five times more common in men than in women (Cohen and Johansson 1992). Thus, in the USA, bladder cancer is the fourth most common malignancy among men, representing 6% of all cancer cases and 2.5% of cancer deaths, and ninth among women, accounting for 2.3% of all cases and 1.4% of deaths (Reznikoff et al. 1993; Kroft and Oyasu 1994). This sex difference is true not only for TCC, but also for squamous cell carcinomas (SCC) and adenocarcinomas (Clayson and Cooper 1970; Price 1971; Kantor et al. 1988). The difference is not entirely explained by variations in cigarette smoking.
and occupational exposure to carcinogenic chemicals, the two most important aetiological factors (Cohen and Johansson 1992) and, thus far, no clear explanation has been found for the difference between men and women (Hartge et al. 1990). Only in Africa, where bladder cancer is frequently associated with schistosomiasis, is the difference between the sexes not apparent, the relative incidences in males and females being generally about equal and, in some areas, being slightly higher in females (Cook-Mozaffari 1982).

Studies of bladder cancer incidence amongst immigrants and their offspring have shown that these populations have a level of risk closer to that of their new surroundings rather than that of their original location, indicating that environmental factors appear to play a more important role than genetic factors in the epidemiology of the disease (Wynder and Stellman 1981; King 1982; Bouffioux 1984).

In addition to the general distribution of bladder cancer in the world, there are specific areas in which there is a consistently elevated incidence of cancer of the lower urinary tract associated with other disease processes (Cohen and Johansson 1992). For example, nephropathy is prevalent in some areas of the Balkans, where it is a significant cause of death and is associated with a high prevalence of TCC of the upper and lower urinary tract (Matanoski and Elliott 1981; Radovanovic 1989). The specific causative factor(s) for the nephropathy or the carcinogenic response have not been identified. In Taiwan, a peripheral vascular disease common in certain parts of the island is associated with an increased incidence of urinary tract carcinoma, as well as cancer of the liver, kidney, skin, lung and colon (Chen et al. 1986). Although there is increasing evidence that the vascular disease - known as 'blackfoot disease' - is due to abnormal levels of arsenic compounds present in artesian water supplies (Chen et al. 1986), the relation of this exposure to urothelial cancer is completely unknown.

Apart from bladder cancer related to bacterial and parasitic (schistosomiasis) infections of the urinary tract, which give rise chiefly to SCC, the majority of bladder cancer cases are TCC. This latter type of tumour makes up more than 90% of bladder cancers in Europe and North America (Reznikoff et al. 1993), although a range of other histological types is also seen. SCC and adenocarcinomas account for 5% and 1%, respectively, of bladder cancers in these parts of the world. Undifferentiated bladder tumours are uncommon (Reznikoff et al. 1993). The distribution of histopathological types of bladder cancer is strikingly different in areas of the world where bladder cancer is chiefly associated with schistosomal infection. For example, in Egypt, bladder cancer is the most common malignancy and SCC is the most frequently observed bladder cancer.
type (Tawfik 1987). These data, according to Reznikoff et al. (1993), suggest that the oncogenic agent(s) may play some role in determining the histological forms of bladder cancer.

2 NATURAL HISTORY OF TRANSITIONAL CELL CARCINOMA

Payne's (1959) analysis of 1420 new bladder cancer cases seen in London gives a very good overall impression of the natural history of the disease. The sex ratio was 4.1 men:1 woman, with a mean age of 62.7 and 66.5 years, respectively. A history of haematuria occurred in over 90% of the patients. This series of observations was notable for the high frequency of both long and short histories (19% less than 1 month, 23% more than 2 years). Detailed examination was made of 1331 patients on first presentation. The duration of the history in relation to the tumour type when first examined suggested that there were two forms of tumour growth: (1) those that, if left, will steadily infiltrate through and beyond the bladder; (2) those that have been and may still remain localised to the epithelium for a long time.

Payne's results showed that the prognosis seemed to be profoundly influenced by the degree of infiltration at first presentation. The crude five-year survival rates for the different levels of infiltration were: epithelial 71%, muscular 35%, perivesical 13% and pelvic fixation 1%. On the basis of the histological type, it was found that of 328 patients with differentiated papillary tumours, 62% survived 3 years, whereas only 31% of 41 patients with anaplastic tumours were alive after the same period of time. These studies seemed to suggest that there were two forms of human bladder cancer - one, a superficial lesion with a very orderly differentiated structure, the other endowed with properties that typify neoplastic cells.

Today, at presentation, human bladder cancer can generally be divided into three categories: superficial, invasive, and carcinoma in situ (cis) (Kroft and Oyasu 1994). This grouping of bladder tumours is defined according to the most commonly used staging and grading systems, those of the American Joint Committee of Cancer and the International Union Against Cancer (Spiessl et al. 1992), and the World Health Organisation (Mostofi et al. 1973), respectively. In the scheme reported by Spiessl et al. (1992), non-invasive, exophytic papillary tumours (stage Ta), carcinoma in situ (Tis) and tumours which invade the sub-epithelial connective tissues (T1) are considered superficial, while lesions invading into the muscle of the bladder wall (T2, T3a) and beyond to the perivesicular fat (T3b) and adjacent viscera (T4) are considered invasive. Many superficial lesions have a papillary form, whereas many muscle-invading lesions are non-papillary (nodular) at presentation (Kroft and Oyasu 1994). In general, prognosis of bladder cancer is profoundly affected by stage (Ta - T4), which is the most important independent predictor (Kroft
and Oyasu 1994). Stage, in turn, is closely correlated with the degree of histological
differentiation (grade) of the lesion. The WHO system of classification recognises 3 grades of
TCC (grades 1-3), the higher the grade, the poorer the differentiation and the greater the likelihood
of invasive behaviour (Friedell et al. 1976).

Kroft and Oyasu (1994) reported that most patients (around 80%) with TCC present with
superficial disease, and most of these are grades 1 and 2. The natural history of these lesions is
one of multiple superficial recurrences in 70 to 80% of patients, with only about 10 to 15%
progressing to muscle invasive or metastatic disease. Various clinical and pathological parameters
have been identified which alter the risk and pattern of recurrence; these are, at initial presentation,
multiplicity, size, grade, stage and presence of carcinoma in situ in the mucosa adjacent to the
gross tumour (Kroft and Oyasu 1994). An important and characteristic feature of superficial
bladder cancer is a tendency for low grade tumours to recur as more life threatening higher grade
lesions. Kunze et al. (1976) reported similar observations, with carcinogen-induced bladder
tumours in rats.

Kroft and Oyasu (1994) pointed out the possibility, based on the relative likelihood of progression
to muscle invasion/metastasis, that exophytic non-invasive papillary tumours (Ta) and lesions
which invade the sub-epithelial connective tissues (T1) could be considered as exhibiting different
biological behaviour from each other. For example, in an analysis of 249 cases of superficial
TCC, Heney et al. (1983) documented progression to muscle invasion/metastasis in 30% of T1
lesions but only in 4% of Ta tumours.

Previously, it had been thought that invasive TCC occurred as the sequelae of more superficial
papillary lesions, after a sequence of recurrences with progressive increases in tumour grade and
ultimate invasion (Brawn 1982). This has been reported for chemically-induced rat bladder
tumours (Kunze et al. 1976), and there is no doubt that this often occurs in humans (Kroft and
Oyasu 1994). However, it has become apparent that the majority of invasive bladder cancers arise
de novo without a history of papillary neoplasms or morphological evidence of papillary
architecture (Kaye and Lange 1982; Brawn 1984).

Support for the idea of two distinct pathological patterns for invasive bladder cancer comes from
studies of cystectomy specimens. Soto et al. (1977), found carcinoma in situ (cis) in continuity
with invasive tumours in 33 out of 45 cystectomy specimens. In 10 cases, there was no cis
adjacent to unifocal invasive lesions. They concluded that there may be two distinct pathogenetic
patterns of bladder cancer: 1) multifocal lesions arising from an extensive 'field effect', and 2) single lesions arising in a focal abnormal area. Similarly, Kakizoe et al. (1984), in a study of 90 step-sectioned cystectomy specimens, divided bladder cancer into 3 types: 1) multiple papillary superficial carcinomas with or without small areas of in situ change, 2) multiple papillary and non-papillary lesions with extensive in situ change, and 3) localised non-papillary invasive tumour without accompanying dysplastic or in situ changes. In a later, larger study of 186 cystectomy specimens, Kakizoe et al. (1988) concluded that nodular invasive carcinoma arises in two ways: 1) progression of low grade papillary carcinomas, and 2) development of invasive lesions directly from cis.

In the carcinogen-treated heterotopically transplanted rat bladder, Oyasu et al. (1987) concluded that invasive bladder tumours may develop by both these mechanisms. However, in their model, the direct development of invasive carcinoma from cis was infrequent. Nodular invasive carcinoma with associated cis can be readily induced in mice after treatment with N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN). Thus, this seems to be a good model system in which to study the relationship between cis and muscle-invasive carcinoma (Kroft and Oyasu 1994). In contrast, tumours which develop in rats after treatment with BBN, or other model carcinogens, resemble human superficial papillary carcinomas and the subsequently developing invasive carcinomas (Erturk et al. 1967; Ito et al. 1969; Fukushima et al. 1976).

The final source of support for this dual pathogenesis model of invasive bladder cancer is molecular genetics (Kroft and Oyasu 1994). Two distinct pathways of molecular alteration involving oncogenes and tumour suppressor genes have been proposed for the development of invasive TCC (Reznikoff et al. 1993; Spruck et al. 1994).

3 AETIOLOGY OF URINARY BLADDER CANCER

The aetiology of urinary bladder cancer has been reviewed by many authors (Clayson and Cooper 1970; Price 1971; Miller 1977; Wynder and Goldsmith 1977; Matanoski and Elliot 1981; King 1982; Bouffioux 1984), the most recent reviews being those of Cohen and Johansson (1992), Silverman et al. (1992) and Shirai (1993). Multiple diverse risk factors have been strongly linked to bladder cancer, both epidemiologically and experimentally. These factors are: occupational and environmental exposure to carcinogenic chemicals including aromatic amines, cigarette smoking, certain bacterial and parasitic infections, and certain analgesics and chemotherapeutic agents. Other factors have been implicated, but now seem less likely to be involved in urinary bladder carcinogenesis; these include coffee and tea drinking, abnormal L-tryptophan metabolism, and
artificial sweeteners such as saccharin and cyclamates. All these aetiological factors are briefly reviewed below.

A Occupational and environmental exposure to carcinogenic chemicals

Of all human neoplastic diseases, cancer of the urinary bladder has historically been one of those most commonly linked with environmental and occupational exposure to chemicals. Today, approximately 40 high risk occupations have been identified, the strongest evidence existing for workers in industries manufacturing or handling dyes, aromatic amines, leather, rubber, paints, as well as lorry drivers and aluminium workers (Matanoski and Elliot 1981; Silverman et al. 1992).

As long ago as 1895, the surgeon Rehn (reviewed by Price 1971) described three cases of bladder cancer in workers from the German dye industry, pointing out a possible causal relationship between the disease and the manufacture of fuchsin (magenta) in which these workers had been actively engaged for many years. Since then, several specific chemicals, chemical mixtures and environmental exposures have been identified as causes of urinary bladder cancer, many of them aromatic amines.

(i) Aromatic amines

It was more than half a century before adequate experimental evidence in animals was obtained to confirm Rehn's suspicions of a causal link between bladder cancer and occupational exposure to chemicals in the dyeing industry. Investigations into the specific chemicals involved in Rehn's 'aniline dye cancers' led to the discovery by Hueper and his associates in 1938 that feeding dogs the industrial arylamine 2-naphthylamine - one of the suspected chemicals - induced bladder carcinomas identical to human lesions (reviewed in 1970 by Clayson and Cooper and by Price in 1971). Following similar results from Bonser's laboratory (Bonser 1943; Bonser et al. 1956) attention was directed towards the possibility that other related environmental aromatic amines might induce bladder cancer. Thus, retrospective epidemiological studies of workers in the British dye industry soon demonstrated that exposure to benzidine, as well as 2-naphthylamine, was associated with a high risk of bladder cancer (Case et al. 1954). Indeed, there was virtually a 100% incidence of bladder tumours among 2-naphthylamine distillers (Case 1966) and a high incidence among benzidine manufacturers (Scott 1952). The mean induction time for occupational bladder cancers in the dye industry was 18 years, but some occurred as early as 2 years after exposure (Case et al. 1954).
In the rubber industry the use of 2-naphthylamine as an antioxidant was expected (Case and Hosker 1954) and later confirmed (Veys 1969) to be associated with a high risk of bladder cancer. Chronic exposure to another antioxidant used in the industry, 4-aminobiphenyl, was also found to be associated with an increased risk (Melick et al. 1955). The latent period to tumour formation was as little as 5 years in some cases but, following the discontinuation of its use, new cases of bladder cancer associated with exposure to this compound were still being reported (Koss et al. 1969).

Thus several aromatic amines have been related to human bladder cancer. Exposure to 2-naphthylamine, benzidine and 4-aminobiphenyl occurs, or occurred, in specific industries (such as the chemical, rubber, cable-making industries and in coke ovens - also a wide range of laboratories in which benzidine was formerly used), while 4-aminobiphenyl, O-toluidine and some other aromatic amines have been detected in cigarette smoke (Clayson and Cooper 1970; Talaska et al. 1991). The discovery of the occupational hazards posed by these carcinogenic compounds has resulted in the elimination or control of these agents in industry and, subsequently, the proportion of bladder cancer cases attributable to occupational exposures, which used to be in the order of 10%, is now reduced (Ferber et al. 1976; Doll 1992). Nevertheless, the list of occupations which have been associated with an increased risk of bladder cancer (Table 1.1) includes many in which no specific chemicals have been implicated (Cohen and Johansson 1992). It must be noted, however, that the degree of risk for some of these occupations, such as petroleum workers, metal industries (drill press operators, metal fabrication etc.), and dusty occupations (blasters and miners) is much lower than in the dye industry (Bouffioux 1984; Cohen and Johansson 1992). Overall, it is estimated that approximately 20% - 25% of the male population in the United States with bladder cancer has the disease as a result of occupational exposure (Silverman et al. 1989).

Although 1-naphthylamine was initially regarded as an animal and human bladder carcinogen, subsequent investigations have yielded considerable evidence that this is not so; rather, exposure to 1-naphthylamine occasionally is related to bladder cancer development because of contamination with 2-naphthylamine (Clayson and Cooper 1970; Price 1971). Indeed, 2-naphthylamine is probably the most potent of all known human bladder carcinogens, despite some difficulty in reproducing this potency in animal models (Harnden 1993).

Some carcinogenic chemicals are derivatives of other aromatic amines. For example, a large number of azo dyes are derived from benzidine and exposure to many of these compounds has been associated with bladder cancer in humans and in animals. This was first demonstrated in a
group of kimono painters in Japan, who licked their brushes in order to obtain the fine points needed for the delicate paintings on the fabrics (Yoshida et al. 1971). The azo dyes are rapidly metabolised to yield free benzidine (Martin and Kennelly 1985).

In addition to the aromatic amines which are proven human bladder carcinogens, a growing list of other arylamines and related chemicals have been identified which are strongly suspected of producing bladder cancer in man. Ames et al. (1975a) showed that about 85% of hair dyes contained mutagenic arylamines and aryl nitro compounds. Subsequently, one of these, 2,4-diaminotoluene was demonstrated to induce liver and mammary tumours in rats whereas 2-methoxy-5-methylaniline (p-cresidine) was found to a bladder carcinogen in both rats and mice (Shirai 1993). Hairdressers, along with workers in the chemical, dye, rubber, leather and painting industries belong to an occupation in which bladder cancer has been particularly associated with occupational exposure to chemicals (Cohen and Johansson 1992). Similarly, 4,4-methylene-bis(2-chloroaniline) or MOCA, used as a curing agent for epoxy resins since the early 1950s, was found to cause bladder carcinomas in dogs (Stula et al. 1977; McQueen and Williams 1990). In the same report, another agent, 3,3-dichlorobenzidine, used in the dyestuffs industry for nearly 40 years and more recently as a curing agent for polyurethane elastomers, was also found to be a bladder carcinogen in dogs and rodents. MOCA and O-toluidine, another aromatic amine which causes bladder cancer in experimental animals (Ward et al. 1991), are metabolised in a similar manner to other known carcinogenic arylamines and there is epidemiological evidence to suggest that these chemicals are related to the development of bladder cancer in exposed workers (Ward et al. 1991). However, as workers are exposed to numerous related compounds it is often difficult to identify a specific aetiological agent in an occupational setting.

There is some evidence to suggest that aromatic amines might have more general relevance to human bladder cancer than that related to occupational exposure to specific chemicals or cigarette smoke. For example, numerous heterocyclic aromatic amines have been identified as pyrolysis products in foods exposed to high temperatures, particularly charcoal broiling of meats and other foodstuffs (Ohkagi et al. 1991; Wakabayashi et al. 1992). Also several aromatic amino compounds, have been identified as environmental pollutants, particularly in diesel exhaust. All these heterocyclic compounds are metabolised to produce reactive intermediates identical to the analogous aromatic amines. Furthermore, they are mutagenic and carcinogenic in experimental animals, where they have produced tumours in several organs including the liver, intestine and urinary bladder. Particular attention has been given to
2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) because it is the most abundant heterocyclic amine produced in cooking processes and it induces rat colon and mammary carcinomas, both of which are very common malignancies in human populations which consume a large amount of meat. However, 3-amino-1-methyl-5H-pyridol(4,3-b)indol (Tryp-P-2) has been shown by Takahashi et al. (1993) to induce bladder tumours in rats. Several mutagenic and carcinogenic heterocyclic amines including PhIP and Tryp-P-2 have been identified in cigarette smoke (Matsumoto et al. 1981; Manabe et al. 1991). Thus, carcinogenic heterocyclic amines in cooked foods and in cigarette smoke, could well be causative factors in human bladder cancer (Shirai 1993).

Occupational and environmental exposure to specific aromatic amines clearly is an important risk factor for human bladder cancer, although the comparative importance of this in relation to other aetiological factors is diminishing with the elimination or control of the specific chemical usage in industry (Doll 1992). Furthermore, the comparatively high incidence of bladder cancer in non-industrialised parts of the world such as Africa indicates that there are other important aetiological factors involved in the development of human bladder cancer (Price 1971).

(ii) Nitroso compounds

Nitrosamines are among the most potent known carcinogens in experimental animals and several are proven bladder carcinogens in animal models (IARC 1977). They are not only ubiquitous in nature but can be generated under acid conditions by nitrosation of secondary amines. Epidemiological and experimental studies have demonstrated an association between urinary tract infection and bladder cancer (Kantor et al. 1984; Johansson et al. 1987), and this may be related to the formation of nitrosamines or other carcinogenic substances in the generally acidic infected urine, or to the increased cell proliferation induced by the inflammation. Although nitrosamines have been found in the urine, particularly in relation to certain infections of the lower urinary tract (Hicks et al. 1977; Radomski et al. 1978), there is no convincing evidence yet that these compounds contribute to the development of human bladder cancer (Cohen and Johansson 1992).

B Tobacco smoking

Substantial epidemiological evidence has existed for more than 20 years demonstrating a strong association between cigarette smoking and the development of urinary bladder cancer, with typical smokers having two to three times the risk of non-smokers (Price 1971; Morrison et al. 1984; Auerbach and Garfinkel 1989; Burch et al. 1989; Clavel et al. 1989; Harris et al. 1990; Hartge et al. 1990). Indeed, Silverman et al. (1992) stated that there are more than 30 case-control studies...
and ten cohort (follow-up) studies demonstrating that cigarette smoking causes bladder cancer. Most of these epidemiological studies demonstrate a dose-response relationship such that risk of bladder cancer increases with increasing intensity of smoking (packs per day), with relative risk estimates for moderate to heavy smokers usually ranging from about 2.0 to 5.0 as compared with non-smokers (Silverman et al. 1992). The association of smoking with bladder cancer holds for both sexes, although it is particularly strong in males, and has been observed in many different geographical areas of Europe, the USA and Japan (Shirai 1993). Furthermore, the association is true for TCC as well as squamous cell carcinoma and adenocarcinoma (Kantor et al., 1988).

Although the relationship of cigarette smoking to bladder cancer is not as strong as for tumours of the respiratory tract, it is estimated that cigarette smoking accounts for 25% to 60% of all bladder cancer cases in industrialised, developed countries (Howe et al. 1980; Morrison et al. 1984; IARC 1986). Clearly, therefore, cigarette smoking is the single most important cause of bladder cancer. Similar results have been found in both case-control studies (Wynder and Goldsmith 1977; Mommsen and Aagaard 1983; Morrison et al. 1984) and follow-up studies (Doll and Peto 1976; Hirayama 1982). The risk not only correlates with the number of cigarettes smoked but also the duration of smoking and the degree of smoke inhalation (Wynder and Goldsmith 1977; Morrison et al. 1984). Furthermore, ex-smokers have a reduced incidence of bladder cancer compared with current smokers (Augustine et al. 1988).

The type of tobacco which is smoked appears to have some influence on the development of bladder cancer (Talaska et al. 1991). Dark tobacco carries the highest risk, whereas cigars or smokeless tobacco appear to be associated with a small risk. The smoking of air-cured tobacco as opposed to flue-cured tobacco results in exposures of 2 to 2.5 times higher doses of aromatic amines. Indeed, there are several reports in the literature suggesting that air-cured tobacco is more carcinogenic for the bladder than flue-cured tobacco (Howe et al. 1980; Clavel et al. 1989).

It is noteworthy that most of the evidence linking tobacco smoking with a high risk of bladder cancer is specifically associated with cigarettes (Matanoski and Elliott 1981). In their 1992 review, Silverman et al. reported that a definite dose-response relationship is rarely observed in studies of pipe smokers, although most studies have found pipe smokers at moderately elevated risk of bladder cancer. Weak and inconsistent relationships have been reported between cigar smoking and bladder cancer, with increased risks being observed in some studies, but not in others (Silverman et al. 1992).
Despite all the evidence linking bladder cancer with smoking, the specific chemical carcinogens responsible have not been identified. However, cigarette smoke contains many carcinogenic chemicals including aromatic amines, nitroso compounds, polycyclic aromatic hydrocarbons and unsaturated aldehydes (Clayson and Cooper 1970; Price 1971; Shirai 1993). As indicated above, there is considerable evidence that aromatic amines, especially 4-aminobiphenyl and O-toluidine, are associated with cigarette smoking and bladder cancer (Kroft and Oyasu 1994). Other aromatic amines including 2-naphthylamines and pyrolysis products such as PhIP and Tryp-P-2 are present in small quantities (Shirai 1993). Neither nicotine, and its metabolites, nor tobacco specific nitrosamines appear to be related to the development of bladder cancer, either in animals or in humans (Cohen and Johansson 1992). Although polycyclic aromatic hydrocarbons have been associated with the development of cancer in various tissues, there is little evidence of a link with urinary bladder cancer; they are rapidly metabolised at the sites of exposure and are excreted in the urine in metabolically inactive forms (Cohen and Johansson 1992). Acrolein, an unsaturated aldehyde is present in cigarette smoke at levels approaching 100 μg per cigarette (Cohen and Johansson 1992). This highly reactive compound is mutagenic and possibly carcinogenic and when administered systemically produces mild, flat hyperplasia in the bladder.

Although many different chemicals in cigarette smoke could induce genotoxic damage in the bladder epithelium, it has also been observed that smokers have an increased proliferative response, as evidenced by hyperplasia of the bladder epithelium (Auerbach and Garfinkel 1989). This effect appears to be dose related and may enhance the carcinogenicity of cigarette smoke (Cohen and Johansson 1992). This idea is supported by the observed dramatic drop (30-60%) in the risk of bladder cancer which is associated with cessation of smoking (Silverman et al. 1992), an observation which holds true irrespective of the type of tobacco smoked (Shirai 1993).

C Bacterial and parasitic infections of the urinary bladder

According to Price (1971), a connection between schistosomiasis (bilharzia) and cancer of the bladder had been recognised in the time of the Pharaohs. However, it was not until Ferguson (1911) attributed the relatively high incidence of bladder cancer in Egypt to chronic infection with Schistosoma haematobium, that the two diseases were linked aetiologically. Despite this aetiological link, convincing epidemiological evidence for this association is lacking (Silverman et al. 1992). It is true that carcinoma of the urinary bladder is the most common malignancy in Egyptians, accounting for 27.6% of all cancers - 38.5% of male cancers and 11.3% of female cancers (Tawfik 1987). However, although some subsequent Egyptian investigations have supported Ferguson's original suggestion (Clayson and Cooper 1970; Price 1971; El-Bolkainy
Bladder cancer incidence rates appear similar in areas of Africa where schistosomiasis is endemic, infrequent or absent (Silverman et al. 1992). Monkeys infected with S. haematobium develop bladder cancers, but these are transitional cell lesions rather than the squamous cell carcinomas seen predominantly in cancer patients with schistosomiasis (Silverman et al. 1992).

The eggs of S. haematobium are deposited in the bladder wall, inciting an inflammatory response that continues over the lifetime of the individual in most cases, particularly if re-infection occurs. Metaplasia of the transitional bladder epithelium to a squamous epithelium, the latter having a much higher proliferative rate, is frequently associated with this inflammation. Consequently, approximately 70% of schistosomiasis related bladder cancer patients develop squamous cell carcinomas rather than TCC (Matanoski and Elliott 1981). Even in individuals with TCCs or adenocarcinomas, the epithelium is rapidly proliferating, due to chronic infection, in contrast to the mitotically quiescent transitional epithelium of normal bladder (Cohen and Johansson 1992).

Although the mechanism of schistosomiasis related bladder cancer remains unknown, two factors appear to be relevant. These are: 1) the increased inflammatory and regenerative process in the bladders of these individuals and, 2) the potential of the inflammatory process to generate genotoxic substances in the urine. Increased cell proliferation over long periods of time has been suggested as a mechanism by which more spontaneous genetic mistakes could lead to a higher incidence of cancer, in the bladder and in other tissues (Cohen and Ellwein 1990). There is some evidence that nitrosamines, including BBN, a known bladder carcinogen in rodents and dogs, are generated in the urine of patients chronically infected with S. haematobium (Radomski and Hearn 1976; El-Bolkainy 1983). Secondary bacterial infection in these patients could play a role in the in situ formation of nitrosamines within parasite infected bladders; the most commonly identified organisms being E. coli, Staphylococcus sp. and Pseudomonas sp. (Shirai 1993). These organisms can convert nitrates to nitrites which can be metabolised further to nitrosamines by reaction with amines in the urine (Hicks et al. 1977). Increasing severity of bacterial infection correlates with elevation of urinary beta-glucuronidase, which may activate potential carcinogens previously detoxified and excreted by the liver as glucuronides (Norden and Gelfans 1972).

There is some epidemiological evidence that recurrent urinary infection is associated with the development of bladder cancer, especially in women (Kantor et al. 1984). This has also been noticed in patients with indwelling catheters or chronic urinary tract stones, although in general there is little correlation between the presence of urinary calculi and bladder cancer in man (Matanoski and Elliott 1981). The majority of the tumours in patients with chronic inflammation...
are squamous cell carcinomas, possibly due to the higher incidence of squamous metaplasia in these patients. Similar to schistosomiasis-related bladder cancer, the inflammatory process is associated with regenerative hyperplasia and an increased rate of cell division continuing over long periods of time (Cohen and Johansson 1992). This type of bladder cancer has also been reported to occur in patients with spinal cord injury who subsequently develop chronic cystitis (Bejany et al. 1987). In experimental animals, chronic infection has also been associated with enhancement of bladder carcinogenesis, although by itself, it has generally not been carcinogenic (Johansson et al. 1987). It is noteworthy that while the majority of bladder cancer cases associated with exposure to chemicals are TCCs with variable biological potential, bladder cancers related to chronic urinary infection (parasitic or bacterial or both) are generally deeply invasive squamous cell carcinomas that exhibit a poor prognosis (Shirai 1993).

D Medicines and medical procedures

A small proportion of bladder cancer cases results from the abuse of analgesic mixtures containing phenacetin. Similarly, bladder cancer has arisen as a side effect of certain anti-cancer treatments including chemotherapeutic drugs and pelvic irradiation.

(i) Phenacetin

In 1968 a possible relationship between analgesic abuse and the development of malignant tumours of the urinary tract was first pointed out by Hultengren et al. in Sweden. The occasional occurrence of bladder cancer as a result of the analgesic phenacetin (now replaced by paracetamol) was cited by the IARC in 1977. Phenacetin was used alone or in combination with aspirin and caffeine. The use of such mixtures led to some difficulty in clearly defining the relationship of phenacetin to urothelial cancers in individuals chronically abusing mixtures containing the drug. These difficulties were further exacerbated by the fact that bladder cancer in these individuals is usually preceded by nephrotoxicity. Nevertheless, there is sufficient epidemiological evidence linking these analgesic mixtures with the development of urinary tract carcinomas, particularly involving the renal pelvis, but also of the ureter and urinary bladder (Johansson and Wahlqvist 1977). Over a long period of continual abuse (10 years), exposure to phenacetin can be considerable and an increased risk of bladder cancer under these conditions appears certain (Piper et al. 1985).

Thus, even as one of the components of these analgesic mixtures, the IARC evaluated phenacetin to be carcinogenic for humans (IARC 1977). The chemical structure of phenacetin is similar to aniline and although in itself it is not nephrotoxic, most of its metabolites and some related
compounds have been found to cause kidney damage in rats. In Sprague-Dawley rats, phenacetin can induce TCCs of the urinary tract (Isaka et al. 1979) and it is also reported to promote nitrosamine induced urinary bladder carcinogenesis in F344 rats (Nakanishi et al. 1978). Johansson et al. (1989) demonstrated that phenacetin could chronically increase cell proliferation in the rat urothelium, suggesting this could be important in individuals exposed to the drug.

(ii) **Cyclophosphamide**

Cyclophosphamide is an important alkylating agent which has been used in the treatment of malignant neoplasms. It is extremely toxic to the bladder mucosa and produces marked cellular abnormalities in the urothelium. In 1971, Worth reported the first cases of bladder cancer related to cyclophosphamide therapy. Since that time at least 39 other urinary tract neoplasms following cyclophosphamide therapy have been described in the literature (Shirai 1993). Hicks et al. (1975) reported that cyclophosphamide could act as a co-carcinogen with N-methyl-N-nitrosourea (MNU) to induce bladder tumours in rats. Later Schmahl and Habs (1983) showed that cyclophosphamide alone could induce bladder carcinomas in rats. Patients treated with cyclophosphamide have up to a nine fold increased risk of developing bladder cancer, although a causative relationship has not yet formally been demonstrated in case-control epidemiological studies (O'Keane 1988). Most of the tumours which develop are TCC and have usually infiltrated the muscle of the bladder wall at the time of diagnosis (Durkee and Benson 1980). The latent period for neoplasia related to the drug is therefore relatively short, ranging from 6 to 13 years (Shirai 1993).

Cyclophosphamide is also an immunosuppressive agent and this effect is recognised as a contributing factor to its carcinogenicity. However, the carcinogenic potential is probably mediated through both the immunosuppressive and toxic properties of the compound, rather than the former alone. Of the various metabolites of cyclophosphamide, acrolein is known to bind to DNA and to be responsible for its toxicity (Calabresi and Parks 1975). Furthermore, this metabolite is thought most likely to be the cause of the associated bladder cancers (Cox 1979). Patients have been exposed to cyclophosphamide for periods ranging from a few months to several years. Tumours have arisen as quickly as a few years after the initial exposure. Cyclophosphamide-exposed individuals appear to exhibit tumours more quickly than those exposed to chemicals such as aromatic amines. It is possible that the extensive regenerative proliferation associated with haemorrhagic cystitis – a significant toxic manifestation of this compound - could be related to the speed of tumour development (Cohen and Johansson 1992).
In 1984, 2-mercapto-ethane sodium sulphonate (Mesna) was used to prevent cyclophosphamide induced haemorrhagic cystitis (Ehrlich et al. 1984). Mesna binds to the double bond of the acrolein molecule thus detoxifying the metabolite. Furthermore, Schmahl and Habs (1983) showed that Mesna could reduce the induction of bladder cancer in rats.

(iii) Chlornaphazine
Chlornaphazine is an alkylating agent which was originally developed as an anti-cancer chemotherapeutic agent and which was used in Denmark for the treatment of Hodgkin’s disease and polycythemia vera (Clayson and Cooper 1970), but was quickly withdrawn from use when a large percentage of the patients treated with it developed bladder cancer (Chievitz and Thiede 1962; Thiede et al. 1964; Thiede and Christensen 1975). It is an aromatic amine which causes human bladder cancer as a result of metabolic conversion within the body to 2-naphthylamine (Cohen and Johansson 1992).

(iv) Radiation
Ionising radiation causes bladder cancer, but this type of exposure is so rare that it contributes very little to bladder cancer incidence as a whole (Silverman et al. 1992). There are some reports that pelvic irradiation for cervical cancer or for benign uterine conditions results in an increased risk of subsequent bladder cancer (Palmer and Spratt 1956; Price 1971; Duncan et al. 1977; Silverman et al. 1992). In 1982, Antonokopoulos et al. reported that a single localised dose of X-rays (20 Gy) to the rat urinary bladder induced a variety of changes including urinary bladder carcinomas.

E Dietary constituents or additives
The role of aromatic amines, cigarette smoking, urinary infection and medicines such as phenacetin and cyclophosphamide in human bladder cancer development is undisputed. Several other aetiological factors, all of which are dietary components or additives, have also been implicated, at sometime, in human bladder cancer. These include the amino acid tryptophan and its metabolites, beverages containing caffeine (especially coffee) and artificial sweeteners (saccharin and cyclamate). Although the relation of tryptophan to bladder cancer remains to be fully resolved, all of these dietary components are no longer thought to be important aetiological factors for the development of human bladder cancer.
Abnormal tryptophan metabolism

With the discovery of the association between environmental aromatic amines and bladder cancer and the realisation that occupational exposure to these chemicals could not possibly account for all cases of the disease, researchers began to search for other aetiological factors. This led Price and his colleagues to investigate the possibility that endogenous urinary aromatic amines might be implicated in the development of 'spontaneous' bladder cancer (Price 1971). Most endogenous aromatic amines, such as kynurenine, are formed from the essential amino acid tryptophan and lie on the niacin pathway (Clayson and Cooper 1970). Furthermore, some of these compounds have been shown to induce bladder cancer when fed to mice (Clayson and Cooper 1970). Thus, this investigation of urinary endogenous aromatic amines, which started in the 1950's, led to several reports suggesting that tryptophan, and its aromatic amine metabolites, might be associated with the development of human bladder cancer (Dunning et al. 1950; Brown and Price 1956). In particular, the report of Dunning et al. (1950), in which dietary tryptophan enhanced the carcinogenicity of 2-acetylaminofluorene (2-AAF) in the rat bladder generated great interest in the possible role of tryptophan in human bladder cancer. Price and Brown (1962) reported that 20 of 41 patients with spontaneous bladder cancer had abnormal tryptophan metabolism (as demonstrated by increased levels of aromatic amine metabolites), whereas none of 16 occupationally exposed and none of 19 control subjects exhibited this abnormality. Similar findings of abnormal tryptophan metabolism in bladder cancer patients were reported by Boyland and Williams (1956), Quagliariello et al. (1961) and Wolf (1973). Furthermore, tumour recurrence rates were shown to be higher in patients with elevated excretion of tryptophan metabolites (Yoshida et al. 1970). However, not all reports are in complete agreement with these findings; Benassi et al. (1963) found abnormal tryptophan metabolism in only 60 of 201 bladder cancer patients.

Although administration of tryptophan to experimental animals does not induce bladder cancer (Shirai 1993), there are several reports, including that of Dunning et al. (1950), that dietary tryptophan or its metabolites can promote bladder carcinogenesis when initiated with known bladder carcinogens such as N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) (Cohen et al. 1979) or BBN (Ito et al. 1983; Fukushima et al. 1983). However, more recently, these results have been contradicted by Birt et al. (1987), who reported that increased dietary L-tryptophan did not promote bladder carcinogenesis in rats treated with FANFT and does not contribute significantly to the development of bladder cancer.
Studies carried out relatively recently in Egypt have pointed to a possible involvement of abnormal tryptophan metabolism in the aetiology of bladder cancer associated with schistosomiasis (Abdel-Tawab et al. 1986; Tawfik 1987).

Although the relation to bladder carcinogenesis has not been completely resolved, Cohen and Johansson (1992) and Shirai (1993), in reviewing the aetiology of human bladder cancer, considered that abnormal tryptophan metabolism is not an important factor in the development of the disease. On the other hand, Shirai (1993) pointed out that one of the pyrolysis products of tryptophan is Tryp-P-2, a heterocyclic aromatic amine known to induce bladder cancer in rats.

(ii) Caffeine

There has been, and continues to be, considerable controversy relating to the urinary tract carcinogenicity of caffeine-containing beverages, particularly coffee (Hartge et al. 1983; Cohen and Johansson 1992). The results of epidemiological investigations have varied greatly, with studies relating coffee drinking to bladder cancer finding either no effect (Sullivan 1982; Bouffioux 1984; Ohno et al. 1985; Nomura et al. 1986; Morrison 1987; Risch et al. 1988; Panelli et al. 1989; Viscoli et al. 1993), or a slight increase in the relative risk (Miller 1977; Morrison et al. 1982; Morrison et al. 1984; Kantor et al. 1988; La Vecchia et al. 1989; Nomura et al. 1991; Kunze et al. 1992). A significant difficulty has been the presence of confounding factors, such as cigarette smoking, occupational exposure, and diet, which have been controlled to a variable extent in the different studies (Morrison et al. 1982; Bouffioux 1984). Little support for the carcinogenicity of caffeine has been gained from experimental studies, with negative results obtained in long-term carcinogenicity trials (Nakanishi et al. 1978). Furthermore, no promoting activity has been demonstrated following caffeine administration to animals initiated with a known carcinogen (Nakanishi et al. 1978). There are problems also in assessing the genotoxicity of caffeine, the assay results being dependent on the type of assay used and the caffeine concentrations that are attained in the assay system. There is no evidence at present that caffeine is mutagenic in humans (Cohen and Johansson 1992).

From the conflicting epidemiological data it is likely that any increased risk of bladder cancer development associated with caffeine in coffee, tea or cocoa is very low (Morrison et al. 1982; Bouffioux 1984; Morrison 1987). Indeed, in 1991 the IARC stated that there was no consistent association in descriptive studies between coffee intake and cancer risk and that in case control studies, the observed weak positive relationships might be due to bias. The same review also
concluded that there was no association between tea consumption and bladder cancer, despite at least one report to the contrary (Slattery et al. 1988).

(iii) Artificial sweeteners: cyclamate and saccharin

In 1970, Price and his associates reported to the National Academy of Sciences, National Research Council, papillary bladder tumours in 8 out of 80 rats surviving more than 78 weeks fed a diet containing a 10:1 cyclamate to saccharin mixture at a dose rate of 2.5 g/kg body weight/day. On the basis of this evidence, sodium cyclamate was withdrawn from the market in the USA as a human food additive. Subsequent workers showed that high doses of cyclamate alone produced tumours in a few animals (Egeberg et al. 1970).

As a result of the report of Price et al. (1970), sodium saccharin also came under suspicion as a possible bladder carcinogen (Hicks et al. 1973; Chowaniec and Hicks 1979). These animal studies showed that very high doses of dietary saccharin result in a higher, but relatively low, incidence of bladder tumours, particularly if administered from birth and for the lifetime of the animal (Cohen and Johannson 1992). However, when saccharin was administered after pre-treatment with relatively low sub-carcinogenic doses of proven bladder carcinogens, malignant tumours developed in a high percentage of the animals (Hicks and Chowaniec 1977; Cohen et al. 1979; Hicks 1980). These results suggested that saccharin was a promoter of weak carcinogens, rather than a carcinogen per se. However, if these studies in animals show the potential of saccharin to promote bladder tumours, epidemiological studies have been unable to verify a relation between exposure to these agents and the development of bladder cancer in humans (Bouffioux 1984; Ohno et al. 1985; Morrison 1987; Risch et al. 1988; Nomura et al. 1991; Cohen and Johannson 1992). Indeed, diabetics and those suffering from obesity, consumers of larger amounts of sweeteners than other groups, do not appear to show an increased risk of bladder cancer (Wynder and Stellman 1981; Sullivan 1982; Bouffioux 1984).

The mechanism in rodents, according to Cohen and Johannson (1993), appears to be related to the formation of silicate-containing crystals, which are cytotoxic to the urothelium and lead to a chronic proliferative state and, ultimately, to tumour formation. These workers considered that the factors necessary for the formation of the silicate-containing crystals are unlikely to be met in human urine and, therefore, it is unlikely that sodium saccharin or any other form of saccharin will be related to the development of bladder cancer. Also, the report of Auerbach and Garfinkel (1989) suggests that the epidemiological evidence for saccharin-related tumour formation is
lacking in humans, and that there is no increase in urothelial proliferative response in individuals exposed to artificial sweeteners.

**Miscellaneous factors**

Various other factors have been, or could possibly be, associated with enhancement of bladder cancer in humans.

*(i) Urinary calculi*

Urinary calculi, which are commonly associated with infections of the urinary tract, have been linked to urothelial malignancy. In rodents, calculi can induce severe urothelial hyperplasia and promote bladder carcinogenesis (Cheng 1980; Shirai *et al.* 1987) and their presence alone can result in the development of bladder carcinomas when exposure is prolonged (Fukushima *et al.* 1992). In man, Kantor *et al.* (1984) reported that bladder stones present an increased risk for bladder cancer. Furthermore, urothelial hyperplasia and dysplasia are commonly observed in association with bladder stones (Beyer-Boon *et al.* 1978). However, other epidemiological studies have found no association between human bladder cancer and a history of bladder stones (Kroft and Oyasu 1994).

*(ii) Drinking water*

There have been reports that exposure to drinking water in areas of high pesticide usage is associated with an increased risk of developing bladder cancer, although the specific chemical and mechanism are unknown (Cantor *et al.* 1987). In the 1980s, several studies in the USA (reviewed by Silverman *et al.* 1992) reported a link between chlorination by-products in drinking water and increased bladder cancer risk.

*(iii) L-Valine and L-Leucine*

Kakizoe *et al.* (1982) found that of 21 amino acids, L-valine and L-leucine increased concanavalin A agglutinability of isolated rat bladder cells, suggesting that both could be possible tumour promoters. Subsequently, in 1986, these workers showed that dietary supplementation with both amino acids promoted the development of bladder cancer in rats treated with the bladder carcinogen BBN; these results led this group to speculate that the Western style diet - rich in protein - could be a risk factor for human bladder cancer development.
Viruses

There have been periodic suggestions of an association between bladder cancer development and various viruses, including retroviruses, papillomaviruses, herpes viruses and adenoviruses (Cohen and Johansson 1992). Bovine papillomavirus appears to be associated with bracken fern-related cancer of the bladder and upper gastro-intestinal tract in cattle (Campo and Jarrett 1986). Recently, some evidence has been obtained, implicating human papillomaviruses (HPVs) in human bladder carcinogenesis (Chetsanga et al. 1992; Anwar et al. 1992; Furihata et al. 1993). However, although HPVs are thought to have an important role in the development of human anogenital cancers, their role in human bladder carcinogenesis is controversial (Kroft and Oyasu 1994).

DNA viruses related to the HPV family, such as simian virus-40 (SV40), have been used in the laboratory to immortalise cultured human cells, a necessary step before malignant transformation in vitro by chemical carcinogens (Reznikoff et al. 1993). SV40 is thought to act through binding of its large T antigen to critical nucleoproteins, particularly p53 and the retinoblastoma gene product (see below), thus inactivating them (Kroft and Oyasu 1994). HPV has also been shown to transform human cells in culture (Willey et al. 1991) and, like the SV40 large T antigen, the HPV E6 and E7 oncoproteins are known to bind p53 (Werness et al. 1990) and the retinoblastoma gene product (Dyson et al. 1989). On the basis of these observations, Reznikoff et al. (1993) have proposed a mechanistic model of bladder carcinogenesis involving inactivation of p53 and the retinoblastoma gene product by viral oncoproteins as the initial immortalising event, with subsequent genetic alterations allowing transformation and tumour progression.

The prevalence of HPV in bladder cancers has been investigated using in situ hybridisation, polymerase chain reaction (PCR) and immunohistochemistry (Bryant et al. 1987; Chetsanga et al. 1992; Anwar et al. 1992; Furihata et al. 1993), with wildly variable results. Thus, the involvement of HPV in bladder carcinogenesis remains uncertain.

Bracken fern

Enzootic bovine haematuria associated with bladder cancer is common in mountainous areas in many parts of the world and can effect up to 90% of cattle over 2 years of age (Clayson and Cooper 1970). No particular breed of cattle appears to be especially susceptible. From Kenya it has been reported that several European breeds contracted the disease as well as the local Zebu cattle (Clayson and Cooper 1970). The disease has been reproduced in cows by feeding dried bracken fern (Pteridium aquilinum) daily for life. This material has been shown to be a potent...
carcinogen in many species, causing bladder cancer in the guinea pig and rat; intestinal cancer in the rat, sheep and, probably, the hamster; caecal and colonic cancer in the Japanese quail, and lung adenomas in the mouse (Evans and Mason 1965; Clayson and Cooper 1970; Evans 1987).

Bracken possesses a number of toxic properties other than carcinogenicity which are reminiscent of the action of radiation and certain alkylating agents. These include the destruction and denudation of the gastrointestinal mucosa, with severe electrolyte loss and diarrhoea.

Apart from its undoubted economic importance to cattle and sheep farmers, the toxicity and carcinogenicity of bracken fern is of interest in man. The young shoots of bracken ('croziers') are eaten as a delicacy in Japan and bracken salads have been a part of the diet in many parts of the world (Clayson and Cooper 1970). In epidemiological studies in Japan, cancer of the oesophagus was 2.7 times more common in people who ate bracken than in those who did not (Hirayama 1979). Villalobos-Salazar and colleagues (1990) demonstrated that milk derived from cows which had grazed on bracken was carcinogenic to mice. In a previous epidemiological survey (Villalobos-Salazar et al. 1989), these workers had shown that the standardised incidence of gastric and oesophageal cancer was almost three times greater in parts of Costa Rica where bracken (and haematuria in cattle) is common than in those areas of the country where the plant was uncommon. The implication of these two studies being that carcinogens derived from bracken were consumed in milk. Similarly, Galpin et al. (1990) also suggested the possibility that carcinogens derived from bracken had been consumed in milk, when reporting a significantly increased risk of gastric cancer among people who had spent their childhood in bracken-infested areas of Gwynedd in North Wales.

Several chemicals, including ptaquiloside and its related illudane-type sesquiterpene glycosides, have been isolated from bracken fern and been shown to be mutagenic and clastogenic in short term tests for genotoxicity (Matoba et al. 1987; Matsuoka et al. 1989). Furthermore, ptaquiloside has been reported to induce both ileal and bladder tumours in experimental animals (Hirono et al. 1987).

Recent research has suggested that bovine papillomavirus (BPV) type 4 may play a role, together with carcinogens derived from bracken in the development of upper gastro-intestinal tract cancer in cattle foraging on bracken (Campo and Jarrett 1986; Campo 1987; Bertone 1990). Similarly, it is possible that latent BPV type 2 infection could be involved in the aetiology of bladder cancer in bracken-eating cattle (Campo and Jarrett 1986; Campo 1987; Campo et al. 1992).
Despite the proven carcinogenicity of bracken fern in many varied animal species and at multiple sites including the urinary bladder, and the limited epidemiological evidence relating it to human gastro-intestinal malignancies, there is little evidence at the present time linking human bladder cancer with exposure to bracken fern (Aoki and Ohno 1982).

(vi) The role of urine in development of urinary bladder cancer

It is now accepted that the effective metabolites of bladder carcinogens reach their site of action by way of the urine rather than the blood (McDonald and Lund 1954; Chapman 1969; Ito et al. 1971). Ito and his colleagues demonstrated that unilateral ureter ligation induced renal pelvic tumours, that normally do not develop, at the site of ligature. Furthermore, Hashimoto and Kitigawa (1974) reported that in vitro malignant transformation of rat urothelial cells required the presence of urea in the tissue culture medium, while Oyasu et al. (1981) demonstrated the significance of urine for carcinoma development in a heterotopically transplanted urinary bladder system.

G Dietary inhibitors of urinary bladder cancer development

Various dietary factors have been observed to influence the development of bladder cancer in humans and in experimental animals, although the specific relationships remain poorly defined (Cohen and Johansson 1992).

(i) Vitamin A and beta-carotene (BC)

Overall, there has been support for the observation that individuals consuming higher than average amounts of vitamin A or BC have a lower rate of bladder cancer than individuals who have a low or negligible intake of these nutrients (Mettlin and Graham 1979; Cohen and Johansson 1992; Michels and Willett 1994). These epidemiological observations have been supported to some extent by experimental studies and have formed the basis for the use of synthetic derivatives of vitamin A (synthetic retinoids) as potential chemotherapeutic agents in patients with a previous papillary carcinoma (Alfthan et al. 1983; Hill and Grubbs 1992; Kelloff et al. 1994).

(ii) Vitamin C

There is some evidence that high doses of vitamin C suppress bladder cancer development, although this has not been supported by experimental studies. Indeed, extremely high doses of sodium ascorbate (5% of the diet) have been reported to enhance rat bladder carcinogenesis (Ellwein and Cohen 1990). However, in this last instance, the mechanism is likely to be similar to
that for sodium saccharin and not related to the antioxidant properties of vitamin C; thus, it is not relevant to human exposure (Cohen and Johansson 1992).

4 URINARY BLADDER CANCER IN EXPERIMENTAL ANIMALS

A Spontaneous urinary bladder cancer in animals

Cancer of the urinary tract in domesticated animals is comparatively rare (Clayson and Cooper 1970). This is due, in part, to the fact that domesticated animals, other than those kept as pets, are generally killed at an early age for food. In 1968, Osbourne and associates estimated that less than 0.5% of canine neoplasms were of the urinary system. The same authors (1968b) reported that the incidence in the cat was considerably lower than that in the dog. As discussed above, bovine bladder cancer is common in animals over 2 years of age in areas of bracken-infested pasture, but comparatively rare in cattle from areas where there is no bracken.

Spontaneous bladder tumours are very rare in laboratory rats and mice. The paucity of spontaneous tumours in these animals is a great advantage in experimental work because it renders significant even low incidences of experimentally induced tumours (Clayson and Cooper 1970). This advantage can be further enhanced by the use of inbred strains to reduce the effects of genetic variation. One such strain, the Fischer (F344) rat is used extensively in research and in the National Cancer Institute/ National Toxicology Program (NCI/NTP) carcinogenicity bioassays. Using the historical data from the NCI/NTP carcinogenicity studies, Haseman et al. (1990) reported upon the incidence of spontaneous transitional cell papillomas and carcinomas in this strain. In a total of 1858 untreated, male, control rats, 3 papillomas (0.2% of the animals) and no (0%) carcinomas were found. Similarly, in 1891 corn oil-treated control males, 3 (0.2%) papillomas and 2 (0.1%) carcinomas were observed. In female F344s, the corresponding findings in 1932 untreated and 1879 corn oil-treated animals were: 3 (0.2%) papillomas, 1 (0.1%) carcinoma, and 5 (0.3%) papillomas and 0 (0%) carcinomas, respectively.

B Chemically-induced urinary bladder cancer in experimental animals

The discovery, at the turn of the century, that industrial chemicals were implicated in the causation of urinary bladder cancer led to an intense effort to reproduce the disease in experimental animals (Clayson and Cooper 1970). However, the majority of these early studies, conducted between 1920 and 1940, were negative. These failures were due to the use of the wrong chemicals, the wrong animal species, inadequate dosage and a lack of realisation of the length of the latent period required (Clayson and Cooper 1970).
Since these early experiments, a very large number of diverse chemical agents (reviewed by Clayson and Cooper 1970) has been shown to induce urinary bladder cancer in a variety of different experimental animal species, including mice, rats, hamsters, rabbits, guinea-pigs, dogs and monkeys (though not necessarily every compound in all species). Each of the three known human bladder carcinogens (2-naphthylamine, benzidine and 4-aminobiphenyl) has been shown to produce bladder tumours in appropriate animal models. Using dogs and a long exposure time, Hueper et al. (1938) were the first workers to reproduce the carcinogenic action of 2-naphthylamine experimentally. Until 1966, the dog was the only animal species in which bladder tumours could be induced with this chemical. However, in 1966, Saffioti et al. obtained bladder carcinomas in the Syrian hamster by increasing the dosage from 0.1% (the dosage used in the dog) to 1.0% and 3 years later similar results were found in the monkey (Conzelman et al. 1969). Early experiments with 2-naphthylamine in the rat were virtually without effect, although Bonser et al. (1956) produced bladder papillomas after 2 years of continuous exposure. Hicks et al. (1982a) reported malignant TCCs in 4 out of 18 rats given 2-naphthylamine (300 mg/kg body weight) each week for 57 weeks.

C Animal models of urinary bladder cancer
Among the many varied compounds that have now been shown to induce malignant neoplasms in the urinary bladder (Clayson and Cooper 1970), some have been found to be valuable model carcinogens. Examples of these model bladder carcinogens, most of which were reviewed by Price (1971), include: 2-AAF, dibutylnitrosamine (DBN), BBN, MNU and FANFT. Most of the experimental data relating to chemically-induced urinary bladder cancer, of which there is a great deal, has been obtained using these five chemicals.

(i) 2-AAF
One of the few compounds known to induce bladder tumours in the rat, mouse and dog, as well as the rabbit and other species, is 2-AAF. The carcinogenic activity of this chemical is affected by a number of factors. Both tryptophan and indole, for example, potentiate its carcinogenic effects on the rat bladder as does vitamin B6 deficiency and the presence of paraffin pellets in the bladder. Furthermore, neonatal rats are more susceptible to its effects than weanling or post-weanling animals. This variability and range of activity (it also induces tumours of the liver, kidney, subcutaneous tissue, stomach and mammary gland in the rat) has made 2-AAF one of the favourite tools of experimentalists (Price 1971).
Several N-nitroso compounds induce cancer in experimental animals after a single dose (Price 1971). These important compounds are active in many species and in nearly all organs of the body (IARC 1978). In 1967, Druckrey et al published the results of a 1967, Druckrey et al published the results of an eight-year study of the carcinogenicity of over 60 N-nitroso compounds in highl...
(superficial papillary tumours and more invasive flat tumours) seen in human bladder cancer (Hicks et al. 1985; Kroft and Oyasu 1994).

Ito et al. (1969) studied the histogenesis of bladder tumours in male Wistar rats treated with BBN. They found that the longer treatment with carcinogen lasted, the greater was (a) the incidence of bladder epithelial hyperplasia, (b) the tendency toward papilloma formation, and (c) the incidence of bladder carcinomas (usually of the transitional cell type). The incidence of hyperplasia increased from 38% in 34 rats treated for four weeks to 100% in 48 animals treated for 12, 16 or 20 weeks. The compound was given to the rats in their drinking water (0.05% solution). The number of rats with papillomas rose from one of 34 treated for four weeks to 26 of 26 treated for 16 or 20 weeks. The incidence of bladder cancer increased from 5% in 40 rats treated for eight weeks to 85% in 13 treated for 20 weeks. Overall, hyperplasia of the bladder epithelium occurred in 99 (81%) of the 122 rats treated for 4-20 weeks, papillomas were found in 66 (54%), and malignant tumours developed in 32 (26%). No hyperplastic or neoplastic changes were seen in the six untreated control animals.

The principal urinary metabolite of BBN was found by Okada and Ishidate (1977) to be N-Nitroso-N-butyl-N-(3-carboxypropyl)amine. This compound, alone among the other more minor metabolites, produces similar bladder tumours, in rats, to those seen after treatment with DBN or BBN (Okada et al. 1975). This led Okada et al. (1975) to suggest that this common metabolite of DBN and BBN is the ultimate carcinogen responsible for the induction of bladder cancer by these compounds.

Of the several experimental models of urinary bladder cancer which exist, the BBN model has been most widely used for chemoprevention studies (Becci et al. 1979; Becci et al. 1981; Hicks et al. 1985; Moon et al. 1992). As originally described by Ito et al (1969), the model involves BBN administration in the drinking water. Although effective, this method of administration presents inherent safety risks to laboratory personnel and makes quantitation of the amount of carcinogen ingested by each animal difficult (Becci et al. 1981). These disadvantages are eliminated by administering BBN via gastric intubation (Becci et al. 1981; Moon et al. 1992). This is the method used in our laboratory, where the BBN model has been used extensively to study the chemoprevention of urinary bladder cancer by a range of natural and synthetic retinoids in F344 rats and in several strains of mice (Hicks et al. 1982b; Hicks et al. 1985). Additionally, the BBN model has been used to examine the effect of dietary beta-carotene (BC) administered to mice after
carcinogen treatment (Hicks et al. 1984). Consequently, BBN was chosen for use in studies of the anti-cancer activity of dietary BC in carcinogen-treated F344 rats.

(iv) MNU
MNU is a direct-acting carcinogen which does not need to be metabolised to an active intermediate, and produces persistent, multiple methylation of the DNA in any tissues with which it comes into contact (Severs et al. 1982). MNU is carcinogenic in all animal species tested: mice, rats, hamsters (Syrian golden, Chinese and European hamsters), guinea pigs, rabbits, gerbils, pigs, dogs and monkeys (IARC 1978). It induces benign and malignant tumours following its administration by different routes, is carcinogenic following prenatal administration and in single doses. MNU produces tumours at different sites including nervous tissue, stomach, oesophagus, pancreas, respiratory tract, intestine, lymphoreticular tissues, skin, kidney and urinary bladder (IARC 1978).

In 1972, Hicks and Wakefield described the development of urinary bladder papillomas and carcinomas in all 100 female Wistar rats 30 weeks after the administration, by urethral catheterisation, of 4 intravesicular doses, on alternate weeks, of 1.5 mg/animal MNU. In 1976 Vaslov et al. (cited by IARC 1978) found bladder papillomas in 16/23 rats within 9-56 weeks of treatment with total doses of 6 mg/animal MNU.

This MNU/rat model was used by Hicks et al. (1975a) and Hicks and Chowaniec (1977) to investigate the effects of various promoters upon bladder carcinogenesis. However, MNU preparations may be unstable if stored for long periods (Severs et al. 1982). This long-term instability could hamper the comparison of results from a series of different experiments.

(v) FANFT
The first nitrofuran derivative found to be a bladder carcinogen, FANFT, was discovered to be almost 100% effective, producing carcinoma of the urinary bladder in nearly all rats tested (Price 1971). The tumours can be transplanted and they metastasise, thereby proving their malignant nature. In most cases, only bladder carcinomas are produced in large numbers; the development of a significant number of carcinomas at other sites is rare. Erturk et al. (1967) fed 0.188% of FANFT to 30 female Sprague-Dawley rats for 46 weeks, and all 29 animals that survived for 34 weeks developed gross carcinomas of the urinary bladder. A few animals also developed carcinomas of the renal pelvis and others benign mammary tumours. The bladder carcinomas often filled the bladder lumen, and the animals usually died with severe hydronephrosis.
Histologically, these tumours consisted of eight squamous cell carcinomas, seven transitional cell carcinomas, one transitional cell papillary carcinoma, and 13 mixed carcinomas. The course of the disease in these animals was similar to that seen in humans with bladder cancer.

In a later report, Erturk et al. (1969) showed that FANFT induces bladder carcinomas in male Sprague-Dawley rats. These tumours were transplantable and could give rise to pulmonary metastases. In the same report, the pathogenesis of FANFT-induced bladder carcinomas was studied in female rats fed the carcinogen for 26 or 46 weeks. Hyperplasia of the bladder epithelium was present by three weeks and squamous metaplasia by eight weeks. Microscopic papillary or sessile transitional cell carcinomas were found after as little as nine weeks of treatment, while gross papillary lesions were evident at 12 weeks. Interim kills suggested that all females fed the carcinogen for 26 or 46 weeks had carcinoma after 20 weeks. By the 25th week, the bladder carcinomas were as large as 2 cm in diameter and one female animal had a metastatic pulmonary TCC.

FANFT has also been shown to induce urinary bladder cancer in mice (Erturk et al. 1970a) and in dogs (Erturk et al. 1970b). The results obtained in Swiss mice demonstrated that FANFT is one of the most potent known murine bladder carcinogens (Price 1971).

5 CARCINOGENESIS: GENERAL CONSIDERATIONS RELATING TO HUMAN AND EXPERIMENTAL CANCER INDUCTION

The cellular and molecular processes involved in the causation and development of cancer are grouped together under the term 'carcinogenesis'. Although literally meaning the development of carcinomas (malignant epithelial tumours), the term is used synonymously with the term 'oncogenic' to describe the development of all other forms of neoplastic disease. This is probably due to the fact that the majority of human malignancies are, in fact, of epithelial origin.

Carcinogenesis is a huge subject, involving a vast amount of research data collected over the last 200 years, and particularly since the 1940s. It is too vast a field to be dealt with in any great depth in this thesis. However, some understanding of cancer causation in general and, in particular, of the mechanisms underlying bladder cancer development, is vital if a model of bladder neoplasia is being used to evaluate the anti-cancer potential of BC. Therefore, the following section consists of a very brief overview of the fundamental principles underlying the carcinogenesis (reviewed by Miller 1978; Weisberger and Williams 1986; Ruddon 1987; Harnden 1992). Although cancer in general may be caused by exposure to chemicals, ionising radiation,
viruses or intrinsic factors such as hormone imbalance, the scope of this overview will be restricted, in the main, to carcinogenesis resulting from exposure to chemicals.

A  **Historical Perspectives**

The evidence that chemicals can induce cancer in humans has been accumulating for more than two centuries (reviewed by Miller 1978 and Ruddon 1987). The first observations linking chemicals with cancer, were made in 1761 by John Hill (reviewed by Redmond 1970), and by Percival Pott (1775). Hill noted that nasal cancer occurred in people using snuff excessively, while Pott reported a high incidence of scrotal cancer in men who had been chimney sweeps in childhood. In 1875, as reviewed by Haddow and Kon (1947), von Volkman, in Germany, and a year later Bell, in Scotland, observed skin cancer in workers whose skin was in continuous contact with tar and paraffin oils. The contribution of Rehn (1895) in elucidating the role of aromatic amines in the induction of occupational bladder cancer has already been mentioned.

Thus, the first observations of chemically-induced cancer were made in humans. These observations led to attempts to induce cancer in experimental animals with chemicals. One of the first successful attempts was made in 1915, when Yamagiwa and Ichikawa (reviewed by Haddow and Kon 1947) induced skin carcinomas by the repeated application of coal tar to the ears of rabbits. As a result of this, and similar work by other investigators (such as Kennaway 1925), a search was made for the active carcinogen in coal tar, resulting in the discovery of the carcinogenic polycyclic aromatic hydrocarbons (Cook *et al.* 1933). Induction of tumours by other chemical and hormonal carcinogens was described in the 1930s, including the induction of liver tumours in rats and mice with 2, 3-dimethyl-4-aminazobenzene by Yoshida (1933), of urinary bladder cancer in dogs with 2-naphthylamine by Hueper *et al.* (1938), and of mammary cancer in male mice with estrone by Lacassagne (1932). In the 1940s, the list of known chemical carcinogens expanded with the discovery of the carcinogenicity of 2-AAF (Wilson *et al.* 1941), halogenated hydrocarbons (Edwards 1941), urethane (Nettleship and Henshaw 1943) and beryllium salts (Gardner and Heslington 1946). Since the 1940s, certain anti-cancer alkylating agents, nitrosamines, intercalating agents, nickel and chromium compounds, asbestos, vinyl chloride, diethylstilbestrol and certain naturally occurring substances, such as aflatoxins, have been added to the list of known chemical carcinogens (Miller 1978; Ruddon 1987).

B  **Definition of a chemical carcinogen**

Weisberger and Williams (1986) defined chemical carcinogens operationally by their ability to induce tumours. These workers highlighted four types of response which have generally been
accepted as evidence of carcinogenicity in experimental animals: 1) (most importantly) the
development of types of tumour not seen in the controls; 2) an increased incidence of the tumour
types occurring in controls; 3) the development of tumours earlier than in controls; and 4) an
increased multiplicity of tumours. According to Weisberger and Williams, even the induction of
benign lesions is regarded as evidence of carcinogenicity, as no chemical has yet been found that
produces exclusively benign tumours. However, this system must be applied with great care,
otherwise artefacts and inadequate results can lead to misclassification of a chemical, an error
described as 'pseudocarcinogenesis' by Roe (1983). The classification system of Weisberger and
Williams is perhaps unfortunate for some chemicals, such as tumour enhancers or promoters.
However, according to their criteria, such a designation is unavoidable, since these chemicals in
specific situations can increase the yield of spontaneously occurring tumours.

C Mode of action of chemical carcinogens

In order to identify properties of chemicals that are necessary or contributory to the development
of cancer, an understanding of the neoplastic process is required. Although this has not yet been
achieved, considerable insights have been gained which point to certain effects of chemicals as
being essential for carcinogenicity (Weisberger and Williams 1986).

The induction of cancer in humans and experimental animals proceeds through a complex series of
reactions which usually involve a long expression time, the latent period, from carcinogen
exposure to the appearance of the cancer. These events are subject to and controlled by a number
of modifying factors. The events were divided by Weisberger and Williams (1986) into two
sequential groups, one in which the normal cell is converted into a neoplastic cell (neoplastic
conversion) and the second in which the neoplastic or transformed cell develops into an overt
cancer (neoplastic development). These authors suggested that the events involved in neoplastic
conversion are: 1) biotransformation (where necessary) of chemicals to ultimate carcinogenic
species; 2) interaction of the ultimate carcinogenic species with cellular proteins, RNA and, in
particular, DNA; 3) fixation of carcinogen-induced damage and, 4) multiplication of the
chemically altered cells. Likewise, the same authors described the events of neoplastic
development as 1) progressive growth leading to cancer formation (promotion) and, 2) tumour
progression. Chemicals can be involved in many different ways in both sequences of events.

(i) Biotransformation of chemical carcinogens

Chemical carcinogens belong to many different chemical classes and have a very broad range of
structures with no obvious unifying chemistry. As many of these chemicals have structures which
appear to be very unreactive, early investigators were puzzled about how such unreactive and water-insoluble compounds could be potent carcinogens. This situation was clarified when it became clear that there are two broad categories of chemical carcinogens, direct-acting and indirect-acting; the latter require metabolic activation to induce cancer. The in vitro studies of the Millers (1981) and other workers had shown that most interactions between carcinogens and cellular macromolecules resulted from covalent bond formation between an electrophilic form of the carcinogen and nucleophilic sites in proteins (e.g., sulphur, oxygen and nitrogen atoms in cysteine, tyrosine and histidine, respectively) and nucleic acids (e.g., purine or pyrimidine ring nitrogens and oxygens). Most direct-acting agents, of which there are only a few (e.g. MNU) are reactive electrophilic chemicals which are activated in aqueous solution at physiological pH. Indirect carcinogens are converted to reactive 'ultimate carcinogens' by the acquisition of electrophilic moieties during metabolism. The majority of chemical carcinogens (e.g., 2-AAF, dialkylnitrosamines such as BBN, aromatic amines such as 2-naphthylamine and polycyclic aromatic hydrocarbons such as benzo(a)pyrene (BP) are amongst those agents which require metabolic activation.

The enzymes responsible for the metabolic activation of chemical carcinogens are those which are involved with the normal process of detoxification (i.e. those enzymes which solubilise water-insoluble xenobiotic chemicals by the addition of hydrophilic groups prior to excretion in the urine). For example, the dialkylnitrosamines, such as BBN, undergo an enzyme-mediated activation step to form the reactive electrophilic intermediate. These agents are metabolically dealkylated by the mixed-function oxidase system (cytochrome P450 system) in the endoplasmic reticulum of cells, primarily in the liver. The monoalkyl derivatives then undergo a non-enzymatic, spontaneous conversion to monoalkyldiazonium ions that donate an alkyl group to cellular nucleophilic groups in DNA, RNA and protein (Lawley 1976).

(ii) Interaction of the ultimate carcinogen with cellular macromolecules
Attempts to elucidate the mechanism of action of chemical carcinogens have focused on their interactions with cellular macromolecules, in particular, DNA. The neoplastic state is heritable at the cellular level (i.e. the daughter cells from the division of a cancer cell inherit the neoplastic nature of the parent cell), and thus theories on the mechanisms by which chemicals convert normal cells to malignant ones must ultimately explain how the conversion becomes permanent (Weisberger and Williams 1986).
In addition to DNA, carcinogens interact with numerous tissue constituents and produce a number of effects. Chemical carcinogens are capable of interacting with a wide variety of cellular macromolecules. This usually involves the alkylation of nucleophilic (electron-rich) groups on nucleic acids or proteins with electrophilic (electron-seeking) groups on the carcinogen. In 1947, the Millers demonstrated the covalent binding of a metabolite of N, N-dimethyl-4-aminoazobenzene to hepatic proteins of rats fed this compound. Since that time, covalent binding of various carcinogens to proteins in target tissues has been shown (Ruddon 1987). Both carcinogenic nitrosamines (Turberville and Craddock 1971) and aromatic amines (Barry et al. 1968) have been shown to interact with nuclear histones, indicating a possible direct effect, in some cases, on chromatin regulatory proteins.

Chemical carcinogens also react with various forms of cellular RNA. It has been reported that injection of dimethylnitrosamine into various experimental animals including rats and mice produces methylation of cellular RNA of the liver, kidney, spleen and pancreas (Ruddon 1987). The highest degree of methylation occurs in the liver, one of the primary targets of the carcinogen, and the principal reaction product is 7-methylguanine. Nitrosamines have been shown, by Muramatsu et al. (1972), to methylate nuclear RNA, transfer RNA (tRNA) and ribosomal RNA (rRNA) of liver after injection into mice. Methylation of the N-7 and O-6 positions of guanine in liver rRNA occurs after treatment of rats in vivo with the same nitrosamine (Ruddon 1987). The potent liver carcinogen 2-AAF also binds to cytoplasmic RNAs in liver when administered in vivo to susceptible species. In this case, however, the major nucleoside reaction product is at the C-8 position of guanine. Fink et al. (1970) demonstrated that binding of AAF to guanine residues in tRNA alters the functional properties of tRNA with respect to amino acid acceptance capacity and codon recognition. In addition, this modification completely inactivates the ability of synthetic mRNAs with guanine-containing amino acid codons to bind to their respective aminoacyl tRNAs.

It appears that 2-AAF modification of synthetic mRNAs interferes with codon-anticodon recognition. Thus, the binding of 2-AAF, and very likely the attachment of other carcinogens as well, to bases in RNA could prevent normal base-pairing during mRNA translation and lead to the biosynthesis of faulty proteins.

In the late 1970s and early '80s some investigators put forward the theory that the effects resulting from interactions between carcinogens and proteins or RNA could eventually be rendered permanent through epigenetic mechanisms on gene expression, creating a new stable state of differentiation (Hiatt et al. 1977; Rubin 1980). This resulted in the epigenetic or non-genotoxic
theory of carcinogenesis, whereby neoplastic development is thought to take place, without direct interaction with DNA.

The discovery that carcinogens interact with DNA, however, provided a basis on which the permanent neoplastic state could be explained by a direct alteration in the genotype. Williams and Wiesberger (1986) cited a number of considerations to support the view that DNA is a critical target for carcinogens. These considerations were: 1) many carcinogens are, or can be metabolised to form, electrophilic compounds that react covalently with DNA. Consequently, such carcinogens are mutagens. These reactions can often be detected by the induction of DNA repair; 2) defects in DNA repair such as xeroderma pigmentosum predispose to cancer development; 3) several heritable or chromosomal abnormalities predispose to cancer development; 4) initiated dormant tumour cells are persistent, a finding which is consistent with a change in DNA; 5) cancer is heritable at the cellular level and, therefore, may result from an alteration of DNA; 6) most, if not all, cancers display chromosomal abnormalities; 7) many cancers display aberrant gene expression; and 8) cells from many cancers contain genes known as activated oncogenes.

Since most chemical carcinogens react with DNA and are mutagenic, interactions with DNA have been seen as the most important reactions of these chemicals with cellular macromolecules. Reaction with DNA is the simplest mechanism that explains the induction of a heritable change in a cell leading to malignant transformation; thus, many investigators have viewed this as the most plausible mechanism for the initiation of carcinogenesis (Ruddon 1987). Compounds from virtually every class of chemical carcinogen have been demonstrated to affect DNA in some way, and a number of distinct reaction products have been identified after treatment of cells in vivo or in culture with carcinogenic agents. As with RNA, the principal reaction products of the nitrosamines and similar alkylating agents with DNA are N-7 and O-6 guanine derivatives. However, the extent of O-6 alkylation of DNA guanine residues correlates better with mutagenic and carcinogenic activity than the quantitatively greater N-7 alkylation of guanine residues (Loveless 1969; Lawley 1976). Reactions also occur with other DNA bases and these may also be important in subsequent mutagenic or carcinogenic events. Aflatoxin also forms nucleic acid reaction products (adducts) of guanine at the N-7 position after metabolic activation. The principal reaction product of 2-AAF with DNA is the C-8 position of guanine, as it is for RNA (Miller 1978). Other carcinogenic aromatic amines, such as N-methyl-4-aminoazobenzene, also produce C-8 substituted guanine residues as their major adduct, while polycyclic aromatic hydrocarbons, after activation, also react with DNA and RNA, forming adducts involving the
2-amino group of guanine (Ruddon 1987). However, in the case of polycyclic aromatic hydrocarbons, reaction products derived from adenine and cytosine have also been observed.

There are several potential consequences of DNA base-adduct formation by chemical carcinogens. In some cases it may stabilise an intercalation reaction in which the flat planar rings of a polycyclic hydrocarbon are inserted between the stacked bases of DNA and distort the double helix, leading to a frame-shift mutation during DNA replication past the point of the intercalation (Hogan et al. 1981). Alkylated bases in DNA can mispair with the wrong base during DNA replication - for example, O-6 methylguanine pairs with thymine instead of cytosine, leading to a base transition (i.e., GC→AT) type of mutation during the next round of DNA replication (Eadie et al. 1984). Point mutations such as these have been shown to be one way in which various growth controlling genes become activated to form oncogenes, a group of genes known to be important in the molecular biology of cancer development (see below). Many of the base adducts formed by chemical carcinogens involve modifications of N-3 or N-7 positions on purines that induce an instability in the glycosidic bond between the purine base and deoxyribose. This destabilised structure can then undergo cleavage by DNA glycosylase, resulting in loss of the base and creation of an apurinic site in DNA (Loeb 1985). This apurinic site can then be filled by any base, but most commonly by adenine, during subsequent DNA replication. This substitution can result in a base transition (purine→pyrimidine base change, but in the same orientation, e.g., GC→TA). Finally, interaction with some carcinogens has been shown to favour a conformational transition of DNA from its usual double-helical B form to a Z-DNA form (Needle 1981). This type of change could alter the transcribability of certain genes, since B→Z conformational transitions are thought to be involved in regulating chromatin structure (Ruddon 1987).

Interaction of chemical carcinogens with DNA or chromatin does not appear to be a random process. In vitro studies with the ultimate carcinogen of BP and cloned chicken DNA have shown that the carcinogen preferentially binds to areas of DNA thought to be involved with regulating gene transcription. Similarly, treatment of the large polytene chromosomes of Chironomus with the same carcinogenic agent has demonstrated that the carcinogen binds preferentially to areas most active in gene transcription. These observations indicate that the specificity of carcinogen binding is determined by the base sequence of DNA and the structure of chromatin, with active, 'open' sites being favoured (Ruddon 1987).
(iii) **Fixation of carcinogen damage**

Following exposure to chemical carcinogens, damaged DNA is subject to removal and restoration by repair enzyme systems (Ruddon 1987). Other damaged macromolecules are removed and replaced. Thus, under normal conditions, the cell can recover from most of the effects of carcinogen exposure. If the cell replicates while DNA damage is persistent, however, permanent alterations in the genetic information can be produced in several ways, including the mispairing of bases leading to point mutations, errors in replication giving rise to frame-shift mutations, transpositions resulting in codon rearrangement, combinations of these changes in sequential steps, and their amplification (Weisberger and Williams 1986). Codon rearrangements may involve oncogenes which emerged during the 1980s as critical genes for neoplastic transformation (Land et al. 1983; Bishop 1985; Reynolds et al. 1986). Point mutation is just one of at least five different mechanisms which have been identified as being responsible for converting cellular proto-oncogenes into active oncogenes (Weisberger and Williams 1986; Bishop 1987; Darnell et al. 1990). Furthermore, most known oncogenes are concerned with the control of cell growth and encode protein products which can induce cellular transformation.

All these alterations can give rise to a permanently abnormal cell with altered genotype and phenotype. The abnormal cell may possess only some of the properties of neoplastic cells or, if the alterations are sufficient, may be fully neoplastic (fully transformed).

(iv) **Multiplication of altered cells**

Abnormal cells that are not fully transformed may be held in check by tissue homeostatic factors or, if the conditions of carcinogen exposure or abnormalities in the cell permit, they may undergo limited proliferation to form 'preneoplastic' lesions (Farber 1982). During this proliferation, further alteration of the DNA as a result of transpositions and other error-prone processes are possible and could lead to the formation of a fully neoplastic cell (Weisberger and Williams 1986).

(v) **Progressive growth leading to tumour formation (promotion)**

Cells that have undergone neoplastic conversion may remain dormant, perhaps under the control of tissue homeostatic factors, for many years. These regulatory factors may be transmitted by intercellular communication. Some neoplastic cells with the requisite alterations may possess the ability to escape the influence of the regulatory factors and undergo progressive growth to form a tumour. In the absence of such ability, suppressed neoplastic cells can be facilitated to proliferate by the action of promoters, possibly by the interruption of tissue growth control, leading to neoplastic development.
Some neoplasms exhibit changes in their phenotypic properties, possibly including the transition from benign to malignant (Weisberger and Williams 1986). These changes probably reflect the selection during growth of a population with a genotype coding for advantageous phenotypic properties. New genotypes could arise through errors in DNA replication, alterations in chromosome constitution, or hybridisation of different cell types. The neoplasm which ultimately emerges is in most cases the progeny of a single cell; that is, it is a clonal population. Nevertheless, neoplasms display abnormalities in expression of numerous gene products.

Many of the steps in carcinogenesis are controlled and modified by numerous endogenous and exogenous factors (Weisberger and Williams 1986). Thus, in experimental animals, species, strain, sex and age affect some of the steps, particularly biotransformation and DNA repair. In addition, hormonal, immunological, and other endogenous factors may enhance or diminish the extent and rate of carcinogenesis. For example, co-carcinogens enhance neoplastic conversion, while promoters enhance neoplastic development. Many exogenous factors are nutritional elements such as the micronutrients: vitamins A, C and E, BC and selenium. Furthermore, many interactions occur between all of these factors as well as between synthetic and naturally occurring agents that can augment or diminish the overall effectiveness of a given chemical carcinogen.

Many chemical carcinogens have been shown to give rise to electrophilic reactants capable of interacting with DNA and proving mutagenic in short-term tests for mutagenicity such as that of Ames (Ames et al. 1975b). These so-called genotoxic carcinogens (Weisberger and Williams 1986) support the idea that interaction with DNA is an essential step in cancer development and provide a basis on which the permanent neoplastic state could be explained by alterations in the genotype. Considering the variety of abnormalities in cancer cells, it seems likely that genotoxic carcinogens would have to produce multiple genetic mutations, alterations in major regulatory genes, or changes in expression of large regions of the genome.

However, the interaction of carcinogenic chemicals with RNA and proteins has also been postulated as being capable of leading to permanent alteration through epigenetic mechanisms on gene expression (Rubin 1980). Support for epigenetic carcinogenesis comes from a growing list of carcinogenic substances (such as plastics, asbestos, hormones) which have structures which do not suggest they could form electrophilic species; none of these substances been reported to alter
DNA nor to be mutagenic (Weisberger and Williams 1986). Thus, it has been recognised that various mechanisms of action seem to be involved in chemical carcinogenesis.

Epigenetic carcinogens, while not demonstrating evidence of DNA reactivity, usually show evidence of another biological effect that could be the basis for carcinogenicity; possible mechanisms may involve cytotoxicity and chronic tissue injury, intracellular generation of reactive species such as singlet oxygen radicals, hormonal imbalance, immunological effects or promotional activity. In some cases, these agents could indirectly cause genetic alterations and neoplastic conversion by means of the production of inaccurate DNA synthesis, reactive oxygen free radicals, aberrant methylation and chromosomal abnormalities (Williams 1983). Alternatively, they could produce neoplastic conversion by epigenetic effects on gene expression (Rubin 1980; Nyce et al. 1983).

Thus, in summary, it seems likely that there is more than one mechanism by which chemical carcinogens induce cancer. Nevertheless, the ultimate effect, the production of a population of neoplastic cells with a permanently altered phenotype, is the same. To elucidate fully the essential effects of a particular carcinogen, it is necessary to determine at what step in the overall process of carcinogenesis the agent functions.

**D Multistage carcinogenesis**

One of the characteristics of carcinogenesis, whether in humans or experimental animals, is the long period of time, the latent period, which elapses between exposure to a stimulus (chemical, ionising radiation or viral) and the appearance of physical tumours. In experimental animals, the latent period can be weeks or months, or even years, depending to some extent upon the species (Hamden 1992). In humans, it is known that cancer may arise many decades after exposure to a suspected carcinogen. So what, if anything, is happening during this latent period of carcinogenesis? Hamden (1992) suggested that there are several possible answers to this question:

1. The causal agent may create one or more cancer cells which lie dormant for many months or years but which eventually begin to divide and cause a cancer.

2. The cancer cell or cells begin to grow immediately, but either this growth is very slow or the increase in cell number is balanced by loss of cells caused by intrinsic instability or by killing of cells by the host immune system.

3. The carcinogenic agent does not induce cells to become cancerous at one step but alters a normal cell, or cells, in some way that makes them more susceptible to further changes. This third model of carcinogenesis is, therefore, a multistage one.
Most arguments point towards a multistage model for carcinogenesis, for which there is much compelling experimental evidence (Peraino et al. 1973; Farber and Solt 1978; Reddy et al. 1978; Witschi and Lock 1978; Hicks 1980). Similarly, an examination of the increase in incidence of particular cancers with age, either in man or in experimental animals, reveals that the rate of increase is not compatible with carcinogenesis being attributable to a single event (Armitage and Doll 1961; Doll 1971; Doll and Peto 1978). Apart from cancers of childhood or early adulthood, such as testicular cancer, the human age incidence curves best fit a model which suggests 4-6 stages being involved in the development of full malignancy (Hamden 1992). This has led to the concept of carcinogenesis (outlined in section 5c) involving an initial stimulus, producing an altered cell which is not an autonomous cancer cell, followed by a period of promotion and progression, during which further changes take place (Figure 1.1).

(i) Initiation and promotion

Much of the experimental evidence which led to the idea of carcinogenesis as a multistage process was derived from early experiments with virus-induced tumours and also from the discovery of the co-carcinogenic effects of croton oil (Ruddon 1987).

Rous and associates found that certain virus-induced skin papillomas in rabbits regressed after a period of time and that papillomas could be made to reappear if the skin was stressed by punching holes in it or by applying irritants such as turpentine or chloroform. These findings led Rous and his colleagues to conclude that tumour cells could exist in a latent or dormant state and that the tumour induction process and subsequent growth of the tumour involved different mechanisms, which they termed initiation and promotion, respectively (Friedewald and Rous 1944).

The term co-carcinogen was first used in 1940 by Sall and Shear (cited by Ruddon 1987) who demonstrated that a basic fraction of creosote oil enhanced the production of mouse skin tumours by benzo(a)pyrene (BP). In 1941, Berenblum found that a single application to mouse skin of a carcinogen, such as methylcholanthrene, resulted in papillomas in only a small proportion of the animals, but if the same area of skin was later repeatedly painted with croton oil, which by itself is not carcinogenic, almost all the animals developed skin carcinomas. The observations of these three sets of investigators, therefore, suggested that carcinogenesis in the skin, at least, was a multistage process.

Following the findings of these early pioneering investigators, further study of the events involved in initiation and promotion were greatly helped by the discovery of agents that have primarily an
initiating activity, such as urethane, and the isolation from croton oil of individual components which have only promoting potential. Understanding of promotion was especially enhanced by the identification of the diesters of phorbol as potent promoting agents (Van Duuren 1969; Hecker 1971). Of these, 12-O-tetradecanoylphorbol-13-acetate (TPA) was found to be the most potent promoting agent (Berenblum 1982).

Typically, initiation-promotion experiments in mouse skin carcinogenesis involve a single application of an initiating agent. This may be a 'pure' initiator such as urethane or a subcarcinogenic dose of a complete carcinogen (i.e., a carcinogen having both initiating and promoting activity), such as benzo(a)pyrene. Promotion is carried out by repeated application of a phorbol ester such as TPA (Van Duuren 1969). Benign papillomas begin to appear at 12 to 20 weeks and by about 1 year, squamous cell carcinomas have developed in 40-60% of the animals. If the promoting agent is given alone or before the initiating agent, usually no malignant tumours occur.

Carcinogenesis has been observed to be a multistage process in other tissues also. For example, Peraino et al. (1973) found only a small number of liver tumours several months after a 3 week exposure of rats to 2-AAF in the diet. However, if the animals were subsequently treated with phenobarbitone for several months after the end of the carcinogen treatment, a high incidence of liver tumours was observed. Thus, in the liver the action of phenobarbitone appears to be analogous to that of TPA in mouse skin; that is, it fixes the damage to cells induced by an initiating agent and causes a clone of cells arising from a damaged cell to proliferate (Ruddon 1987). In addition to skin and liver, evidence supporting the multistage theory of carcinogenesis has been observed for mammary gland, thyroid, lung and urinary bladder (see below) as well as in cell culture systems (reviewed by Miller 1978).

Several of the characteristics of initiation and promotion provide a few clues as to the mechanisms involved in these processes. Initiation can occur after a single brief exposure to a potent initiating agent. The events leading to transformation into a dormant tumour cell appear to occur within one mitotic cycle, or about 1 day for the mouse skin model (Berenblum 1982). Initiation appears to be irreversible; the promoter can be administered up to 1 year later and a high percentage of tumours will be still obtained. Thus, initiation only requires a short period of time, it is irreversible, and it must be heritable because the initiated cell conveys the transformed alteration to its daughter cells. All these observations are consistent with the theory that initiation involves genetic mutation (although other explanations are also possible). On the other hand, promotion is a slow, gradual
process which requires a more prolonged exposure to the promoting agent. Promotion occupies the greater part of the latent period in carcinogenesis, is at least partially reversible, and can be arrested by certain anticarcinogenic agents such as vitamin A and its synthetic analogues (Sporn et al. 1976). Furthermore, tumour promotion also requires at least two distinct processes. Firstly, a cell proliferation phase propagates the initiated damage to produce a clone of altered cells. Most promoting agents are mitogens for the tissue in which promotion occurs. The second phase of tumour promotion is tumour progression. This term was initially coined by Foulds in 1954 to describe the processes within neoplastic development which led to the procurement by a tumour of higher degrees of autonomous growth and malignancy, processes which were still seen as part of tumour promotion. More recently, however, tumour progression has been defined as a distinct phase of carcinogenesis, occurring after promotion (Pitot 1989). This later phase requires continued clonal proliferation of altered cells, during which a loss of growth control and an escape from the host defence mechanisms become dominant phenotypic properties. This allows growth to progress to a clinically detectable tumour (Ruddon 1987).

(ii) Multistage carcinogenesis in the urinary bladder

As in other organs, there is now much evidence demonstrating that urinary bladder carcinogenesis also is a multistage process (Hicks et al. 1973; Hicks and Chowaniec 1977; Cohen et al. 1979; Hicks 1980 and 1981).

As described previously, prolonged exposure to solitary carcinogens, such as 2-naphthylamine, will induce bladder cancer in humans. Similarly, a 100% incidence of bladder carcinomas will be found in experimental animals after treatment with a sufficiently high dose of a carcinogen such as BBN, MNU or FANFT without any other treatment (Hicks 1981). If bladder carcinogenesis is a multistage process, then complete carcinogens such as these must be able to initiate and promote tumour development. In general, however, even these potent complete carcinogens have to be applied more than once, or continually over a period of time, in order to complete the process of tumour development (Hicks 1981). Thus, BBN (0.05%) administered to rats in drinking water is not carcinogenic if withdrawn within 4 weeks but will produce a 100% incidence of bladder tumours if maintained in the diet for 16 weeks or longer (Ito et al. 1969).

In humans, it is possible that many bladder cancers arise from either exposure to low doses of two or more carcinogens, with an additive or synergistic effect, or from initiation by a low dose of a carcinogen followed by promotion with various cofactors which may not be complete carcinogens themselves. Thus, administration of phenacetin or BBN alone to rats induced urothelial...
hyperplasia and bladder tumours, and given together they showed an additive effect on bladder tumour incidence (Nakanishi et al. 1978). Alternatively, Ito et al. (1974) reported that the simultaneous administration of BBN and 2-AAF to rats, at doses which were sub-carcinogenic when given alone, together produced bladder cancer. Thus, the report of Ito and his associates (1974) demonstrated that two bladder carcinogens can exert a synergistic effect; that is, their effect can be more than additive if they both act on the same target cell(s) in such a way that one compound promotes the effect of the other (Hicks 1980). Incidentally, Ito's paper also reported that pre-treatment of the rats with 2-AAF did not enhance the incidence of tumours found after a subsequent dose of BBN, suggesting that 2-AAF acts at a later stage in the carcinogenic process than does BBN.

Hicks et al. (1978) successfully demonstrated that the artificial sweeteners saccharin and cyclamate could promote the development of bladder tumours in rodents following a sub-threshold or threshold dose of intravesically-instilled MNU. When a carcinogenic dose of MNU producing a bladder cancer incidence of about 30% was used, no significant increase in tumour incidence resulted from subsequent administration of saccharin in the drinking water (Hicks 1980). This finding was similar to that seen in the early initiation-promotion studies in mouse skin carcinogenesis, when croton oil failed to increase the tumour incidence after a high carcinogenic dose of benzo(a)pyrene (Berenblum 1941).

The tumour promoting capabilities of saccharin in the rodent urinary bladder were confirmed by Cohen et al. (1979), who demonstrated that both saccharin and tryptophan could promote tumour development in rats given a threshold dose of the carcinogen FANFT. These workers also showed that a similar number of bladder tumours was found if a time interval of six weeks was interposed between treatment with FANFT and the start of saccharin administration, thereby broadly supporting the idea of persistence of initiated cells in the target tissue after cessation of carcinogen treatment. The mechanism of saccharin-induced tumour promotion in rodents is not thought to be relevant to humans (Cohen and Johansson 1992). However, repeated exposure to cigarette smoke, which contains many potential carcinogens and promoters, and/or recurrent urinary tract infections could have a promoting effect in the development of human bladder cancer (Cohen and Johansson 1992; Shirai 1993).

6 RECENT ADVANCES IN MOLECULAR CARCINOGENESIS
Since the mid-1980s, when the experiments described in this thesis were first conceived, cancer research has been greatly aided by the techniques of modern molecular biology and, in particular,
by the discovery of two groups of genes, the cellular oncogenes and the tumour suppressor genes. Much research in the past 15 years has led to the discovery that in normal humans and experimental animals, the proliferation of cells appears to be regulated by growth promoting proto-oncogenes counterbalanced by growth restraining tumour suppressor genes (Wolff and Habib 1993). Alterations in these genes results in protein products with altered activities which in turn initiate a cascade of molecular and cellular events leading to the progressive transformation of normal cells into fully malignant cells.

None of the experimental work described in thesis involves directly the assessment of oncogene or tumour suppressor gene activity. Nevertheless, it is important that relevant aspects of these significant advances in modern carcinogenesis be described in overview. This will allow the experimental work of the thesis to be discussed in the light of these developments.

A Oncogenes

An oncogene is a gene the product of which is involved either in transforming cells in vitro or in inducing cancer in animals; many of these genes are believed to play important roles in human cancer (Darnell et al. 1990). Exogenous or viral oncogenes (v-onc) are introduced into cells by tumour viruses. Two categories of viral oncogenes can be distinguished, those which show no obvious relation to normal cellular genes and those which are clearly derived from them (Paul 1987). Endogenous or cellular oncogenes (c-onc) arise within cells as altered (activated) forms of normal cellular genes, referred to as proto-oncogenes. These proto-oncogenes are important for normal cellular processes, but can be altered, often in very simple ways, to become oncogenes or cancer-causing genes.

Much of what is known about oncogenes has been derived from studies of those proto-oncogenes which can give rise, on the one hand, to viral oncogenes and, on the other, to activated cellular oncogenes (Bishop 1983). Some endogenous oncogenes do not appear to be related to viral oncogenes but most viral oncogenes of oncogenic (cancer-causing) retroviruses are derived from proto-oncogenes which can, in other circumstances, be activated to give rise to cellular oncogenes. Oncogenic retroviruses can incorporate sequences from normal genes into their genome and carry them by infection to other cells, a process called transduction (Paul 1987). The transferred viral oncogenes undergo mutations, thus altering their properties and bringing them under new types of control; as a result of these changes, transferred oncogenes behave abnormally. On the other hand, proto-oncogenes can undergo alterations in situ by insertional mutagenesis, rearrangement or point mutations. These changes can occur naturally at extremely low frequency but this can be
greatly increased by mutagens such as chemical carcinogens, radiation, both ionising and non-ionising and viral infection. When activated, oncogenes contribute to malignant behaviour (Varmus 1984).

Oncogenes were first discovered within the genetic material of oncogenic viruses (Bishop 1983). The first to be discovered was the src oncogene, found in chicken cells infected with Rous sarcoma virus (Stehelin et al. 1976). Later experiments showed that the normal cellular gene (c-src) had been appropriated by the retrovirus, as the same nucleic acid sequence was found to be present in uninfected chicken cells and in uninfected cells from a variety of other vertebrates (Spector et al. 1978).

B Classification of oncogenes and their products

Most known proto-oncogenes encode proteins that appear to come from one of four types of proteins involved in the control of normal cellular growth. These four important types of proteins are: 1) growth factors e.g. sis oncogene which encodes a form of platelet derived growth factor (PDGF), 2) growth factor receptors e.g. neu or erbB-2 oncogene which encodes a protein related to epidermal growth factor receptor, 3) intracellular signal transducers (second messengers) e.g. the Ras proteins (Ha-ras, Ki-ras and N-ras which encode guanine nucleotide-binding proteins with GTPase activity), and 4) nuclear transcription factors e.g., the fos and jun oncogenes which encode Transcription factor API and the myc oncogene which encodes a protein possibly involved in regulating transcription (Darnell et al. 1990).

In the normal situation, the chain of events which leads to growth is started by ligand-receptor interactions, either at the cell surface or within the cytoplasm or nucleus. The cell interprets this signal and then often sends-intracellular transducers that alter transcription (although not all second messengers are targeted at transcription), either by causing new genes to be expressed or by modifying levels of expression of already active genes. Transcription is controlled through two types of DNA sequence: promoters, which are found close to the start site of transcription, and enhancers, which are located farther from the start site. Both elements function through specific binding proteins (nuclear transcription factors) that recognise short sequences (motifs) within the promoters and enhancers. These bound factors then accelerate or retard the rate of initiation of transcription by RNA polymerase II (Darnell et al. 1990).

In terms of oncogene action, the transcriptional response to a growth signal is of paramount importance because many oncogene proteins are transcriptional modifiers. The transcriptional
response alters the protein composition of the cell, providing the critical proteins needed for cell growth.

C Oncogenes and carcinogenesis

Relatively few human cancers are known to have a viral component in their aetiology. Once it was realised that oncogenes from DNA viruses ('early phase genes') or from RNA transducing viruses (incorporated cellular proto-oncogenes) were not heavily involved in human cancer, attention was focused on the non-viral, non-transmissible cellular oncogenes (Darnell et al. 1990). This was regarded as pertinent in view of the ability of transducing retroviruses to induce tumours by converting cellular proto-oncogenes into oncogenes. As these conversions involved relatively simple alterations such as deletions or losses of parts of the normal cellular gene, or single point mutations or other small changes, it seemed possible that agents such as chemical carcinogens could also activate proto-oncogenes into oncogenes (Darnell et al. 1990). Since the mid-1980's, activated oncogenes have indeed been discovered in many tumours of laboratory animals, both spontaneous and chemically-induced, as well as in many different forms of human cancer, including urinary bladder cancer (Reznikoff et al. 1993).

Point mutation is one of at least five distinct molecular mechanisms which have been demonstrated to convert proto-oncogenes to active oncogenes after exposure to chemicals. Another process by which cellular proto-oncogenes can become activated to produce oncogenes is through chromosomal translocations, whereby an oncogene is translocated to the proximity of a very active cellular gene (Klein 1981). Many tumour cells contain chromosomal translocations. Generally, these unusual features are not reproducible from tumour to tumour. However, there are some notable exceptions, such as the Philadelphia chromosome which is found in virtually all patients with chronic myelogenous leukaemia (Shtivelman et al. 1985) and Burkitt's lymphoma, which is nearly always associated with translocations between chromosome 8 and chromosomes 14 or 20 or 22 (Lenoir et al. 1982). In both these diseases, it is now known that oncogenes are present at the breakpoints within one of the participating chromosomes - the c-abl proto-oncogene in the case of the Philadelphia chromosome and c-myc in Burkitt's lymphoma. Furthermore, these translocations activate the associated proto-oncogenes to become oncogenic.

Among the most common oncogenes found in human tumours are the members of the c-ras family of intracellular transducers (Darnell et al. 1990). Experimentally, more than one activated oncogene is frequently required before cells, in vivo or in vitro, show all the signs of malignant transformation. Thus, when primary rat embryo fibroblasts are transfected with a ras oncogene,
they show the morphological changes associated with transformation but they continue to exhibit a
limited life span in culture (i.e. they are not immortalised, and therefore lack a key property of
malignant cells in vitro). Transfection with ras plus myc, however, leads to fully transformed,
immortal, tumourigenic cell lines. As ras proteins are cytoplasmic and myc protein is nuclear,
transformation would appear to be a two-event process, a cytoplasmic alteration of cell behaviour
plus a possible change in gene transcription (Darnell et al. 1990). These in vitro findings and also
the presence of more than one activated oncogene in many human tumours suggests that multiple
genetic changes contribute to the malignancy of a given cell. This, in turn, adds support to the
multistage model of carcinogenesis.

D Oncogenes and urinary bladder cancer
The molecular events underlying neoplastic development in the urinary bladder are thought to
represent multiple genetic alterations, contributing to the malignant transformation of normal
bladder epithelium (Sandberg and Berger 1994). Such events implicate the activation and
inactivation of oncogenes and tumour suppressor genes, respectively.

Some of the earliest research into the relevance of oncogenes in carcinogenesis was performed
using samples from bladder cancers, particularly cell lines (Sandberg and Berger 1994), the ras
oncogene being the first oncogene to be investigated (Nagata et al. 1990). Indeed, ras is the only
oncogene which has been seen to be altered with any frequency. Transforming ability is conferred
upon ras through point mutations involving either the 12th, 13th or 61st codons (Perucca et al.
1990). The frequency of H-ras mutations detected in bladder cancer appears to depend upon the
type of assay utilised. For example, transfection assays have produced frequencies ranging from
5-10% (Fujita et al. 1984; Fujita et al. 1985), whereas frequencies of 12-16% have been detected
using the polymerase chain reaction (PCR) and dot blot hybridisation (Visvanathan et al. 1988;
Nagata et al. 1990). Czemiak et al. (1992), who also used PCR and dot blot hybridisation
detected mutations in 45% of tumours, a frequency far higher than those achieved by any other
workers. In a study of more than 150 bladder cancers, using PCR-single-strand conformation
polymorphism (SSCP) with confirmation by direct sequencing, no correlation was found between
tumour stage and/or grade and the presence of Ha-ras mutations (Knowles and Williamson 1993).
This finding led the authors to conclude that although Ha-ras mutations may contribute to bladder
tumourigenesis, they did not have a role in the development of the majority of bladder cancers,
either as an early event or as an essential molecular event. Mutations of the other ras genes in
bladder cancer are rare events. Nagata et al. (1990) detected a mutation in codon 61 of the Ki-ras
oncogene in 1 of 26 bladder tumours; no mutations have been detected in N-ras. These findings
are strikingly different from the frequency of ras mutations found in other human tumours, such as carcinomas of the lung or colon, in which Ki-ras mutations predominate (Bos 1989) or haematopoietic tumours, in which N-ras mutations predominate (Bos 1989); Ha-ras mutations are rare in these malignancies.

Knowles et al. (1987) investigated the role of Ha-ras activation in the transformation by MNU of rat urothelial cells. Using a transfection assay these authors concluded that ras oncogenes were not involved in MNU-induced transformation of rat bladder epithelial cells in vitro. Similar results were reported by Yura et al. (1991) who found Ha-ras mutations by transfection assay in only 1 of 18 MNU-induced rat bladder carcinomas. However, when the same tumours were investigated using an immunoblot technique, Ha-ras mutations were found in 7 of 18 tumours (39%). In this study, the presence of Ha-ras mutations did not correlate with the aggressiveness of the tumours, leading the authors to conclude that ras mutations may be involved in development but not progression of rat bladder carcinomas.

Reznikoff and co-workers (1993) have demonstrated that transfection of a mutant ras oncogene (isolated from a human bladder carcinoma - EJ human bladder cancer cell line - with point mutations at the 12th codon and the last intron) failed to immortalise human bladder epithelial cells, and also only rarely transformed human bladder epithelial cells which had been immortalised by SV40 (Christian et al. 1990; Pratt et al. 1992). However, expression of EJ/ras was associated with tumour progression when transfected into human bladder epithelial cells immortalised by SV40 and at various stages of transformation induced by in vitro exposure to arylamines (Pratt et al. 1992). Based on these results, this group have suggested a working model of human bladder cancer in which mutant ras oncogene, in combination with another rare event such as HPV infection, contributes to neoplastic progression (Reznikoff et al. 1993).

E Tumour Suppressor Genes
Interest in the role of oncogenes in carcinogenesis grew in the mid to late 1980's (Bishop 1987), but began to recede by the end of that decade (Paul 1989). More recently, interest has begun to focus on another group of important growth regulating genes that have been found to be associated with the progressive transformation of normal cells into fully malignant cancer cells. These are the 'anti-oncogenes' or tumour suppressor genes (Paul 1989). As their name suggests, these genes are thought to be involved in controlling the activity of cancer-causing oncogenes (Ruddon 1987). Interest in these genes was in part triggered by the observations of Knudson (1971) relating to the patterns of inheritance associated with the retinoblastoma gene (Rb). Knudson suggested that a
single inherited mutation is not sufficient to induce a retinoblastoma and proposed that a second mutation is required, the so-called 'two hit' hypothesis. In heritable cases, one event is germinal the other somatic (acquired); in non-heritable cases both events are somatic. Somatic loss of the alleles in a cancer is considered to be a hallmark of tumour suppressor gene inactivation (Wolff and Habib 1993).

A number of experimental approaches have indicated that cancer is a genetically recessive disease (Ruddon 1987). For example, when tumour cells in tissue culture are fused to normal cells the resulting hybrids are usually non-tumourigenic (Harris et al. 1969; Sager 1986). The proposed explanation for this is that genes from the normal cells complement deletions or mutations in tumour cells (Paul 1989).

Other evidence of the recessive nature of cancer and of the suggested role of tumour suppressor genes in carcinogenesis comes from the study of at least 50 familial cancers. In some of these tumours notably retinoblastoma, Wilms' tumour and familial adenomatous polyposis coli, it has emerged that homozygous deletions or mutations at specific genetic loci are frequent and possibly invariable features of the tumour cells. These mutations are commonly nonsense mutations that cause instability or truncation of the protein product (Harris 1993). Retinoblastoma, for example, has been shown to occur in individuals who inherit one normal and one defective allele of a gene (the Rb gene), i.e. they are heterozygous for the defect. It has been suggested that the normal allele acts as a tumour suppressor gene and that its loss precipitates tumour development (Knudson 1971; Benedict et al. 1983; Cavanee et al. 1985; Knudson 1985). These findings from human hereditary cancers provide strongly suggestive evidence for the existence of tumour suppressor genes. Moreover, evidence is accumulating that the loss of both alleles of a specific locus, or multiple loci, is a regular feature even in non-hereditary cancers such as those of the lung and breast (Paul 1989).

Cytogenetic studies have shown that gene loss or inactivation are involved in the development of cancer (Wolff and Habib 1993). Non-random chromosome deletions are known to be associated with specific tumour types, including urinary bladder tumours (Kroft and Oyasu 1994). Changes in specific genetic loci on homologous chromosomes, such as the Rb loci, lead to loss of heterozygosity within tumours. The association of loss of heterozygosity with the progression of certain types of cancer strongly implicates gene loss in the process of oncogenesis (Wolff and Habib 1993). Furthermore, the finding that the most common chromosomal defects seen in human solid tumours are deletions strengthens the idea that loss of genetic material may be as important
as (or more important than) activation of genes in causing progressive cancer (Ruddon 1987). These findings have led some workers to propose mechanistic models for the development of specific cancers involving the stepwise activation of oncogenes acting in concert with the loss of tumour suppressor genes; such models have been proposed for colorectal cancer (Fearon and Vogelstein 1990) and urinary bladder cancer (Reznikoff et al. 1993; Cordon-Cardo et al. 1994; Kroft and Oyasu 1994; Spruck et al. 1994).

In Drosophila melanogaster, many tumours have been identified which are clearly due to homozygous recessive mutations (Paul 1989). Some of the genes responsible have been cloned and reversion to normality has been observed by introducing a cloned normal gene into affected zygotes (Opper et al. 1987). In this instance, therefore, the evidence is very strong that the normal gene suppresses tumour formation.

Yet further evidence for the existence of anti-oncogenes comes from tissue culture observations of tumourigenic cell lines produced by transfecting cells with oncogenes such as ras or src (Paul 1989). There is good evidence that the transformed phenotype of the transfected cells is maintained by the presence of the oncogene protein product. However, non-tumourigenic 'revertants' can arise from these cells (Noda et al. 1983). In some, the oncogene is lost or inactive, but in others the oncogene product continues to be produced. It is therefore suggested that in these cases other genes act to suppress the effect of the oncogene. Thus, there is much evidence supporting the concept of anti-oncogenes with the ability to suppress the malignant phenotype.

Some tumour suppressor genes appear to be associated with tissue-specific differentiation mechanisms (Paul 1989). This contrasts with most oncogenes which appear to be associated with pathways which, after stimulation by growth factors, lead to DNA synthesis and cell division. Much of the strongest evidence for this comes from work with Drosophila in which tumour genes under study produce blocks to normal differentiation and each gene causes a characteristic lesion affecting a specific cell type (Gateff 1982). Many of the genes identified with human familial cancers display similar features - inheritance of the Rb gene results mainly in retinoblastomas and of the Wilms' gene (WT-1) in renal tumours. However, the correlation is not as absolute as in Drosophila. A variety of other tumours in addition to retinoblastomas can subsequently occur in children with the Rb gene (Knudson 1985). These include osteosarcomas, melanomas and epithelial tumours such as bladder tumours and ductal breast cancers. Nevertheless, as the tumour cells show characteristics of cells which have not proceeded to terminal differentiation, suggesting that the normal alleles of the Rb gene are concerned with differentiation.
The human \( Rb \) gene behaves as an autosomal dominant; that is, individuals who inherit a single mutant gene almost invariably develop the disease (Paul 1989). However, in order for a retinoblast to become tumourigenic it must, in many cases, first become homozygous for the \( Rb \) gene (Knudson 1971). Homozygosity can occur through the loss of a normal chromosome 13, on which the \( Rb \) gene resides (Paul 1989). Hence, while the gene behaves as an autosomal dominant in familial inheritance, it behaves in a recessive manner in individual tumour cells. The \( WT-I \) gene appears to behave similarly. Thus, tumourigenesis associated with these genes is provoked by complete absence of a normal gene (Benedict et al. 1983; Cavenee et al. 1985; Knudson 1985). It has become customary to refer to the deleted or mutated gene as a tumour gene and the normal allele as a tumour suppressor gene. The deleted or mutated tumour genes behave in a recessive manner in somatic cells whereas the normal alleles, the tumour suppressor genes, behave in a dominant manner (Paul 1989).

Besides putative 'differentiation genes', a distinct class of tumour suppressor genes exists. Evidence for these genes was first obtained from cultured fibroblasts transfected with oncogenes such as members of the \( ras \) family (Paul 1989). In some instances, normal revertants of transformed cells have been isolated which retain and express the activating oncogene (Noda et al. 1983). This other class of tumour suppressor genes includes the most important to date, \( p53 \).

\( p53 \)

In recent years, the most studied tumour suppressor gene has been a gene known as \( p53 \). This tumour suppressor gene, named after the 53- kilodalton nuclear protein it encodes, has become so important in cancer research that it was named molecule of the year in 1993 (Culotta and Koshland 1993).

The \( p53 \) gene is located on the short arm of chromosome 17 (17p). The gene is expressed in most cells of the body; the encoded protein is unstable and has a half-life of only 20 to 30 minutes (Wolff and Habib 1993). During the G1 phase of the cell cycle, p53 protein accumulates in the cytoplasm and enters the nucleus at the start of the S phase. The protein remains in the nucleus for about 3 hours and then accumulates again in the cytoplasm (Shaulsky et al. 1990).

The \( p53 \) gene is the most frequently altered gene in human cancers (Vogelstein 1990). Of the 6.5 million people diagnosed with cancer each year worldwide, about half have \( p53 \) mutations in their tumours (Culotta and Koshland 1993). Furthermore, similar mutations have been found in
experimental tumours as well. Found in both inherited and spontaneous cancers, p53 is likely to play an important role in human carcinogenesis (Nigro et al. 1989, Levine et al. 1991).

Wild-type p53 protein is thought to be a transcription factor which suppresses cell proliferation (Harris and Hollstein 1992). As such, inactivation of p53 protein would allow neoplastic growth to proceed unchecked. Direct evidence for the regulatory function of p53 was obtained by Baker et al. (1990) who demonstrated that wild-type p53 could suppress the growth of colorectal carcinoma cell lines containing mutated p53. Further evidence indicating suppressor function comes from the finding that there is often deletion of the other 17p allele in cases with p53 mutations in various tumours (Sidransky et al. 1991; Kikuchi-Yanoshita et al. 1992; Oda et al. 1992; Dalbagni et al. 1993b), satisfying Knudson's 'two-hit' hypothesis of suppressor gene inactivation (Knudson 1971). However, it is also apparent that p53 may also in some cases function as a dominant oncogene, with mutation in one allele providing a selective advantage (Jones et al. 1991); the mutant p53 protein may either complex with and inactivate the wild-type p53 (Wright et al. 1991), or some mutants may actually gain oncogenic function (Kroft and Oyasu 1994).

The p53 gene was originally thought to be an oncogene as transfected p53 increased the tumourigenicity of established cell lines and converted non-transformed lines into a more malignant phenotype (Wolff et al. 1984). Subsequently, these studies were shown to have been done with a mutant form of the wild type p53 gene. More recent studies have shown that wild type p53 can prevent malignant transformation (Finlay et al. 1989). Unlike many tumour suppressor genes, however, mutated p53 seems not only to have lost its normal function, but also to have gained the ability to act as an inhibitor of any wild type p53 in the cell (Gannon et al. 1990). Mutant p53 forms an oligomeric complex with the wild type protein and keeps it in the cytoplasm of the cell where it is unable to block cell replication. The mutant form has a greatly extended half-life, resulting in the build up of up to one hundred-fold elevated levels in transformed cells and tumours (Levine 1990).

The prevention of DNA synthesis following DNA damage is crucial for avoiding genetic lesions that could contribute to cellular transformation. In this respect wild type p53 acts as a "molecular policeman", monitoring the integrity of the genome (Lane 1992). If DNA damage is present, accumulation of wild type p53 mediates the arrest of the cell cycle at G1 and switches off replication to allow extra time for DNA repair. If repair fails, wild type p53 may trigger apoptosis (Kastan et al. 1991; Wolff and Habib 1993). In contrast, cells with a mutant p53 can not arrest
the cell cycle and are, therefore, more susceptible to the mutagenic effects of genotoxic chemicals, as they replicate through the damage.

More than 90% of \( p53 \) mutations are missense mutations which change the identity of an amino acid. Such mutations can alter the conformational shape and increase the stability of the protein product (Harris 1993). Furthermore, they can also indirectly alter the sequence specific DNA-binding and transcription factor activity of \( p53 \) (Harris 1993). Since 1989, mutations in \( p53 \) have been reported in over 51 human cancers, including tumours of the urinary bladder, breast, cervix, colon, liver, lung, prostate, skin, stomach and thyroid (Culotta and Koshland 1993). Among common tumours, about 70% of colorectal cancers, 50% of lung cancers and 40% of breast cancers carry \( p53 \) mutations. Many clinical studies have indicated that \( p53 \) status is a good indicator of prognosis. Aberrant forms of \( p53 \) protein are correlated with more aggressive tumours, metastasis and lower 5-year survival rates. Such findings have been reported for cancer of the bladder as well as cancers of the colon, lung, cervix, prostate breast and skin (Culotta and Koshland 1993).

**F Tumour suppressor genes and urinary bladder cancer**

\( p53 \) mutations are the only molecular alterations found in a large proportion of invasive bladder tumours (Kroft and Oyasu 1994), having been detected in more than 50% of these tumours (Sidransky et al. 1991; Spruck et al. 1994). It is clear that they occur at a much higher frequency in invasive, high grade tumours than in superficial, low grade lesions (Fujimoto et al. 1992), implying that these mutations may be important in the conversion from superficial to invasive bladder cancer (Kroft and Oyasu 1994). Consistent with the hypothesis that \( p53 \) functions as a tumour suppressor is the observation that almost all bladder tumours which contain \( p53 \) mutations or overexpress \( p53 \) protein also show loss of heterozygosity on the short arm of chromosome 17, indicating that only mutant protein is present in these cells (Sidransky et al. 1991; Dalbagni et al. 1993b). Interestingly, Dalbagni et al. (1993b) found overexpression of \( p53 \) in both low and high stage tumours but found loss of heterozygosity on the short arm of chromosome 17 only in invasive tumours, underlining the recessive nature of the defect.

Loss of heterozygosity of this chromosome is the most frequent abnormality (up to 67% of cases) seen in studies of allelic loss in bladder cancer (Olumi et al. 1990; Tsai et al. 1990; Dalbagni et al. 1993a; Spruck et al. 1994; Knowles et al. 1994). Spruck et al. (1994) analysed a number of bladder tumours of various stages and grades for the presence of \( p53 \) mutations and loss of heterozygosity of chromosome 9. Loss of heterozygosity of chromosome 9 was the predominant
alteration in non-invasive papillary tumours (Ta) and was present in only a few cases of carcinoma in situ (TIS). In contrast, the reverse was true for p53 mutations, the frequency of which in TIS (65%) was comparable to that observed in muscle-invasive carcinomas (51%). Furthermore, analysis of several cases of TIS with mutated p53 indicated that progression to invasive carcinoma was associated with loss of chromosome 9 heterozygosity. From these observations, they suggested that two distinct pathways of genetic alteration exist. Loss of heterozygosity of chromosome 9 is the event involved in the development of superficial TCC, whereas p53 mutation is the principal genetic alteration in carcinoma in situ, and the later is the principal source of invasive carcinoma in which chromosome 9 defect is likely to be involved also.

Inactivation of the Rb gene has been reported in 28 to 38% of bladder carcinoma cell lines (Ishikawa et al. 1991), while the frequency in primary tumours has varied widely (Kroft and Oyasu 1994). Loss of heterozygosity of the Rb gene on the long arm of chromosome 13 has been observed in about 25% of bladder cancers (Ishikawa et al. 1991; Dalbagni et al. 1993a; Dalbagni et al. 1993b), but it is not associated with Rb gene inactivation, suggesting the possibility of a second tumour suppressor gene on this chromosome (Ishikawa et al. 1991).

Chromosomal abnormalities of the short arms of chromosomes 5 and 11 and the long arm of chromosome 18 are also found in bladder cancer (Kroft and Oyasu 1994). The adenomatous-polyposis-colon (APC) tumour suppressor gene, implicated in polyposis coli, resides on chromosome 5. Allelic loss of chromosome 11 is also found in Wilms' tumours and a candidate tumour suppressor gene, Wilms' Tumour 1 (WT1) has been located in the short arm of this chromosome (Kroft and Oyasu 1994). Furthermore, this is also the location of two putative anti-oncogene loci (BWS and WAGR) (Hopman et al. 1991) as well as the Ha-ras oncogene locus (Gibas 1986). Allelic loss in the long arm of chromosome 18 has been implicated in colorectal carcinoma; a tumour suppressor gene, DCC (the deleted-in colorectal-cancer gene) resides on the long arm of this chromosome (Fearon and Vogelstein 1990).

7 CHEMOPREVENTION OF HUMAN CANCER
There are currently three major approaches to cancer prevention as distinct from cancer treatment modalities: primary prevention, secondary prevention and chemoprevention (Szarka et al. 1994). Primary prevention involves identification and elimination of cancer causing agents, while secondary prevention relies on the screening of individuals at high risk of malignant disease with the hope that early detection and treatment will beneficially affect survival. Chemoprevention of cancer, a phrase first coined by Sporn et al. (1976), involves reducing cancer risk in susceptible
individuals by the administration of a natural or synthetic compound to reverse or suppress the
process of carcinogenesis before the development of invasive cancer (Lippman et al. 1993). This
third and relatively new branch of cancer prevention has its foundations in studies involving
laboratory animals and also in human epidemiology (Bertram et al. 1987; Szarka et al. 1994).
The first potential chemopreventive agents were suggested by epidemiological studies in which
substances naturally occurring in the diet, such as vitamin A, vitamin E and selenium, were shown
to be associated with decreased cancer incidence (Greenwald et al. 1990).

In a previous section of this thesis, carcinogenesis has been described as a multistage process
involving several distinct phases from the first exposure to a carcinogenic stimulus to the
development of invasive metastatic cancer (refer to Figure 1.1. for a diagrammatic summary of
this process). Furthermore, in humans, the latent period for the development of invasive cancer
may be 20 years or more (Sporn et al. 1976). It follows, therefore, that there are several stages
during the process of carcinogenesis, occurring over a long period of time, at which it might be
possible to intervene chemically in order to block or reverse further neoplastic development. This
is the aim of chemoprevention.

There is some evidence to believe that chemopreventive mechanisms operate naturally in man
during preneoplastic conditions (Auerbach et al. 1962), but that they are relatively ineffective if
exposure to carcinogenic stimuli is excessive. Sporn and associates (1976) foresaw that a
nutritional, physiological or pharmacological enhancement of these natural protective mechanisms
was required in order to prevent invasive cancer.

A Types of chemopreventative agent

More than 1000 compounds have been reported to demonstrate some cancer inhibitory properties.
However, the number of compounds which have been fully evaluated for efficacy and toxicity
before advancing to clinical trials in humans is much smaller (Szarka et al. 1994). Certain agents,
because they were considered safe and because significant published data suggested a role in
chemoprevention, were not screened for efficacy or toxicity and have progressed directly into
clinical trials under the auspices of the National Cancer Institute (NCI), USA Among these
agents were BC and vitamin A (Boone et al. 1990).

Boone et al. (1990) classified candidate chemopreventative agents into four mechanistic
categories: 1) agents that block or suppress initiating DNA damage (mutation), 2) agents that
block promotion or cell proliferation, 3) agents that block mutation and promotion and 4) agents of
undetermined mechanism. The bulk of potential chemopreventative agents are those which affect promotion or cell proliferation (second category), with some of these, such as BC and non-steroidal-anti-inflammatory drugs (NSAID) also showing potential to block mutation (third category).

Some mutation blocking agents act by preventing the formation of a carcinogen from a pro-carcinogen, for example the inhibition by vitamin C and vitamin E of nitrosamine formation in the stomach (Bertram et al. 1987). Most of this class of agents, however, act via their effects on phase I and/or phase II detoxification enzymes. Thus, they exert their protective effect by either removing the carcinogen before it reacts with its target (DNA or key cellular receptors) or by altering the form of the carcinogen such that it can not interact with its target (Szarka et al. 1994). Metabolic activation of a pro-carcinogen to yield the ultimate carcinogen and subsequent reaction with cellular macromolecules (DNA bases) occurs within a few hours of exposure. Many tissues have the ability to repair this DNA damage. If replication occurs before such repair has taken place, however, the damage is converted (fixed) into a stable genetic lesion. Thus, chemical intervention to prevent the initiation phase of carcinogenesis must occur prior to, or immediately after, exposure (Bertram et al. 1987).

Agents that suppress mutation, such as BC, vitamin C, vitamin E, selenium, oltipraz, ellagic acid and phenolic compounds, exert their effect at a later stage in carcinogenesis (Szarka et al. 1994). They function after the carcinogen has reacted with the target site by a mechanism which, in many instances, is not well understood (Wattenberg 1985).

A diverse group of chemopreventative agents function by inhibiting tumour promotion and proliferation. The conversion of an initiated cell to a pre-neoplastic or fully malignant cell, either spontaneously or induced by promoting agents, is a prolonged process lasting decades in humans. Inhibition of this phase of carcinogenesis at any time prior to the onset of frank malignancy can be expected to delay the onset of malignant disease. According to Szarka et al. (1994), chemopreventative agents that act at this stage of carcinogenesis include BC, vitamin A and the synthetic retinoids, steroidal compounds such as tamoxifen and finasteride, and non-steroidal anti-inflammatory drugs such as indomethacin, piroxicam, ibuprofen and aspirin. Some of these agents may function by preventing attack by singlet oxygen radicals (Wattenberg 1985), while others, such as the retinoids, may exert their effect on epithelial tissue by inducing differentiation and maturation thus leading to suppression of proliferation (Boone et al. 1990).
Chemopreventative agents differ from cytotoxic chemotherapeutic compounds in that the latter are used in the treatment of invasive cancers (Sporn et al. 1976). In chemotherapy, cytotoxic agents are used in an attempt to kill invasive tumour cells, by blocking key metabolic pathways. Being cytotoxic, these agents also kill normal cells. In contrast, chemoprevention is undertaken during the period of preneoplasia (before the development of invasive cancer), in an attempt to delay or reverse the process of carcinogenesis (Sporn et al. 1976). Furthermore, chemopreventative agents are not intended to be cytotoxic, but rather are designed to prevent initiating DNA damage, or arrest or delay the promotion of such damage into frank malignant disease.

B Clinical intervention trials of potential chemopreventative agents

In an extensive programme set up to identify and evaluate potential chemopreventative agents, the NCI uses a battery of in vivo and in vitro assays to assess toxicity and efficacy before the agents progress to clinical trials in humans (Boone et al. 1990; Kelloff et al. 1994). Intervention trials in humans, according to Szarka et al. (1994), particularly phase III trials, are time-consuming (lasting 2-10 years) and very costly to administer (they require sample populations of 2000-16000 individuals, depending on the agent and the end-point). However, even if laboratory studies are highly suggestive of a beneficial effect, intervention trials in humans with specific pathologies are essential to provide definitive evidence of efficacy against human cancer (Bertram et al. 1987).

Recently, Kelloff et al. (1994) reviewed progress with 22 agents and 3 combinations which have reached an advanced stage of development as chemopreventative agents in the NCI’s chemoprevention programme. Those agents which had progressed the furthest, including BC, vitamin A, several synthetic retinoids, tamoxifen and finasteride, were at this time in phase II and phase III clinical trials. Similarly, Szarka et al. (1994) reported a total of 17 on-going studies involving beta-carotene. These investigations involved, a) populations at risk of recurrences of cancers of the head and neck, skin or colon, b) populations with previous pre-malignant lesions in the oral cavity, colon or uterine cervix and, c) populations at high risk of developing a first lung cancer, such as heavy smokers or individuals with a history of asbestos exposure. However, only one of these studies was designed to examine the effect of BC against cancer at all sites, including the urinary bladder. This is the double blind randomised study of BC and aspirin in 22000 US physicians (Hennekens and Eberlein 1985).

Among other on-going clinical trials reported by Szarka et al. (1994) were eleven trials involving pre-formed vitamin A (retinol) including one study in a population that had had a previous skin cancer, one trial in individuals with pre-malignant oral lesions and eight trials in people at high...
risk of developing a first lung cancer (cigarette smokers or those previously exposed to asbestos). Synthetic retinoids, because of relatively high toxicity, are restricted to clinical trials involving individuals with previous cancers or those at high risk of developing cancer (Kelloff et al. 1994). Thus, 4-hydroxyphenyl retinamide (4-HPR or fenretinide) was being evaluated in 4 trials: one in individuals with actinic keratoses, one in patients with cervical intraepithelial neoplasia, one in patients that have had breast cancer, and one in patients following resection of a superficial urinary bladder tumour. Other clinical trials involving synthetic retinoids included four trials with 13-cis-retinoic acid, in individuals with a previous head or neck cancer (one trial), in patients with a previous skin cancer (one trial), in patients with a previous small cell cancer of the lung (one trial), and in chronic smokers (one trial). A further three trials were underway with vitamin E, one trial each in individuals with pre-malignant oral or colonic lesions, and one trial in women smokers at risk from a first lung cancer. Finally, several trials were being undertaken in populations at risk of various cancers to evaluate the chemopreventative potential of other agents including wheat bran, calcium carbonate, piroxicam, aspirin, tamoxifen and finasteride.
CHAPTER 2
VITAMIN A and CANCER

1  HISTORICAL PERSPECTIVES
Vitamin A has been linked with neoplastic development for 70 years. Between 1922 and 1925, it was discovered that vitamin A-deficiency lead to squamous metaplastic changes in the epithelia of the respiratory, gastro-intestinal and urino-genital tracts (Mori 1922; Wolbach and Howe 1925). These seminal experiments demonstrated that vitamin A had potent effects in the control of epithelial differentiation and proliferation. In 1926, Fujimaki observed the formation of gastric carcinoma in vitamin A-deficient rats. Taken together, these reports suggested that vitamin A might have a protective role against neoplastic development (Hennekens et al. 1984). The significance of these observations with regard to cancer were neglected for almost 30 years until the work of Lasnitzki in the 1950s. Working with mouse prostate gland in organ culture, she reported pre-cancerous changes in the secretory epithelium after addition of a carcinogen, methylcholanthrene (MCA), to the medium (Lasnitzki 1951). Of greater significance, however, was her observation that these pre-cancerous changes were inhibited and reversed by addition of vitamin A (Lasnitzki 1955). In 1957, Moore, working in the same institution as Lasnitzki (Strangeways Research Laboratory, Cambridge), commented upon the possible significance of vitamin A in cancer development and prevention. Between 1959 and 1967, several groups of workers demonstrated a prophylactic effect of vitamin A in vivo in arresting the progression of pre-cancerous lesions such as metaplasias and benign epithelial tumours to carcinomas (reviewed by Bollag and Matter 1981). One of these groups (Saffioti et al. 1967) reported the preventative effect of vitamin A palmitate on the formation of pre-cancerous tracheobronchial squamous metaplasias, as well as tumours, induced by intratracheal instillations of (BP) and iron oxide in hamsters.

All the above experiments had been performed with natural forms of vitamin A such as retinol or retinyl esters. The ultimate aim of this work was to provide the experimental rationale upon which to base a strategy for the use of high dose vitamin A as a human cancer chemopreventative agent (Spom et al. 1976; Pitt 1985). However, such a strategy is difficult to put into practice, as giving large doses of vitamin A acts only to increase the liver reserves and has little effect on the plasma level and, therefore, the amount of the vitamin which reaches the tissues (Pitt 1985). Furthermore, high doses of vitamin A are toxic, resulting in pathological changes (hypervitaminosis A) in both man and experimental animals (Pitt 1985). Thus, a search for less toxic analogues was
undertaken (reviewed by Bollag and Matter 1981). The first compound which was thoroughly investigated was all-trans-retinoic acid (RA) (Bollag 1970, 1972, 1979), as it is not stored in the liver, and also it is metabolised rapidly, thus avoiding prolonged hypervitaminosis A (Pitt 1985). RA gave encouragingly positive results in preventative and therapeutic experiments against chemically-induced tumours in experimental animals and against preneoplastic and neoplastic conditions in humans (Bollag and Matter 1981). However, RA was found to induce hypervitaminosis A also, leading to the search for synthetic analogues of vitamin A possessing high activity and tumour specificity, but low toxicity (Bollag and Matter 1981). Subsequently, a range of new synthetic derivatives of vitamin A were developed and extensively evaluated in various laboratories for anti-carcinogenic activity against experimentally-induced cancers in various organ systems including the skin, lung, urinary bladder and mammary gland (Bollag 1974; Port et al. 1975; Sporn et al. 1976; Sporn and Newton 1979; Moon and McCormick 1982; Hicks et al. 1985). Sporn et al. (1976) first used the term retinoid, an analogy with the word carotenoid, to refer to the enlarged family of compounds which now includes all synthetic analogues of vitamin A as well as the natural forms of the vitamin. To date, more than 2,500 synthetic retinoids have been synthesised and tested for biological efficacy (Bollag and Holdener 1992). At the present time, some synthetic retinoids are undergoing clinical trials (Szarka et al. 1994), although, due to toxicity, such trials are usually restricted to patients with previous cancers or who are at high risk of developing cancer, such as heavy smokers (Kelloff et al. 1994).

At about the same time as the initial experimental evaluation of synthetic retinoids was underway (1975 to 1982), a series of early epidemiological studies reported inverse associations between human cancer risk and vitamin A. These investigations were undertaken both from a dietary consumption viewpoint (Bjelke 1975; Mettlin 1984) and also by measurement of serum vitamin A in stored blood samples drawn prior to the development of disease (Wald 1980; Kark et al. 1981). Although later studies have tended not to support the initial association of blood retinol with human cancer risk (Peleg et al. 1984; Willett et al. 1984; Wald et al. 1986), there is strong epidemiological evidence to suggest that dietary BC, the main provitamin A carotenoid (Stahelin et al. 1984; Nomura et al. 1985; Menkes et al. 1986; Malone 1991; Willett 1994; Michels and Willett 1994) as well as possibly other dietary carotenoids (Zeigler 1989; Willett 1994), are inversely associated with cancer risk.

Thus, almost 50 years after the findings of Wolbach and Howe, and Fujimaka, a new era of vitamin A research began, examining the link between the vitamin and cancer (Pawson 1981). This link is still very much alive today, as exemplified by the many clinical trials currently
underway examining the chemopreventative potential of natural vitamin A, synthetic retinoids and beta-carotene against several important human cancers (Szarka et al. 1994). The relatively recent report by Huang et al. (1988), of complete remission of a form of human acute promyelocytic leukaemia in 23 out of 24 patients treated with RA, has focused attention again on the chemopreventative potential of this compound as well as on the synthetic retinoids in general. This interest in RA has increased with the discovery of the nuclear retinoic acid receptors. In understanding the mechanisms by which this diverse family of receptors operate, it is hoped that the long hidden processes through which vitamin A controls cell differentiation and proliferation may soon be fully elucidated. Furthermore, such knowledge might also highlight new targets for improved chemopreventative drug design.

2 THE DISCOVERY OF VITAMIN A AND ITS NUTRITIONAL IMPORTANCE

In 1913, both McCollum and Davis, and Osborne and Mendel reported that a fat-soluble substance was responsible for the xerophthalmia observed in rats fed on lard as the sole source of dietary fat. This xerophthalmia could be reversed by a diet that contained butter, egg yolk and cod liver oil (Goss and McBumey 1992). Later, McCollum and Davis (1915) found that all essential dietary growth factors (vitamins) were divided into two classes according to solubility, called by McCollum and Kennedy (1916) fat soluble A and water-soluble B. In 1920, fat-soluble A was renamed vitamin A by Drummond. All animals, including man, require this nutrient in their diet (Moore 1957), as they can not synthesise it for themselves from smaller molecules.

Vitamin A exhibits a multiple and diverse range of essential physiological functions including vision, reproduction, control and maintenance of epithelial and mesenchymal differentiation and proliferation (thus affecting virtually every organ system in the body) and growth (Ganguly et al. 1980; Wolf 1984; Pitt 1985; Sklan 1987; Goss and McBumey 1992). In addition, vitamin A appears to perform important potentiating functions in the immune system (Sklan 1987), and, through its role in epithelial differentiation, may protect humans against the development of various epithelial cancers (Sporn et al. 1976; Hicks 1983; Hennekens 1984).

Lack of vitamin A leads to a deficiency syndrome in both man and animals (Wolbach and Howe 1925; Moore 1957, 1960). There are many manifestations of vitamin A deficiency, with considerable variations between species (Pitt 1985). In the main, however, the clinical signs of vitamin A deficiency in experimental animals and man, as described by Moore (1957) and Pitt (1985), are loss of appetite and cessation of growth, loss of low-intensity vision (night blindness in man) which eventually leads to complete loss of sight, keratinisation of epithelia (particularly in
the eye, where it leads to keratomalacia and xerophthalmia, and in the respiratory, gastro-intestinal and urino-genital tracts), greater susceptibility to infections, impaired bone remodelling with possible secondary effects upon the nervous system, reproductive sterility and ultimately death.

The pathological feature which is most characteristic of vitamin A deficiency is squamous metaplasia and keratinisation of various non-squamous epithelia. This involves the replacement of the normal tissue with a squamous epithelium which eventually produces keratin (i.e. failure of normal differentiation) (Pitt, 1985). The original epithelium atrophies after which there is regenerative proliferation of the basal cells, with growth and differentiation of the new cells into a stratified squamous epithelium comparable histologically with epidermis (Capurro et al. 1960; Hicks 1968, 1969). The epithelia involved are usually, but not always, mucous epithelia, such as those lining the respiratory and gastro-intestinal tracts (Pitt 1985); other affected epithelia are those lining the urino-genital tract including the urinary bladder (Wolbach and Howe 1925; Hicks 1975a), cornea and salivary glands (Mori 1922), and prostate gland (Wolbach and Howe 1925). This extensive pathological change, which is accompanied by a high degree of mitotic activity, is completely reversed when the supply of vitamin A is restored (Wolbach and Howe 1933).

In the UK, and in the western world in general, where most people have an adequate diet, the effect of acute vitamin A deficiency is seldom seen. In the developing world, however, severe vitamin A deficiency is a major problem, leading to loss of sight due to xerophthalmia and death from bacterial and parasitic infections in literally millions of people (Pitt 1985). Even in the UK, however, analyses of diets show a substantial percentage of the population with multiple, marginal vitamin deficiencies, including vitamin A, particularly in the older age groups (Hicks 1983). Although these mild deficiencies are not likely to produce overt symptoms of deficiency, early epidemiological studies (to be reviewed elsewhere) demonstrated a tendency for some human cancer risks to be inversely proportional to plasma vitamin A levels (Bjelke 1975; Wald et al. 1980; Kark et al. 1981; Peto et al. 1981).

3 AN OVERVIEW OF THE CHEMICAL NATURE, DIETARY SOURCES AND RELEVANT PHYSIOLOGY AND BIOCHEMISTRY OF VITAMIN A

The chemical nature, functional physiology and metabolic biochemistry of vitamin A have been reviewed by many workers including Moore (1957), Goodman (1979), Ganguly et al. (1980), De Luca (1983), Goodman (1984), Wolf (1984), Pitt (1985), Sklan (1987), and Goss and McBurney (1992). Of these, the reviews of Wolf (1984) and Pitt (1985) are probably the most detailed and useful, although that of Moore (1957), despite its age, remains the most comprehensive.
A Chemical nature of vitamin A

In contrast to most other vitamins, vitamin A exists in a multiplicity of chemical forms. The chemical structures of the some of the natural forms of vitamin A (natural retinoids), as well as the structures of a few synthetic retinoids are shown in Figure 2.1. Within animal tissues there are a number of biologically active forms of vitamin A, the most important of which are retinol (vitamin A alcohol), retinyl palmitate (vitamin A palmitate) (VAP), retinal (vitamin A aldehyde or retinaldehyde), and retinoic acid (vitamin A acid) (RA). In some freshwater fish and amphibians, 3,4-didehydroretinol, another form of vitamin A is found (Pitt 1985). Retinyl acetate (vitamin A acetate) (VAA) does not exist naturally. However, as a pure and stable synthetic derivative with high vitamin A activity, VAA has been used, and continues to be used, in vitamin supplements for humans and for agricultural animals, as well as a pure source of vitamin A for research purposes.

Retinol is the parent molecule from which retinal, RA, and VAP are metabolically derived. Retinal is the form found in the eye and is essential for the visual process. RA is a potent metabolic form of retinol capable of fulfilling all the physiological functions of vitamin A except vision and those concerned with reproduction (vitamin A is required for spermatogenesis in male animals and also to prevent foetal resorption in females). Indeed, apart from vision and reproduction which require the alcohol/aldehyde form, RA can often be regarded as the most important active form of vitamin A at the cellular level (Morriss-Kay 1992; Allenby 1995). VAP is the chief storage form of vitamin A within the mammalian body. As such, VAP is found mainly in the Ito cells (also known as fat-storing or stellate cells) of the liver. All the different forms of natural vitamin A vary slightly in their vitamin A activity.

Retinol, RA, retinal and VAP are readily inter-converted by animals. Retinal can be oxidised to RA, but this reaction is apparently irreversible (Lloyd et al. 1978). The inability of animals to convert the acid back to the alcohol or aldehyde explains why RA is effective against many of the symptoms of vitamin A deficiency, but is ineffective against night blindness and reproductive failure (Lloyd et al. 1978).

All the forms of vitamin A and the provitamin A carotenoids (plant pigments which can act as dietary precursors of vitamin A) exist in a variety of isomeric forms, although these isomers exhibit reduced vitamin A activity (Lloyd et al. 1978). The term vitamin A, as described in 1978 by the International Union of Nutritional Sciences, excludes the provitamin A carotenoids and includes only the derivatives of the parent molecule retinol which have the same B-ionone ring and which demonstrate the biological activity of retinol (Pitt 1985). In practice, the contribution of
provitamin A carotenoids is often included as dietary 'vitamin A', for example, in epidemiological studies of total dietary 'vitamin A intake' (Pitt 1985; Michels and Willett 1994).

B  **Vitamin A activity of biologically active forms**

Any molecule which demonstrates the ability to prevent vitamin A deficiency in experimental animals is said to exhibit vitamin A activity (Moore 1957). In practice, such *in vivo* bioassays measure the ability of the active compound to promote growth in vitamin A-deficient animals (Moore 1957), one of the first clinical signs of vitamin A deficiency in experimental animals being cessation of body weight gain.

Vitamin A activity was traditionally measured in international units (iu). When pure vitamin A became available, the international unit was defined as being equivalent to 0.344 µg of all-trans-retinyl acetate, 0.3 µg all-trans-retinol or 0.55 µg of retinyl palmitate (Lloyd *et al.* 1978; Pitt 1985). However, it is more convenient to express vitamin A activity in retinol equivalents (µgRE) (Pitt 1985). These units are used throughout this thesis and refer to the weight of any form of vitamin A (e.g. VAA or VAP) in terms of retinol. With regard to biological activity, 1 µg of retinol is equivalent to 1.1466 µg of VAA or 1.833 µg of VAP, respectively. Therefore, to express a given weight of VAA or VAP in µgRE, the given weight is divided by 1.1466 or 1.833, respectively. For example, 2000 µg of VAA or VAP would be expressed as 1744.28 µgRE, or 1091.1 µgRE, respectively.

Recently, in contrast to the time-consuming *in vivo* assays of vitamin A activity, synthetic derivatives of vitamin A have been assayed *in vitro* for vitamin A activity by their ability to maintain normal epithelial differentiation in cultured mouse epidermis or in organ cultures of hamster trachea (Clamon *et al.* 1974; Sporn *et al.* 1975). In the case of the hamster trachea system, which resembles that used by Lasnitzki (1951, 1955) in prostate organ culture experiments, the hamster tracheas are first cultured in the absence of vitamin A to induce squamous metaplasia of the epithelium. Addition of synthetic retinoids with vitamin A activity reverses these changes and replaces the squamous epithelium with columnar ciliated cells and mucus secreting cells (Sporn *et al.* 1976).

C  **Daily requirements for vitamin A**

In man the dietary requirement for vitamin A is small. It should be available in the diet in sufficient quantities to maintain normal health and provide adequate liver stores. Watson (1984)
stated that one large carrot provides about 200% of the recommended daily amount (RDA) of vitamin A. The RDA of vitamin A varies widely between different countries due to differences in convention. Thus, Pitt (1985) reported that RDAs ranged from 600 µgRE in Korea to 1500 µgRE in the former Soviet Union. Most RDAs, however, are from 750-1000 µgRE (International Union of Nutritional Sciences, 1982).

Normal healthy laboratory rats are maintained with adequate vitamin A reserves by feeding conventional maintenance diets. The amount of vitamin A reported to be present in maintenance diets varies greatly depending on the supplier. The maintenance diet used routinely in the experiments described in this thesis (Rat and Mouse No. 1) was calculated by the supplier (SDS Ltd, Witham, Essex, UK) to contain about 1800 µgRE/kg, mainly in the form of supplemental VAP. However, calculated dietary vitamin A values can be up to 50% inaccurate. Analyses of 11 previously used batches of this diet demonstrated a wide variability in the amount of vitamin A present, with values ranging from about 900 µgRE/kg to 1890 µgRE/kg and a mean value of 1216.7 µgRE/kg. Based on the mean vitamin A content, and assuming that an adult healthy rat consumes about 15 g of diet per day, this diet provided each animal with about 18.25 µgRE/day.

Without a supplementary source of vitamin A, rats fed a vitamin A-free diet will die about two weeks after developing clinical signs of deficiency (cessation of growth and loss of appetite, eye lesions etc.). In investigations into the effects of cigarette smoke in vitamin A-deficient male F344 rats, Shields and Jeffery (1987) fed a semi-synthetic vitamin A-free diet from weaning until cessation of growth. Once growth ceased, the animals were transferred to a diet containing enough supplemental vitamin A, as VAP, to provide each rat with between 1.2-1.8 µgRE/day, assuming a daily consumption of 10-15 g of diet. This level of vitamin A supplementation was reported to be just sufficient to maintain a growth rate of about 1 g/rat/day (Shields and Jeffery 1987). In contrast, assuming a similar daily consumption, the control rats in these experiments were fed from weaning with the same semi-synthetic diet, but supplemented with about 12-18 µgRE/day VAP.

**D Dietary sources of vitamin A**

The major dietary sources of natural vitamin A are retinyl esters (chiefly palmitate), from animal tissues and, from plants, the provitamin A carotenoid pigments. However, vitamin A, as such, is only found in animals. Pitt (1985) lists the best sources of vitamin A as liver and fish liver oils,
dairy products, kidney and eggs. Some foodstuffs, such as margarine, are supplemented with synthetic retinyl esters.

Provitamin A carotenoid pigments are widely found in plants. In particular, they are found in large amounts in orange, green and yellow vegetables, and in orange and yellow fruits (Pitt 1985). Of the large family of carotenoid pigments, about 50 can serve as dietary precursors of vitamin A, but to varying degrees (Wolf 1984). The chemical structures of three common provitamin A carotenoids (BC, a-carotene, and cryptoxanthin) and the structures of two carotenoids with no provitamin A activity (lutein and canthaxanthin) are shown in Figure 2.2. For all provitamin A carotenoids, it is a requirement for activity that after splitting the molecule through the central double bond, at least one intact molecule of retinol is obtained (Wolf 1984). The most common and effective provitamin A carotenoid is BC (Moore 1957). As carotenoids are usually found associated with chlorophyll (Wolf 1984), the best dietary sources of BC are leafy green vegetables (Goss and McBumey 1992). BC is the most effective provitamin A carotenoid because both halves of the molecule on each side of the central double bond correspond to retinol (Pitt 1985). None of the other provitamin A carotenoids has more than half the vitamin A activity of BC and all are less widespread in nature. Therefore, most of the vitamin A derived from carotenoids is obtained from BC (Pitt 1985). Plant carotenoids provide over 80% of the vitamin A in the human diet in developing countries, whereas about equal quantities come from animal and plant sources in developed countries (Pitt 1985).

BC is also synthesised commercially and, as the compound is bright red/orange in colour, it is added to many human foods and animal feeds as a colouring agent (Pitt 1985).

**E Conversion of provitamin A carotenoids to vitamin A**

The intestine is the major site of the conversion of provitamin A carotenoids to vitamin A, although a little conversion can take place in the liver (Pitt 1985). The efficiency of conversion is dependent on many factors including levels of dietary lipids and protein, the presence of bile salts and the existence of vitamin A deficiency (Pitt 1985). Furthermore, the quantity of provitamin A carotenoids ingested also affects the efficiency of conversion, with small dietary amounts being converted better than large amounts. This last factor is important and means, for example, that it is not possible to induce vitamin A toxicity by the administration of BC (Willett et al. 1983; Pitt 1985). The efficiency of conversion and absorption is also dependent on species differences (Moore 1957; Pitt 1985). For example, some species, such as the rat are very efficient converters of BC to vitamin A, whereas others such as man are less efficient converters (Moore 1957; Lloyd
Thus, by a somewhat simplistic convention, assuming all other variables are constant, rats can convert 0.6 μg of all-trans-BC to 0.3 μg of retinol, while the amounts of BC required to give 0.3 μg of retinol in humans and cattle are 1.8 μg and 3.0 μg, respectively (Lloyd et al. 1978). It should be noted that in terms of retinol equivalents, in man, 6 μg of BC, and 12 μg of other provitamin A carotenoids, are equivalent to 1 μg of retinol (Pitt 1985). Species, such as man and cattle, which are relatively poor converters of carotenoids to vitamin A, absorb unconverted carotenoid which can be subsequently deposited in various tissues in the body, particularly adipose tissue, colouring them yellow (Moore 1957). For this reason, such species are sometimes referred to as 'yellow-fat' species (Moore 1957). Colouration of the tissues is especially obvious in the adipose tissue of cattle which have grazed on pasture rich in BC. In man, a high consumption of carotenoids can result in carotenaemia and pigmentation of the skin (Simpson and Chichester 1981). In contrast, rats, mice, hamsters and other efficient converters of carotenoids to vitamin A are referred to as 'white-fat' species. This is because, under normal physiological conditions, they convert virtually all the carotenoid in their diet to vitamin A in their small intestine. Thus, almost no unconverted BC enters the blood and no BC is stored in the body fat (Moore 1957).

Digestion, absorption and storage of vitamin A and provitamin A carotenoids

In mammals, most dietary pre-formed vitamin A is in the form of retinyl esters, chiefly retinyl palmitate (Wolf 1984). Retinyl esters are hydrolysed to retinol, probably within the intestinal lumen, although some evidence has suggested that the mucosal brush border is the site of hydrolysis (Wolf 1984). The resultant retinol is actively absorbed by the enterocytes, a process which is dependent on the presence of intestinal bile salts, dietary protein, fats and vitamin E (Moore 1957).

In contrast, BC and other dietary provitamin A carotenoids pass from the gut lumen into the cells of the small intestine by passive diffusion, at a rate which is directly proportional to the intestinal luminal concentration (Hollander and Ruble (1978). The absorption of carotenoids by the intestine requires the presence of fats to form micelles. Micelle formation and the subsequent absorption of carotenoids is facilitated by the presence of lecithin and bile salts. The presence of vitamin E also aids in the absorption process, probably by acting as an antioxidant (Wolf 1984). Within the enterocytes, BC and other dietary provitamin A carotenoids are split to yield retinaldehyde (retinal) (Pitt 1985). Carotenoids enter the enterocytes of all species requiring vitamin A, but transfer of unchanged carotenoid out of the enterocyte into the body varies among species (Wolf 1984). This is because different species vary with regard to the efficiency of
conversion of carotenoids to vitamin A (Moore 1957). In species such as man which absorb appreciable amounts of unconverted carotenoids (Goodman et al. 1966), the unchanged pigments are taken up by chylomicrons and enter the bloodstream via the lymph. In this case, the blood level of carotenoids, such as BC, is directly proportional to the dietary intake (Peto et al. 1981). Ultimately, these carotenoids are laid down in body fat and in various organs, such as the liver, colouring them yellow/orange (Wolf 1984). In man, about 80% of absorbed carotenoid is found in adipose tissue while about 10% is associated with the liver (Olson 1984).

Whether obtained by hydrolysis of retinyl esters or by reduction of retinal from carotenoids, retinol is re-esterified within the intestinal mucosal cells (Wolf 1984), the main products being retinyl stearate and retinyl oleate (Pitt 1985). A little retinol may also be converted to RA which is transported directly to the liver via the portal vein (Goss and McBurney 1992). The retinyl esters formed in the mucosal cells are incorporated into lymph chylomicrons, a process which is enhanced in the presence of high concentrations of bile salts (Wolf 1984), and transported to the liver. On arrival within the hepatocytes, the absorbed retinyl esters are initially hydrolysed and then re-esterified (Goodman et al. 1965). Newly absorbed vitamin A in excess of immediate tissue requirements is stored mainly in the liver, esterified with long-chain fatty acids, chiefly palmitic acid (Goodman et al. 1965; Wolf 1984; Pitt 1985). This excess vitamin A is transferred from the hepatocytes and stored in high concentration in the stellate cells of the liver (Knook et al. 1982). The effective storage of vitamin A in the liver is dependent upon the presence of vitamin E as an antioxidant (Wolf 1984). Thus, Robison et al. (1979) found that hepatic vitamin A in rats fed a vitamin E-free diet was 50% that of animals given a vitamin E-adequate diet.

G Mobilisation of liver vitamin A stores and transport of vitamin A to target tissues

The retinyl esters in the liver make up about 90% of the total vitamin A within the body (Wolf 1984) and, as such, serve as the body's vitamin A reserve (Pitt 1985). Vitamin A is unique as a vitamin because it is so massively stored in the liver (Wolf 1984). This liver reserve is used to supply vitamin A, in the form of retinol, to the tissues via the blood. The amount of vitamin A in blood plasma is equivalent to about 1% of the total quantity of vitamin A in the body (Olson 1984). The amount of retinol released into the blood from the liver is under strict homeostatic control, to provide a fairly constant plasma level (Pitt 1985). Without such control, insufficient retinol might be released for the needs of target tissues, or conversely, excessive release would lead to toxicity (Wolf 1984). Plasma retinol, therefore, only falls when the liver reserve is very low (less than 10% of normal values, or about 20 μg/g of liver in man) (Underwood et al. 1979; Olson 1984; Wolf 1984). Similarly, plasma retinol only rises when hepatic reserves are extremely high.
(more than 300 µg/g of liver in man) (Olson 1984). Thus, it is only in conditions of marginal vitamin A deficiency, when liver vitamin A is virtually exhausted, or in situations of extremely high intake, that plasma retinol levels accurately reflect vitamin A status (Olson 1984). In this way, when liver vitamin A levels lie within normal physiological limits (i.e. 20-300 µg/g of liver in man), they have little influence on plasma retinol concentrations (Loerch et al. 1979; Underwood et al. 1979; Olson 1984).

In contrast to retinol, as there is no mechanism to control the level of plasma carotenoids, blood levels of these components in man are directly related to dietary intake (Peto et al. 1981; Willett et al. 1983; Olson 1984; Wolf 1984). In non-smoking humans, even a single measure of blood BC is a potentially good index of dietary intake (Michels and Willett 1994).

Prior to release into the blood, retinyl esters stored in the liver are hydrolysed to retinol by a cytosolic enzyme which forms part of a high molecular weight protein aggregate (Heller 1979). The retinol is then coupled to a specific binding protein, plasma retinol-binding protein (RBP), which is synthesised in the liver and acts as a carrier protein in the blood (Pitt 1985). This carrier protein protects the labile and water-immiscible retinol molecule from chemical or biological damage within the aqueous environment of the plasma (Glover 1973). Each molecule of RBP has a single binding site for one molecule of retinol. RBP interacts with another plasma protein, prealbumin, with which it usually circulates as a 1:1 molar complex.

RBP and retinol usually appear in the blood together in the form of the holoprotein (i.e. apoprotein plus retinol); unbound retinol is never found in the blood (Pitt 1985). Apo-RBP continues to be biosynthesised in the liver even in the absence of retinol. However, lack of retinol inhibits secretion of most of this apo-RBP, which subsequently builds up in the liver. Repletion of vitamin A-deficient rats with retinol causes a rapid release of holo-RBP, as the stockpiled apo-protein is loaded with retinol and secreted into the plasma (Peterson et al. 1973). As retinol is never released into the blood without RBP, reduced RBP biosynthesis in the liver will adversely affect the level of retinol in the plasma. Thus, if protein intake is too low, insufficient RBP is produced, resulting in falling plasma retinol values. This is true regardless of the size of the hepatic vitamin A reserve (Pitt 1985).

In well nourished individuals, only a relatively small proportion of the vitamin A stored in the liver is ever released to the tissues. The remainder is slowly catabolised, at a rate proportional to the hepatic concentration, by the liver and excreted via the bile as glucuronides (Pitt 1985).
Thus, mobilisation of vitamin A from the liver to the peripheral tissues involves tight homeostatic regulation of the rate at which holo-RBP is formed and secreted by the liver. The exact mechanisms involved are poorly understood, but some knowledge has been gained about the factors influencing it. Wolf (1984) and Pitt (1985) have reviewed and summarised this knowledge and the experimentation by which it was gleaned.

The specific delivery of retinol to the target tissues is another important function of plasma RBP (Pitt 1985), in addition to its protective role in the plasma. The target tissues possess receptors on their surfaces which recognise RBP (Rask and Peterson 1976). Retinol is taken up by the target cells from the holo-RBP; the apo-protein does not enter the cell but is released immediately from the receptor (Rask and Peterson 1976).

Once within the target cells, retinol combines with another binding protein, cellular retinol binding protein (CRBP) (Chytil and Ong 1979). Within cells, all-trans-retinol can be oxidised, via retinaldehyde, to retinoic acid (Pitt 1985). Many cells contain a binding protein, cellular retinoic acid binding protein (CRABP), for this highly reactive form of vitamin A (Ong and Chytil 1979). RA is metabolised firstly, by hydroxylation, to produce 4-hydroxy metabolites and then oxidised to form 4-oxo-metabolites (Goss and McBurney 1992). Following oxidation, the resultant metabolites are conjugated with glucuronic acid and subsequently secreted in the bile.

Interestingly, a small proportion of vitamin A is released into the blood from the liver as RA (Goss and McBurney 1992). Although RA can bind to apo-RBP as efficiently as retinol, the binding protein does not appear to facilitate in RA transport (Goss and McBurney 1992). Instead, like many fatty acids, RA is transported in the blood bound to albumin. Synthetic analogues of RA have also been shown to compete for the RA binding site on albumin, suggesting that these compounds are also transported in the blood bound to serum albumin (Goss and McBurney 1992).

H Toxicity of vitamin A (hypervitaminosis A) and BC
Animals are able to consume moderate excesses of dietary vitamin A safely, increasing their hepatic reserves in the process (Pitt 1985). However, very large single doses may be too much for the hepatic storage mechanisms and if this intake persists, the holding capacity and catabolic capability of the liver may be overwhelmed. Under these conditions, the normal strict homeostatic control of plasma retinol concentration is evaded, resulting in the release into the blood of large amounts of retinyl esters attached mainly to lipoproteins (Mallia et al. 1975; Smith and Goodman 1976), with the transport of excessive amounts of the vitamin to the tissues (Pitt 1985). This
condition, known as hypervitaminosis A, has toxic consequences which, in some cases, can be very severe (Moore 1957; Bauernfeind 1980).

Pitt (1985) lists the main signs of hypervitaminosis A in man as being observed in the skin (e.g. erythema, desquamation and hair loss) and mucous membranes (e.g. cheilitis, conjunctivitis), with liver dysfunction, muscle and joint pains, and headache. In experimental animals, a characteristic lesion is the thinning and fracture of the long bones (Harrison et al. 1977). Vitamin A is also a powerful teratogen (Kistler 1986; Turton et al. 1992; Wan 1993), inducing specific malformations such as craniofacial abnormalities in animals and humans. Indeed the administration of very high doses of vitamin A supplements to pregnant women, and perhaps even to women of child-bearing age, is to be strictly avoided.

In in vitro systems, hypervitaminosis A induces changes which parallel those observed in vitamin A deficiency, such as the differentiation of chick embryonic skin to form a mucus-secreting and ciliated epithelium rather than the normal squamous epithelium (Fell and Mellanby 1953).

As a direct result of the toxicity of pharmacological doses of natural vitamin A, many synthetic retinoids have been developed as potential cancer chemopreventative agents, in the hope of obtaining a less toxic molecule which retains the anti-cancer efficacy of the parent vitamin. However, toxicity remains a problem, despite the development of over 2500 different retinoids (Bollag and Holdener 1992). Nevertheless, Allenby (1995), commenting on a recent report by Fanjul et al. (1994), suggested that it may soon be possible to design synthetic retinoids which preferentially act through antagonism with the nuclear transcription factor AP-1 to induce repression of proliferation-related gene expression (the desired effect), without inducing gene expression leading to the characteristic toxic side-effects.

In sharp contrast to the retinoids (natural or synthetic), there is no evidence of overt toxicity from BC, either in animals or in man (Bagdon et al. 1960; Peto 1983; Heywood et al. 1985; Greenberg et al. 1985; Greenberg, 1993). There are occasional reports of individuals with carotenaemia (Simpson and Chichester 1983), who exhibit a characteristic yellow pigmentation of the skin due to storage of unconverted BC in the epidermal adipose tissue. Vakil et al. (1985) noted that carotenaemia is caused by excessive dietary intake of fruits and vegetables rich in BC, but that it is also associated with several diseases including, diabetes mellitus, hypothyroidism, hypopituitarism, liver disease, nephrotic syndrome, and anorexia nervosa. Furthermore, McLaren and Zekian (1971) have described an inborn error of metabolism with failure to convert BC to
vitamin A as a cause of carotenaemia. According to Sklan (1987), there have been several reports of carotenaemia with detrimental effects. In a single case report, Vakil et al. (1985) described symptoms of abdominal pain, nausea, headaches and amenorrhoea in a 32 year old nurse who had eaten a diet of raw carrots and tomatoes for two months in order to lose weight. As in this case reported by Vakil et al (1985), however, individuals with carotenaemia usually exhibit unusual dietary habits, for instance sufferers of anorexia nervosa (Crisp and Stonehill 1967; Robboy et al. 1974), or diabetics who inadvertently eat too many fruits and vegetables (Mathews-Roth et al. 1976). Such people often consume very large quantities of foodstuffs containing BC, such as carrots or spinach, without necessarily eating adequate amounts of carbohydrates, proteins or fats. It is highly likely that any detrimental effects observed in carotenemic subjects would be due to imbalances in other more important nutrients, or due to any concomitant disease, rather than high BC levels per se.

There are no reports of toxic effects induced by the administration of high levels of BC to people with normal dietary habits, such as patients receiving BC therapy for photosensitisation (Mathews-Roth et al. 1976), or individuals taking part in any of the clinical trials investigating the chemopreventative activity of BC (Malone 1991; Greenberg 1993). Furthermore, BC has not been reported to induce any evidence of toxicity in animals in chronic toxicity tests in rats, mice and dogs (Bagdon et al. 1960; Heywood et al. 1985), in embryotoxicity tests in rats and rabbits (Heywood et al. 1985), or in mutagenicity tests in bacteria and in mouse bone marrow cells (Heywood et al. 1985).

I Nuclear retinoic acid receptors and mechanisms of vitamin A action

In 1973 Bashar et al. proposed that the mechanism by which natural retinoids control cell growth and differentiation is mediated in a manner similar to that of the steroid hormones. For this premise to be true, natural retinoids must bind to a specific cytosolic receptor and the resultant complex must be translocated to the nucleus, with subsequent modification of gene transcription (Lotan 1980).

The existence of the specific cytosolic retinoid binding proteins (CRBP and CRABP), that bind retinol and RA, respectively, was demonstrated by Ong and Chytil (1979) and Chytil and Ong (1979). Indeed, in their review Goss and McBurney (1992) point out that there are four such cytoplasmic binding proteins, two binding retinol (CRBP-I and CRBP-II) and two binding RA (CRABP-I and CRABP-II). These proteins are expressed widely in adult and embryonic tissues. The function of these proteins remains unclear (Goss and McBurney 1992). However, there is
some evidence that CRABP translocates into the nucleus when bound to RA (Grippo and Sherman 1990), suggesting that the major function of these proteins is intracellular transport of their ligands. Both CRBP and CRABP are frequently referred to as being definitely involved in the translocation process (Tallman and Wiemik 1992), a fact which appears to be unproven. Another function which these proteins may serve, as reported by Morriss-Kay (1992), could be in controlling local RA concentrations at the cellular level. She stated that such a mechanism must exist since, for example, the RA requirements of different embryonic tissues are tissue specific. Furthermore, she stated that there is good evidence to suggest that CRBP and CRABP are involved in such a mechanism.

The idea that retinoids act in a similar manner to steroid hormones gained further support in the late 1980's with the discovery of retinoid nuclear receptors (Giguere et al. 1987; Petkovich et al. 1987), belonging to the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors (Levin et al. 1992; Allenby 1995). The retinoic acid receptors have been reviewed by many authors, among them Goss and McBurney (1992), Morriss-Kay (1992), Tallman and Wiemik (1992), Bollag and Holdener (1992), Holdener and Bollag (1993), Wan (1993), and Allenby (1995).

Two classes of nuclear retinoic acid receptors, termed RAR and RXR, have been identified and cloned, with each receptor class consisting of three receptor subclasses called a, b and c (Holdener and Bollag 1993), and each subclass existing in multiple isoforms that arise from alternative initiation sites (Leid et al. 1992). These receptors are differentially expressed in tissues (Wan 1993), giving rise to a multiplicity of diverse signalling pathways utilising retinoid responsive genes to control differentiation and cell proliferation (Allenby 1995). The diversity of receptor subtypes and isoforms helps to explain the wide range of physiological functions performed by vitamin A (Holdener and Bollag 1993; Allenby 1995). These functions primarily are performed by RA, acting in a similar fashion to a classical steroid hormone, to induce gene expression (Wan 1993; Allenby 1995). Bollag and Holdener (1992) succinctly explained a putative mode of function for these receptors. RA or other retinoids first bind to the ligand-binding domain of the receptor, perhaps inducing a conformational change of the receptor. The receptor, thus activated, then induces or represses transcription of genes which contain a RA response element (RARE), the RARE being recognised by the DNA-binding domain of the receptor. These transcriptional events result in the synthesis of mRNAs and proteins which might be responsible for biological functions, such as cell proliferation or differentiation.
The ligands for the receptors are formed from the intracellular metabolism of all-trans-retinol, via a series of oxidative and isomerisation reactions, to at least three retinoids (all-trans-RA, 3,4-didehydro-RA and 9-cis-RA) which bind to the two classes of nuclear receptors (Holdener and Bollag 1993). The three RARs bind both all-trans-RA and the newly identified 9-cis-RA with high affinity. The RXRs, however, only bind 9-cis-RA (Holdener and Bollag 1993; Wan 1993; Allenby 1995).

RARs require heterodimerisation with RXRs for appreciable DNA binding and function, whereas RXRs are able to bind not only as heterodimers but also as homodimers (Holdener and Bollag 1993). Interestingly, other members of the nuclear hormone receptor superfamily, including the thyroid hormone receptor, the vitamin D receptor, and the peroxisome proliferator-activated receptor, also show enhanced function when heterodimerised with RXR (Holdener and Bollag 1993; Allenby 1995).

As well as gene induction, steroids and retinoids also repress gene expression. This repressive activity is in part mediated through interference with the activity of another transcription factor, AP-1, which is involved in mediating cell proliferation signals (Allenby 1995). It is possible that all the therapeutic effects of retinoids, including the anti-cancer effects, could be due in part to the inhibition of AP-1 and certain AP-1 target genes (Allenby 1995).

(i) Retinoic acid receptors and cancer

In humans the genes for RARa, RARb and RARc are situated on chromosomes 17, 3 and 12, respectively. Acute promyelocytic leukaemia (APL), which is a result of a chromosomal (15;17) translocation, involves the RARa gene (Wan 1993). Administration of RA induces in vivo differentiation of the APL cells into mature granulocytes, leading to complete morphological remission (Huang et al. 1988).

The gene for RARb has been speculated as playing a role in human hepatocarcinogenesis (Wan 1993), as this gene was found as a hepatitis B viral integration site in a human hepatoma case, and also the expression of the rat RARb gene is decreased or inactivated in several rat hepatoma cell lines.

Lung cancer cells are characterised by a non-random deletion in the short arm of chromosome 3 (Wan 1993). This has led to the hypothesis that one or more tumour suppressor genes normally present in the deleted region is/are responsible for the development and/or progression of the
malignant phenotype. A high frequency of abnormalities in the structure and expression of the RARb gene has been detected in human lung cancer (Wan 1993). Similarly, Wan (1993) reported that several squamous lung cancer-derived cell lines have also been found to express low or undetectable levels of RARb mRNA. These data suggest that RARb is situated very close to the putative tumour suppressor gene or that RARb contains DNA sequences prone to carcinogen-induced breakage (Wan 1993).

In urinary bladder cancer, rearrangements of chromosome 3p14 have been frequently observed. This change is associated with aggressive tumour behaviour (Kroft and Oyasu 1994). The RAR-b gene is located at 3p24 (Mattei et al. 1988), and this gene has been suggested as a possible target for alteration in bladder cancer cases showing 3p14 alterations (Kroft and Oyasu 1994). Bolla et al. (1985) reported that human bladder tumours which showed a lack of RAR expression, deduced from the absence of binding of detectable tritium-labelled RA, were more likely to recur and more likely to become invasive than tumours which did bind labelled RA.

4 THE ASSOCIATION OF VITAMIN A WITH CARCINOGENESIS

Since Fujimaki (1926) observed stomach tumours in vitamin A-deficient rats, the neoplastic process has been linked to vitamin A. Over the years, a great deal of research has been done in relation to vitamin A and cancer. The main areas of investigation that are of relevance to this thesis include: a) the effect of vitamin A deficiency on cancer incidence in (i) animals and (ii) in humans and, b) cancer prevention with natural and synthetic retinoids in (i) animals and (ii) man. These fields of research are vast in themselves and to cover each in depth would be clearly beyond the scope of this thesis. Nevertheless, a brief outline of main findings in each of these areas is essential.

A Vitamin A deficiency and cancer

(i) Experimental animals

For many years it has been known that vitamin A deficiency enhances the development of chemically-induced tumours in experimental animals. Sporn et al. (1976) and Pitt (1985) reviewed several studies supporting this premise including those of Davies (1967), Newberne and Rogers (1973), Rogers et al., (1973), Nettesheim and Williams (1976), and Cohen et al. (1976). Other studies with similar findings include those of Newberne and Suphakarn (1977), Dogra et al. (1985) and Bansal and Gupta (1987).
However, Ong and Chytil (1983) commented that the effect of vitamin A deficiency upon the susceptibility of experimental animals to carcinogens was perhaps less clear than the above reports might suggest. This was because such studies were made technically difficult to perform in truly vitamin A-deficient animals, due to the long time scale required for the induction of tumours and the generally poor health and rapid death of the animals.

In reviewing the effects of various nutrients, including vitamin A, on carcinogenesis, Birt (1986) drew attention to two studies in which vitamin A deficiency had no effect upon carcinogen-induced carcinogenesis. In these studies, vitamin A deficiency had no influence in salivary gland carcinogenesis in hamsters treated with locally applied dimethylbenz(a)anthracene (DMBA) (Chaudhry et al. 1961), or colon carcinomas in rats treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Narisawa et al. 1976). The results of Narisawa et al. (1976) were in sharp contrast to those obtained in the same species by Newberne and Suphakarn (1977) after treatment with aflatoxin. Indeed, in the study of Narisawa et al. (1976), there was clear evidence that vitamin A-deficient rats developed significantly fewer tumours than normally-nourished controls, suggesting that vitamin A deficiency might affect colon carcinogenesis quite differently depending upon the carcinogen used (Ong and Chytil 1983). In addition to these two investigations highlighted by Birt (1986), vitamin A deficiency was also reported to have had no effect in two other studies, namely that of Nauss et al. (1987), involving experimental oesophageal carcinogenesis in rats treated with methylbenzylnitrosamine (MBN), and that of Zile et al. (1986) who studied low dose DMBA-induced rat mammary carcinogenesis.

It is clear, therefore, that the effects of vitamin A deficiency in experimental carcinogenesis are varied and may depend upon the particular carcinogen used and, perhaps, the tissue of interest, as well as other variables such as carcinogen dose or rat strain.

(ii) **Epidemiology of vitamin A and human cancer**

As vitamin A deficiency can increase susceptibility to tumour formation in experimental animals, it was natural for epidemiologists to investigate whether vitamin A deficiency might be associated with increases in human cancer risk (Pitt 1985). There is now a large number of reports examining the relationship between vitamin A status and human cancer risk (reviewed by Peto et al. 1981; Basu 1983; Ong and Chytil 1983; Mettlin 1984; Willett et al. 1984; Pitt 1985; Menkes et al. 1986; Rogers and Longnecker 1988; Michels and Willett 1994; Willett 1994; Willett and Hunter 1994).
Several different approaches have been used to investigate the relationship of vitamin A to human cancer. 'Retrospective' investigations have been performed comparing blood retinol levels, or dietary vitamin A intake determined from questionnaires, in cancer patients and comparable normal controls (Michels and Willett 1994). These investigations are termed retrospective because they are carried out after the diagnosis of cancer. Although retrospective studies are relatively simple to perform, there are several drawbacks to this type of study. Firstly, because dietary intake is assessed after cancer has occurred, differential recall by cases and controls, in case-control studies, is a serious potential problem (Michels and Willett 1994). Secondly, if vitamin intake is determined via blood samples, the cancer itself might affect metabolism and influence blood levels of the vitamin (Metlin 1984; Pitt 1985). Furthermore, cancer patients often change their dietary habits including reducing their total energy intake due to loss of appetite (Michels and Willett 1994). In case-control studies, the choice of an appropriate control group is vital to the validity of the study; to avoid bias, controls must be sampled from the population that gave rise to the cases (Michels and Willett 1994).

In contrast to retrospective investigations, 'prospective cohort studies' involve the assessment of vitamin intake once or repeatedly before the diagnosis of cancer, while the subjects are followed over several years. The vitamin intakes of the cancer patients are then compared with suitable normal controls from the same population. This allows rates of cancer among individuals with different levels of dietary intake to be determined (Michels and Willett 1994). Furthermore, since information on dietary patterns is obtained before the disease occurs, a prospective study will not be biased by differential recall between cases and controls. A few investigations have been reported which include case-control studies nested within a cohort, using information collected prospectively, and thus with greater validity. To assess vitamin levels in individuals who subsequently develop cancer and in selected controls, most such studies use blood samples which have been collected at the study beginning for the entire cohort and stored at low temperatures (Michels and Willett 1994).

In randomised intervention studies, such as that of BC in the Physicians' Health Study (Hennekens and Eberlein 1985), choice of exposure (control or test) is under the control of the investigator. If diet has an affect on cancer, it will probably act long before diagnosis. Therefore, dietary intervention studies have to be continued for several years. This makes dietary intervention in humans difficult, since it is not feasible to dictate to individuals what they should eat over an extended period of time. Such trials, therefore, appear most appropriate to study the effect of vitamin supplements on cancer risk (Michels and Willett 1994).
Any potential protective effect of vitamins on cancer is likely to be rather modest. Therefore, to obtain a valid estimate of such an effect, a sufficiently large study sample is essential (Michels and Willett 1994).

When any investigation is undertaken into a potential association between vitamin intake and cancer, several confounding factors may bias the association. These factors are specific to the type of cancer being studied, and in a particular study the list of potential confounding factors to control for is dependent on several considerations. These include the state of knowledge about other risk factors for that cancer at the time the study is conducted and the investigators' judgement and availability of relevant data.

The confounding factors in blood-based assessments of vitamin intake might differ from those in dietary comparisons. Blood levels of vitamins can be affected by a number of factors that must be accounted for. Cigarette smoking, for example, lowers the plasma level of carotenes (Stryker et al. 1988). When a possible association between one vitamin and cancer is examined, the relationship might be confounded by another micronutrient, such as another vitamin present in the same foods as the nutrient under study. Optimally, analyses should be published with and without adjustment for a potentially confounding effect of other vitamins (Michels and Willett 1994). Many of the earliest epidemiological investigations into the association of vitamin A and cancer risk did not take into account confounding factors such as cigarette smoking. If the confounding factors had been included in the analyses, many of these early studies, that frequently reported inverse associations between vitamin A and cancer, might not have found such relationships. Indeed, most subsequent investigations have, in the main, failed to demonstrate a strong relationship between vitamin A and the risk of cancer at virtually every site (Michels and Willett 1994).

Notwithstanding the potential sources of bias, comparison of blood retinol concentrations of cancer patients with those of control patients (retrospective study) is perhaps the simplest method of studying the association between cancer and vitamin A (Peto et al. 1981; Ong and Chytíl 1983). In seven out of eight of the early studies using this approach (reviewed by Peto et al. 1981), cancer patients demonstrated significantly lower retinol levels. As the cancers themselves might have affected blood retinol, these studies do not show, however, that a lowered vitamin A status has any significant influence on cancer incidence. Nevertheless, while blood levels of retinol are not directly related to dietary intake of vitamin A, data indicating lower levels of the vitamin in the
blood of cancer patients would at least be consistent with the hypothesis that vitamin A has some protective action (Mettlin 1984).

To overcome the problem of a possible effect of the disease itself on the blood levels of retinol, two large studies (prospective studies) were performed in which blood samples were available from large numbers of individuals among whom diagnoses of cancer were made several years later (Wald et al. 1980; Kark et al. 1981). In the UK, serum samples from 16,000 men were collected and stored by Wald and his associates (1980). After 5 years, 86 of these men were identified as having developed cancer. Retinol levels were analysed in the blood of these 86 men and of another 172 individuals from the sample population who had not developed cancer. In this study, low retinol levels were associated with an increased risk of cancer. In the USA, Kark and associates (1981) used a similar design to follow 3102 men from Evans County, Georgia, for 12-14 years. Blood samples had been obtained from these individuals as part of a prospective study of coronary heart disease risk factors. Individuals from this population who subsequently developed cancer (a total of 129 cases in all) were found to have had significantly lower mean serum retinol levels at least 12 months prior to the diagnoses of cancer. This effect was found in all age and sex groups investigated, but was most prominent amongst males. The lower serum retinol levels in the eventual cancer patients were consistent for all cancers across all sites and cell types apart from leukaemia and Hodgkin’s disease (Kark et al. 1981; Mettlin 1984).

The publication of these two investigations did much to increase interest in the link between vitamin A and human cancer, and many further epidemiological studies have been reported subsequently. However, when these two particular studies were extended, the significant associations between vitamin A and cancer were no longer present (Peleg et al. 1984; Wald et al. 1986). For instance, in the study of Wald et al. (1980 and 1986), the authors observed a diminishing difference between the cases and the controls with increasing time between blood collection and cancer diagnosis. If only subjects were included in the analysis whose cancer developed three or more years after blood was collected, their mean retinol level was even higher than in the controls. The authors concluded that the inverse relationship which they had previously reported was likely to have occurred because low serum retinol is a metabolic consequence of cancer rather than a precursor of malignancy (Michels and Willett 1994). In the extension of the Evans County Study (Peleg et al. 1984), no relationship was found between cancer and serum retinol levels after controlling for several confounding variables including smoking, thus failing to confirm the strong dose-response relationship between baseline retinol levels and subsequent cancer reported in the earlier study by Kark et al. (1981).
The fact that serum retinol levels in cancer patients studied retrospectively or prospectively appear to be associated with cancer risk does not necessarily indicate that the intake of vitamin A influences such risk (Mettlin 1984). Some of the observed variations in serum retinol in the populations studied is attributable to the intrinsic regulatory mechanisms which control the level of vitamin A in the blood, rather than to different levels of vitamin A in the diet (Mettlin 1984). According to Mettlin (1984), the only method of determining whether vitamin A intake influences risk is by observing the levels of retinol or provitamin A carotenoids in the diets as well as vitamin supplements ingested by populations at different risks. While dietary intakes are difficult to study epidemiologically, vitamin A is one of the most easily studied nutrients by this approach because it tends to occur in predictable amounts in a limited number of dietary items (Mettlin 1984).

Assessment of vitamin A intake can be obtained either through food frequency questionnaires (FFQ), dietary records, or dietary recalls (Michels and Willett 1994). For cancer epidemiology, dietary intakes over the past decade(s) rather than the past few days is clearly of importance. FFQs usually cover dietary patterns of at least the past year and are generally thought to come closest to being representative of longer and more remote periods of time (Michels and Willett 1994). Any assessment of food intake is difficult and associated with imprecisions and biases, such as recall bias. Micronutrient intake is calculated from the FFQs based on the nutrient composition of the food consumed as stated in food composition tables. Such calculations do not take into account, however, that foods might differ in their nutrient content depending on season, origin and storage of the food as well as handling and preparation (Michels and Willett 1994).

Blood-based measurements can be used instead of assessments of intake in case-control and cohort studies. Since the cancer may itself may have affected the plasma level of the nutrient, however, such measurements have to be interpreted with caution in case-control studies. Blood-based determinations are also used in nested case-control studies where blood samples are drawn at the start of the cohort follow-up, and then frozen at low temperatures. Only samples from individuals who subsequently develop cancer and selected controls are then thawed and analysed. The interval between sample collection and cancer diagnosis must be sufficiently large, however, to exclude with certainty that a difference in nutrient plasma level is not a consequence of the malignancy rather than its predictor (Michels and Willett 1994).

One of the first dietary studies of vitamin A and cancer was conducted in 1975 by Bjelke who carried out a prospective study of 8278 men in Norway. Using a rather crude index of vitamin A intake, Bjelke found a significant negative correlation between the vitamin index and cancer mortality. Mettlin (1984) reported increasing cancer risk with decreasing vitamin A intake, in a
more carefully performed investigation involving a total of 24,000 patients questioned about their dietary habits over the period from 1954 to 1965. In this study, like many others of this type carried out up to about 1986, dietary vitamin A intake was taken as the sum of pre-formed vitamin A and provitamin A carotenoids.

In their recent extensive review of the epidemiological data Michels and Willett (1994) stated that there is very little epidemiological evidence demonstrating any significant protective effects of dietary vitamin A when all potential cancer sites are combined, either from blood-based studies or from dietary assessments. For example, Smith and Hershal (1978) found no convincing evidence in their dietary study that the regular consumption of vitamin A-containing preparations protected against the development of cancer. Shekelle et al. (1981) found no association between vitamin A and cancer incidence in the 'Western Electric Study'. Shibata et al. (1992) did not detect a protective effect of vitamin A supplementation for cancer at all sites in the 'Leisure World' cohort study. Similarly, the early promising results of the blood-based studies of Wald et al. (1980) and Kark et al. (1981) were later corrected to show no significant association between serum vitamin A and subsequent cancer risk (Peleg et al. 1984; Wald et al. 1986). Willett et al. (1984) reported that the mean values for retinol were similar for cases and controls in the study nested within the Hypertension Detection and Follow-up Program. In the Japanese ancestry study in Hawai‘i (Nomura et al. 1985), no association was detected between blood retinol and cancer. Similarly, no important differences between blood retinol in cancer cases compared to controls were reported in three other nested case-control studies reviewed by Michels and Willett (1994); however, in one of these three investigations, that of Salonen et al. (1985), serum retinol levels were lower in male smokers compared with smoking controls. Unlike BC, plasma levels of vitamin A were not found to be different in individual members of the Basel Study who subsequently died of cancer when compared to levels of the rest of the cohort in any of the three follow-up analyses (Stahelin et al. 1984, 1989 and 1991). However, in the twelve year follow-up (Stahelin et al. 1991), low BC levels, combined with low retinol levels seemed to increase the risk of death from cancer.

There have been many studies, both blood-based and dietary, of the association between vitamin A and cancer at specific sites (Rogers and Longnecker 1988; Michels and Willett 1994). The list of specific cancers which have been studied in relation to vitamin A include cancer of the lung, breast, cervix, ovary, endometrium, colo-rectum, stomach, oesophagus, pharynx, larynx, mouth, pancreas, prostate, urinary bladder and skin (Michels and Willett 1994). Of all these forms of cancer, only prostate and urinary bladder have shown relatively consistent inverse associations between dietary intake or blood retinol and cancer risk (Michels and Willett 1994). Increased risk
of cancer at oral, pharyngeal or laryngeal sites has also been a fairly common finding when these sites have been investigated (Michels and Willett 1994). In contrast, investigations of vitamin A and cancer of the lung, breast and colorectum have tended to show no strong associations, although a few occasional reports have detected some effect at these sites (Hunter et al. 1993). There is virtually no evidence of any association between vitamin A and cancers of the skin, pancreas, stomach, endometrium, cervix and ovary, while retrospective case-control studies have tended to show an increased risk of oesophageal cancer with high retinol intake (Michels and Willett 1994).

It is important to note that many early studies indicated an increased risk of some cancers, such as stomach cancer, which are mentioned above as not being associated with vitamin A. This is most probably because the early studies in question did not account for confounding factors when calculating the reported associations. As a result of this, recent reviewers such as Michels and Willett (1994) do not now regard such reported findings as significant.

As previously mentioned, there is relatively strong epidemiological evidence to suggest that vitamin A is associated with urinary bladder cancer. Shibata et al. (1992) reported a lower, though not significant, incidence of bladder cancer in men who consumed vitamin A supplements. Out of six retrospective dietary studies (Mettlin and Graham 1979; Kolonel et al. 1985; Risch et al. 1988; La Vecchia et al. 1989; Nomura et al. 1991; Riboli et al. 1991), those of La Vecchia et al. (1989) and Mettlin and Graham (1979) reported significantly decreased risk associated with high dietary retinoids or vitamin A. However in the study of Mettlin and Graham (1979), smoking was not adjusted for in the analysis. A weak protective effect of total vitamin A intake was reported by Kolonel et al. (1985). Steineck et al. (1990) observed a borderline significant decrease in risk of bladder cancer with intake of vitamin A supplements, with a dose response relationship for frequency of consumption. Finally, there have been three nested case-control studies (Nomura et al. 1985; Helzlsouer et al. 1989; Knekt et al. 1991a), all of which have shown that pre-diagnostic blood levels of retinol were not significantly different in bladder cancer cases and controls.

B Cancer prevention with vitamin A and its synthetic analogues (retinoids)

(i) Cancer prevention in experimental animals

There have been mixed results from animal experiments (reviewed by Sporn et al. 1976; Ong and Chytil 1983; Birt 1986; Rogers and Longnecker 1988) investigating the prophylactic effects of high doses of vitamin A (VAP or RA), or the synthetic retinoid VAA, against tumours induced by
various carcinogens, at many sites, and in several different species of laboratory animals. Some reports have indicated an inhibitory effect; for example, feeding VAP inhibited tumours of the stomach and uterine cervix in hamsters treated with benzo(a)pyrene or DMBA (Chu and Malmgren 1965), feeding VAA inhibited the development of urinary bladder tumours in rats treated with BBN (Miyata et al. 1978) and in mice given FANFT (Dawson et al. 1979), feeding vitamin A (exact form not stated) inhibited the appearance of skin papillomas in rhino mice treated with DMBA (Davies 1967), and, similarly, high doses of VAP or RA inhibited the development of DMBA-induced mouse skin papillomas and carcinomas (Bollag 1971). Other results indicating an inhibitory effect of high doses of vitamin A against experimental tumours were reported by Saffioti et al. (1967), who demonstrated inhibition of tracheobronchial carcinomas and forestomach papillomas in hamsters treated with benz(a)pyrene, Cone and Nettesheim (1973), who showed inhibition by VAA of MCA-induced lung carcinomas in rats, and Moon and associates (Moon et al. 1976; Moon et al. 1977), who have reported inhibition by VAA of rat mammary carcinomas induced by DMBA or MNU.

In contrast, high doses of VAP had no effect against urinary bladder tumours induced in rats by FANFT, despite vitamin A deficiency enhancing urinary bladder carcinogenesis in this model (Cohen et al. 1976). Similarly, VAA was reported to have no effect in the 3-methylcholanthrene lung carcinogenesis model (Nettesheim et al. 1979), despite the enhancing action of vitamin A deficiency in this system, and an earlier report indicating an inhibitory effect of vitamin A supplementation on the induction of early lung tumours (Cone and Nettesheim 1973).

Skin papillomas, whether induced by chemicals or viruses (but not those induced by ultraviolet light) have been generally very sensitive to the preventative actions of natural and synthetic retinoids (Ong and Chytil 1983) when given orally, parenterally, or topically. However, an enhancing effect of RA was demonstrated by Hennings et al. (1982) in two-stage skin carcinogenesis in mice treated with DMBA and TPA. Other enhancing effects of vitamin A on carcinogenesis were reported in ultraviolet light-induced skin papillomas in mice (Forbes et al. 1979). Similar enhancing effects of vitamin A were found in the hamster/benzo(a)pyrene-ferric oxide model (Smith et al. 1975), in contrast to the marked inhibitory effects reported in this model by Saffioti et al. (1967).

There is a vast literature detailing experiments to test the efficacy of synthetic retinoids, including VAA, against a wide range of tumours in experimental animals. Among the many reviews of this area are those of Sporn et al. (1976), Bollag (1979), Sporn and Newton (1979), Bollag and Matter...

The structure of the basic retinoic molecule and those of several commonly used synthetic retinoids are shown in Figure 2.1. Chemical substitution of the polar terminal group and alteration of the aliphatic carbon skeleton can render synthetic analogues of vitamin A less toxic than the parent molecules (Sporn and Newton 1979). Many synthetic retinoids are neither stored in the liver nor under the same physiological regulation as natural retinoids; consequently tissue levels can be influenced directly by their dietary intake (Hicks and Turton 1986).

Synthetic retinoids have been tested mainly in animals treated with chemical carcinogens (Pitt 1985; Hill and Grubbs 1992). In these systems, retinoids are often organ/tissue specific with regard to their anti-cancer activity (Hill and Grubbs 1992). A number of retinoids have been shown to inhibit the development of tumours in various tissues of experimental animals including skin, oral cavity, blood, mammary gland, pancreas and urinary bladder (Hill and Grubbs 1992). Convincing data are lacking to indicate that synthetic retinoids have substantial activity in preventing cancer of the lung, oesophagus, or colon; data for the liver and forestomach are equivocal (Hill and Grubbs 1992). The effects of synthetic retinoids in skin, mammary gland and the urinary bladder will now be used to illustrate inhibition of experimental tumours by these agents.

In the early 1970s, Bollag had shown that the systemic administration of RA exerts a prophylactic and a therapeutic effect upon skin papillomas and carcinomas in mice treated with DMBA and croton oil (Bollag 1971, 1972). Furthermore, Bollag and Ott (1971) noted a favourable response to topical or oral RA in human pre-cancerous conditions such as actinic keratoses, leukoplakia, and urothelial papillomas. However, to be effective in man, RA had to be used at dosages which produce symptoms of hypervitaminosis A in the form of headache, liver injury, and lesions of the skin and mucous membranes (Editorial 1980). To overcome this problem, derivatives of RA were sought that exhibited greater anti-tumour activity and less toxicity (i.e. with a better therapeutic ratio). In 1974, Bollag reported that an aromatic analogue of RA, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraanoate (TMMP), possessed a therapeutic ratio ten times more favourable than RA in the mouse DMBA/croton oil model, although it still produced symptoms of hypervitaminosis A. TMMP was the first synthetic
retinoid, with a substantially different molecular structure to that of natural forms of vitamin A, to
demonstrate anti-cancer activity.

In Bollag's system, retinoids were given orally or intraperitoneally to mice bearing established skin
tumours. Retinoids which were effective in this system include: etretinate, motretinide, ethyl
retinoate, acitretin and various aromatic and fluorinated aromatic retinoids, as well as the natural
retinoids RA and VAP (Hill and Grubbs 1992). Other retinoids tested in this system have shown
little or no effect. In another similar system that is still widely used to evaluate retinoids for
prevention of papillomas and basal cell carcinomas, the test retinoid is applied at the same time as
the promoter (croton oil or the active component of croton oil, TPA). Retinoids active in this
system include etretinate, 13-cis-RA, 5,6-epoxy-RA, various aromatic retinoids, and various
3-substituted 4-oxoretinoic acids (Shealy 1989), as well as VAA and the natural retinoids retinol,
retinal, VAP and RA (Hill and Grubbs 1992). Other retinoids tested have shown no effect in this
system.

When administered orally to mice previously exposed to ultraviolet light, neither VAP, etretinate,
RA, nor 13-cis-RA had any effect on the incidence of papillomas and squamous cell carcinomas
(Hill and Grubbs 1992). Furthermore, when RA was applied to the skin of these mice, an
increased incidence of squamous cell carcinomas was noted (Forbes et al. 1979). Thus, retinoids
show little promise in preventing skin cancers caused by ultraviolet light (Hill and Grubbs 1992).

Recently, enhancement in vitro of anti-cancer activity has been reported for combinations of
retinoids with cytokines, such as interferon alpha (Bollag and Holdener 1992), and also for
combinations of retinoids with vitamin D and its analogues (Bollag et al. 1994). These positive
findings have led to clinical trials in humans of retinoid/interferon alpha combination therapy
against cutaneous SCC (Holdener and Bollag 1993; Bollag et al. 1994).

Retinoids have been reported by various laboratories to prevent mammary tumours induced in rats
by one of several different carcinogens (Hill and Grubbs 1992). When DMBA is the carcinogen,
VAA and retinyl methyl ether are reasonably effective. Other retinoids that have been reported to
cause a reduction in incidence of mammary carcinomas are 4-HPR (Grubbs et al. 1990),
temarotene (an aromatic retinoid without a functional end group on the sidechain) and two other
aromatic retinoids (Hill and Grubbs 1992). One report, that of Silverman et al. (1983), indicated
that 4-HPR was not effective against mammary tumours induced by DMBA or MNU, but Hill and
Grubbs (1992) suggested that these negative results might be due to the fact that Silverman and associates (1983) used a different rat diet from that used by the other workers.

With MNU as the carcinogen, VAA and retinyl methyl ether are effective in preventing mammary carcinomas (Moon et al. 1977; Welsch et al. 1984). Other active retinoids are 4-HPR (Moon et al. 1979; Grubbs et al. 1990), retinyl propynyl ether (Shealy 1989) and axerophthene (Hill and Grubbs 1992). Of these, 4-HPR is the most effective for reducing tumour incidence and also for delaying tumour onset (Tallman and Wiemik 1992). Retinoids reported to be without activity against mammary tumours are the methyl ether analogue of etretinate (Shealy 1989) and 13-cis-RA (Thompson et al. 1978).

The time and extent of retinoid dosing is often important in determining the outcome of anti-cancer studies with these compounds, irrespective of the type of cancer under study. The report of Grubbs et al. (1990) demonstrated that VAA fed prior to, but not after, MNU, caused an increase in the number of adenocarcinomas. Nevertheless, when this retinoid was fed continuously, the number of adenocarcinomas was greatly reduced relative to administration of no retinoid.

Retinoids are most effective as inhibitors of mammary carcinogenesis when administered shortly after carcinogen treatment (Moon and McCormick 1982). However, retinoids are still effective in this regard even if administration is delayed for some time after carcinogen dosing. The length of time that retinoid treatment can be delayed appears to be related to tumour latency, the longer the latency, the longer retinoid treatment can be delayed (Moon and McCormick 1982). In life-time feeding studies of the activity of retinoids in urinary bladder carcinogenesis, Hicks and associates (1982b) demonstrated that retinoids inhibit the development of bladder cancers by extending the latent period between carcinogen exposure and the appearance of tumours. However, these workers showed that retinoid-induced inhibition of tumour development was only apparent in the early stages of tumour growth. Towards the end of these life-time feeding studies, the incidence of bladder tumours became similar in retinoid-fed and placebo-fed carcinogen-treated animals (Hicks et al. 1982b).

In addition to MNU-induced mammary tumours, VAA is also effective in reducing the incidence of adenocarcinomas that are induced in rats exposed to benzo(a)pyrene, X-rays or oestrogens, as well as those tumours which occur spontaneously (Hill and Grubbs 1992). In contrast to the rat, mice develop mammary tumours after exposure to DMBA that are not inhibited by either VAA or 4-HPR (Hill and Grubbs 1992). In addition, VAA is not able to prevent the spontaneous mammary carcinomas which develop in the C3H-A mouse strain (Hill and Grubbs 1992). These
observations are indicative of a degree of species specificity with regard to mammary cancer among those retinoids which have demonstrated anti-cancer activity in animal experiments (Hill and Grubbs 1992; Tallman and Wiemik 1992).

Sporn and associates (Grubbs et al. 1977; Squire et al. 1977; Sporn et al. 1977) were the first investigators to demonstrate inhibition by a synthetic retinoid of experimental urinary bladder cancer, when they reported that dietary 13-cis-RA reduced the incidence and severity of bladder cancer in Wistar rats treated with intravesicular MNU. The following year, the same workers repeated these experiments with 13-cis-RA in C57BL/6 mice treated with the specific bladder carcinogen BBN and found similar results (Becci et al. 1978). Similarly, 13-cis-RA reduced the incidence of BBN-induced TCCs in F344 rats (Becci et al. 1979a and 1979b).

In sharp contrast to bladder cancer induced by MNU or BBN, there is virtually no evidence to suggest that TCCs induced in rats or mice by FANFT are inhibited by retinoids (Lower and Kanarek 1981). FANFT-induced TCCs in rats were not responsive to 13-cis-RA (Croft et al. 1981a), nor were these lesions inhibited by two amide derivatives of RA, N-ethyl-retinamide and N-(2-hydroxyethyl)retinamide (Croft et al. 1981b). Neither were these lesions responsive to the natural retinoid VAP (Cohen et al. 1976). Although FANFT-induced bladder tumours in C3H/He mice were inhibited by a low level of VAA (Dawson et al. 1979), there was no effect in groups of mice given higher doses of VAA in this experiment. These findings suggest that inhibition of experimental tumours by retinoids could be related to the type of carcinogen employed (Lower and Kanarek 1981).

A high proportion of the carcinomas found in C57BL/6 mice after BBN treatment were SCCs. As the majority of human bladder cancers are TCCs rather than SCCs, the BBN/mouse model was refined (Becci et al. 1981) to yield mainly carcinomas of the transitional type, by replacing the C57BL/6 mouse strain with C57BL/6 x DBA/2 F_1 hybrid mice, (subsequently referred to as BL6D2F_1 mice). Using this system, Becci et al. (1981) reported that dietary supplementation with 13-cis-RA significantly reduced the incidence and severity of TCC. The BBN/BL6D2F_1 model has been used to identify a large number of synthetic N-alkylretinamide derivatives of RA which, when administered in the diet at non-toxic levels, possess a greater activity to toxicity ratio than that seen with 13-cis-RA (Becci et al. 1981; McCormick et al. 1981; Moon et al. 1982). In reviewing these investigations, Moon and McCormick (1982) pointed out that modifications of the basic retinoid molecule can have a significant effect upon anti-cancer activity. For example, addition of an ethylamide or a hydroxyethylamide group to all-trans-RA produces an effective
chemopreventative agent while the parent compound is inactive. Similarly, addition of these same
groups to 13-cis-RA results in the formation of less toxic retinoids, with equal or greater
inhibitory activity (Thompson et al. 1981). In a comparative study of the potential anti-cancer
activity of 15 retinoic acid amides in the BBN/BL6D2F model, Moon et al (1982) reported that
4-HPR was superior to other compounds tested, combining maximum anti-carcinogenic activity
with little or no toxicity. These findings were later confirmed by Hicks et al (1985), in life-time
retinoid feeding studies with the same model.

As in the mammary gland, administration of 13-cis-RA can be delayed for some time, with no loss
of anti-cancer effect in BBN-treated F344 rats (Becci et al. 1979b). The fact that retinoid
administration can be delayed without loss of anti-cancer activity is very important if future, less
toxic, retinoids can be developed for clinical use in cancer prevention.

All the early reports (Grubbs et al. 1977; Sporn et al. 1977; Squire et al. 1977; Becci et al.
1979a, 1979b, 1981; Thompson et al. 1981; Moon et al. 1982) of inhibition by 13-cis-RA and
other synthetic retinoids of urinary bladder cancer in rodents pre-treated with MNU or BBN
involved assessment of the carcinogenic response at a single time-point some months after the
animals were placed on a retinoid-containing diet. In 1982, Hicks and associates reported that the
anti-cancer activities of 13-cis-RA or N-ethylretinamide, in life-time retinoid feeding experiments,
were entirely attributable to an increase in the latent period, compared with placebo-fed controls,
before neoplastically transformed urothelial hyperplasias began to grow rapidly into tumours
(Hicks et al. 1982b). In the early stages of these experiments, both retinoids appeared to hold the
urothelium in the latent period so that, at any point in time, the tumours in the retinoid-fed animals
appeared 'younger' than those of the placebo-fed animals and consequently were both smaller and
better differentiated (Hicks 1983). With continued time on the experimental diets, however, it was
found that retinoid restraint was overcome and the bladder cancer incidence eventually reached the
same level as in the placebo-fed controls, thus, after long-term administration, neither of the
retinoids prevented cancer development. Neither compound reduced the rate of tumour growth
once the cancers had started to grow, nor did they prevent the animals from dying from bladder
cancer (Hicks et al. 1982b; Hicks 1983 Hicks et al. 1985). Nevertheless, retinoid treatment
conferred a real advantage on carcinogen-treated rats, by increasing the average survival time by
the same amount as it increase the latent period between carcinogen exposure and tumour growth
(Hicks et al. 1982b; Hicks et al. 1985; Hicks and Turton 1986).
As the majority of human bladder cancer patients are in the sixth decade of life, a delay in the development of further bladder tumours as a result of retinoid therapy could prove extremely beneficial for the management of cancer in these patients. On this basis, Hicks (1983) suggested that 13-cis-RA or N-ethyl-retinamide could be useful in cancer patients who have already had one bladder neoplasm and thus are at high risk of developing subsequent tumours at other bladder sites. However, in a subsequent long-term retinoid feeding study in the BBN/BL6D2F1 mouse model, Hicks and Turton (1986) reported that N-ethyl retinamide treatment was associated with a high incidence of liver adenomas and carcinomas. In the light of this finding, Hill and Grubbs (1992) noted that the testing of retinamides in humans should not be carried out until it has been determined if retinamide-induced liver tumours are limited to rodents. The most active and least toxic retinamide yet tested in animals is 4-HPR (Moon et al. 1982). This compound was not found to be associated with mouse liver tumours (Hicks and Turton 1986), and a clinical trial of 4-HPR in patients at high risk from a subsequent bladder tumour is currently underway (Szarka et al. 1994). Another type of retinoid, etretinate, has already been used successfully to prevent recurrences of new superficial lesions in patients who have had a primary tumour removed by transurethral resection (Alfthan et al. 1983).

Hill and Grubbs (1992) have classified retinoids with chemopreventative activity into three distinct classes, each with its own biochemical properties. One class of retinoids is characterised by a terminal carboxylic acid group (necessary for binding to CRABP and to RARs) and is active in preventing skin cancer, another class is characterised by a hydroxyl or non-polar terminal group and is active in preventing mammary cancer, and a third group with a terminal amide group is active in preventing urinary bladder and mammary cancer. According to Hill and Grubbs (1992), of nearly 400 reported tests for retinoid activity in preventing cancer, almost 75% have involved one of only six common retinoids (both natural and synthetic): VAP, RA, VAA, 13-cis-RA, etretinate, or 4-HPR, each of which has some undesirable toxic effects. Thus, although synthetic retinoids have shown great promise as potential chemopreventative agents against certain forms of cancer, new retinoids with improved activity/toxicity ratios, or other means of achieving higher efficacy are sought (Hill and Grubbs 1992).

Significant reductions in the incidence and multiplicity of carcinomas have been achieved with retinoids in experimental animals. However, the anti-carcinogenic activity of all of the presently available compounds is incomplete, that is, cancer incidence is not reduced to zero when these agents are administered at non-toxic levels (Moon et al. 1992). Furthermore, the toxicity of retinoids remains an important factor and limits their potential use. If such toxicity could be
reduced, higher doses of retinoids could be administered, raising the possibility of greater anti-cancer activity (Moon et al. 1992). To address this problem, new compounds related to active agents continue to be designed. In addition, another means by which the efficacy of retinoids may be increased is to co-administer these compounds with other anti-carcinogenic agents (Moon et al. 1992). By combining chemopreventative agents in this way, it is hoped that the anti-cancer efficacy of retinoids can be increased, either through additive or synergistic interaction between two chemopreventative agents, or through the reduction of agent toxicity. For example, the combination of 4-HPR and tamoxifen (an anti-oestrogen) acts synergistically to afford greater protection against mammary cancer than that of either agent alone (Moon et al. 1992; Tallman and Wiemik 1992). These positive findings have led to clinical trials in humans of combined 4-HPR/tamoxifen treatment against breast cancer (Tallman and Wiemik 1992; Kelloff et al. 1994).

(ii) Cancer prevention in man with natural and synthetic retinoids

In the 1970's, natural forms of vitamin A (RA and VAP) were evaluated in several clinical trials as therapeutic agents for various human malignancies including basal cell carcinomas, urinary bladder papillomas, bronchial carcinomas, cervical carcinomas, melanomas, and squamous cell carcinomas of the head and neck (reviewed by Ong and Chytil 1983). In addition, natural retinoids were also evaluated for their potential to prevent the development of cancer in a variety of pre-cancerous lesions such as actinic keratoses and oral, cervical and vulval leukoplakia (Bollag and Holdener 1992). The success rate in all these studies was low because the dose levels required proved too toxic (Ong and Chytil 1983; Bollag and Holdener 1992). More recently, RA treatment has been reported to lead to complete remission (though not a cure) of a form of human acute promyelocytic leukaemia (Huang et al. 1988).

In contrast to natural retinoids, there has been much more success in preventing and treating human cancer with certain synthetic retinoids (Hill and Grubbs 1992; Holdener and Bollag 1992). In particular, retinoids such as 13-cis-RA have proved effective in preventing cancers of the skin and/or head and neck (Lippman and Meyskens 1987; Kraemer et al. 1988), and urinary bladder (Alfthan et al. 1983; Studer et al. 1984). Unfortunately, there is still significant potential toxicity associated with synthetic retinoids. Synthetic retinoids, like their natural analogues, can
accumulate in the liver and cause liver damage; they can also result in eye damage, and they are teratogens (Kelloff et al. 1994). Although the most efficacious synthetic compounds, such as 4-HPR, appear to be less toxic than vitamin A, there is still cause for concern. As a result of this potential toxicity, the use of synthetic retinoids has been limited to patients who have had a previous cancer and to those who are at high risk of developing cancer (Bollag and Holdener 1992; Kelloff et al. 1994). People at high risk of developing cancer, according to Bollag and Holdener (1992), are individuals with a high genetic or environmental cancer risk (e.g. patients with xeroderma pigmentosum, individuals with a family history of high susceptibility to cancer, cancer patients with an increased chance of developing second primary tumours within a relatively short time after removal of the first primary, or people who are constantly exposed to carcinogenic substances). Other individuals at high risk of developing frank carcinoma are those presenting with certain pre-malignant conditions (e.g. actinic keratosis of the skin, oral leukoplakia, bronchial metaplasia and dysplasia, cervical dysplasia, and urinary bladder papillomas). Owing to retinoid toxicity, it has been in these categories of high risk individuals only, that prophylactic treatment with these agents has been considered (Bollag and Holdener 1992). Although results of pre-clinical studies and clinical trials have been encouraging so far, there is still an urgent need to synthesise new retinoids with a more favourable therapeutic index, to make these treatments more favourable (Bollag and Holdener 1992).

C Possible mechanisms for the role of vitamin A and its analogues in carcinogenesis

Cancer may be regarded as a family of diseases in which neoplastic transformation, by whatever means this is induced, results in an altered phenotype, such that cancer cells express certain properties which differ from those of the normal cells from which they are derived (Hicks 1978). Hicks (1978) suggested that the logical conclusion of this argument is to regard cancer as a disease, not necessarily of differentiation, but one in which some concomitant phenotypic change in differentiation is obligatory. Indeed, others, referred to by Sporn and Roberts (1984), have regarded cancer as a disease of abnormal differentiation, while Sporn and Roberts themselves regarded the role of retinoids in carcinogenesis or differentiation as essentially the same.

As outlined above, vitamin A has been linked with neoplastic development since the early observations of Fujimaki (1926), who reported the formation of gastric carcinoma in vitamin A-deficient rats. Four years earlier, Mori (1922) and later Wolbach and Howe (1925) had reported that vitamin A deficiency lead to squamous metaplastic changes in the epithelia of the respiratory, gastro-intestinal and urino-genital tracts. These observations indicated that vitamin A deficiency leads to failure of normal differentiation, thereby demonstrating the importance of
vitamin A in the control of epithelial cell differentiation (Hicks 1983). Associated with the squamous metaplastic changes seen in vitamin A deficiency, there is often increased proliferative activity leading to hyperplasia of the affected epithelium (Wolbach and Howe 1925; Hicks 1983; Pitt 1985). These profound changes in differentiation and proliferation in vitamin A deficiency are completely reversed by the restoration of normal vitamin A levels (Hicks 1983), a finding which is true both in vivo and also in vitro in organ culture (Wolbach and Howe 1933; Lasnitzki 1955).

Indeed, it was Lasnitzki's (1955) observation that vitamin A reversed hyperplastic and anaplastic changes induced in mouse prostate organ cultures which renewed interest in the role of vitamin A in differentiation, and its possible role in the development of neoplastic disease (Sporn and Roberts 1984). Lasnitzki's experiments showed that the effects of vitamin A were to suppress the abnormal cellular differentiation that had been induced by the carcinogen in the prostate epithelium, and to restore a more normal pattern of differentiation (Sporn and Roberts 1984).

Interest in the link between cancer and vitamin A increased still further in 1978 when Harisiadis et al. showed that a synthetic retinoid could suppress the malignant transformation by radiation of non-neoplastic mesenchymal cells grown in continuous culture. Similar results were obtained by Merriman and Bertram (1979) in retinoid-treated non-neoplastic cells which had been previously exposed to the chemical carcinogen MCA. These experiments showed that retinoids could suppress the expression of the transformed phenotype in cells which had been previously initiated by a carcinogenic stimulus (Sporn and Roberts 1984).

Sporn and Roberts (1984) stated that any hypothesis to explain the mechanism by which retinoids control differentiation, and, therefore, affect carcinogenesis, must take into account that RA will support growth in the intact animal as effectively as retinol, that RA is more active than retinol or retinal in many in vitro test systems (Lotan 1980), and that RA can not be reduced to retinol.

Sporn and Roberts (1984) proposed their own hypothesis, although they were not the first to think of it, that the mechanism of vitamin A action in controlling cell differentiation was through effects upon gene expression. This hypothesis has received much support from the recent discovery of the retinoid nuclear receptors (Giguere et al. 1987; Petkovich et al. 1987), belonging to the steroid/thyroid hormone receptor superfamily of ligand-dependent transcription factors. Through the interaction of these receptors with DNA, vitamin A is thought able to induce and/or repress the expression of target genes, specifically to induce cell differentiation and/or to inhibit cell proliferation (Bollag and Holdener 1992). The multiplicity of the retinoic acid receptors allows for the diversity of responsiveness to vitamin A, as different cell types express different receptors (Holdener and Bollag 1993).
Theoretically, the alteration of gene expression by retinoids could account for the many biological effects which have been linked with the anti-cancer activity of these agents including: diminished expression of oncogenes such as *myc*, regulation of cytoskeletal elements, enhancement of macrophage phagocytic and tumouricidal activity, regulation of cell entry into phases of the cell cycle, production of gap junctions; permitting cell-to-cell interaction, inhibition of tumour promoter-induced ornithine decarboxylase activity and carcinogen-induced DNA synthesis (Tallman and Wiemik 1992).

To support their hypothesis that vitamin A controlled differentiation and proliferation through gene expression, Sporn and Roberts (1984) pointed to evidence which indicated that vitamin A was involved in controlling the expression of many proteins associated with the cellular cytoskeleton and the extracellular matrix, including the keratins, collagen, collagenase and laminin. Cytoskeletal and matrix proteins are now used as markers of cellular differentiation, illustrating the association of vitamin A with the expression of differentiation specific markers. Other genes known to be controlled by vitamin A included plasminogen activator, the receptor for epidermal growth factor and the proliferation-associated *myc* oncogene (Sporn and Roberts 1984).

More recently, Roberts and Sporn (1992) have highlighted another possible mechanism by which retinoids could exert a cancer chemopreventative action, namely through their ability to stimulate the local production of the cytokine, transforming growth factor-beta (TGF-b). This growth factor is a potent inhibitor of epithelial cell proliferation, and there is growing evidence that loss of responsiveness of cells to TGF-b is often associated with neoplastic progression (Roberts and Sporn 1992). However many tumours remain responsive to inhibition by TGF-b, especially in the early stages of malignant progression. Retinoids have been demonstrated to induce expression of TGF-b as well as TGF-b receptors (Glick *et al.* 1989, 1990), suggesting that local enhancement of responsiveness to TGF-b may have a role in the mechanism of cancer chemoprevention by retinoids (Roberts and Sporn 1992).

There is some evidence that immuno-potentiation by retinoids may be involved in their anti-cancer action (Hicks 1983; Goss and McBurney 1992). RA and other retinoids stimulate T-killer-cell induction *in vitro* and increase cytotoxicity *in vivo*. Clinically, retinoids have been claimed to have an immuno-potentiating effect in the treatment of lung cancer (Micksche *et al.* 1977). Other effects of retinoids on the immune system include the enhancement of skin holograft rejection and the enhancement of antibody response to a variety of antigens (Goss and McBurney 1992). These
effects upon the immune system might well account, in part, for the anti-tumour effects of retinoids (Goss and McBurney 1992).
CHAPTER 3

BETA-CAROTENE and CANCER

1  HISTORICAL PERSPECTIVES

In 1981, Peto et al. published a pioneering review in which they focused attention on the possibility that provitamin A carotenoids, in particular BC, might have a beneficial effect upon human cancer. At the time of this 1981 review, interest had been growing in the potentially beneficial role of vitamin A in human cancer. This interest had been awakened primarily as a result of early prospective epidemiological studies suggesting that lower than average plasma levels of vitamin A were linked to a subsequent higher than average incidence of cancer (Wald et al. 1980; Kark et al. 1981).

Peto et al. (1981) reviewed the prospective studies of Wald et al. (1980) and Kark et al. (1981), and also the literature of the day detailing all retrospective studies of blood retinol levels in cancer patients, eight out of nine of which had reported significant reductions in blood retinol levels. Peto et al. (1981) also reviewed 5 prospective and 15 retrospective studies in which the intake of dietary BC had been examined in relation to the risk of developing cancer. The majority of these dietary studies indicated an inverse correlation between intake of BC and cancer incidence. Peto and associates pointed out that due to pharmacokinetic differences between BC and retinol in the human body, the possible relevance of these two components of 'dietary vitamin A' to cancer prevention should be assessed separately (at the time epidemiological analyses of the association between dietary vitamin A and cancer risk tended to combine BC and pre-formed vitamin A together). Although the epidemiological data at the time indicated that both retinol and BC were associated with a lower cancer risk, Peto et al. (1981) commented that the apparent protective effects were not great, and that the only sure method of determining whether BC and retinol did protect against cancer would be by reproducing these protective effects in randomly controlled prospective intervention studies. In addition to pointing out the need for human intervention studies, Peto et al. (1981) also suggested that there was an urgent need for further laboratory animal investigations into the possible mechanisms whereby BC might exert anti-cancer activity. In particular, these authors suggested that it would be useful to discover whether dietary (not topical) BC started some time after weak (not massive) initiation of animals with DNA-binding carcinogens, has any material inhibitory effects, analogous to the inhibitory effects induced by
natural and synthetic retinoids, on the later stage(s) of carcinogenesis, especially in tissues where
dietary BC is known to accumulate or to cause accumulation of retinol.

As a direct result of the report by Peto and his associates, there was a rapid increase during the 1980's in the number of epidemiological investigations in the literature of the possible association between retinol and/or BC, and a wide range of human cancers. During this time period, improvements in epidemiological techniques have tended to negate the promising findings of early studies relating vitamin A to human cancer risk (Michels and Willett 1994). In contrast, in both dietary studies and in studies of blood levels, the association of BC with cancer has been strengthened for almost every site investigated (Michels and Willett 1994). There is now almost overwhelming epidemiological evidence supporting the thesis that foods containing BC are protective against the development of human cancer (Steinmetz and Potter 1991a; Block et al. 1992; Willett 1994a). However, evidence confirming BC as the actual protective agent, rather than as a 'marker' for protective foods, or beneficial lifestyles, is lacking, and in recent years there has been growing uncertainty as to whether the protective effects attributed to many carotene-rich foods (pigmented fruits and vegetables) are, in fact, due to BC, or to some other factor(s) common to these fruits and vegetables (Steinmetz and Potter 1991b; Block et al. 1992; Greenberg 1993; Rogers et al. 1993; Willett 1994a).

As suggested by Peto et al. (1981), a large number of intervention studies have been established to examine the role of BC in human cancer (Malone 1991; Kelloff et al. 1994; Szarka et al. 1994), the largest of which is a double-blind random cross-over study of both BC and aspirin in 22,000 US Physicians (Hennekens and Eberlein 1985).

In contrast to the relatively large numbers of human intervention studies that have been established as a result of the review by Peto and associates (1981), to date there have been few reports of experimental investigations into the potential mechanisms of BC anti-cancer activity (Moon 1989; Rogers et al. 1993; Kelloff et al. 1994). The reason for this shortage of laboratory investigations has been the belief that the efficient conversion of dietary BC to retinol by experimental animals renders it very difficult to produce animals with appreciable tissues levels of BC (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994).
2 DISTRIBUTION OF BC AND RELATED CAROTENOID PIGMENTS IN NATURE AND IN THE HUMAN DIET

BC is one member of a family of more than 500 naturally occurring pigments known as the carotenoids (Steinmetz and Potter 1991b). These pigments are widely distributed in nature (Karrer and Jucker 1950), where they provide colouration to both plants and animals (such as arthropods, molluscs and fish).

In the human diet carotenoids are present in yellow and orange vegetables and fruits and in dark green leafy vegetables (Steinmetz and Potter 1991b). BC is the most well known carotenoid. BC is found in most orange vegetables and fruits and in dark green leafy vegetables. However, according to Steinmetz and Potter (1991b), BC is not the predominant carotenoid in most vegetables. Sweet potatoes, carrots and red palm oil are especially high in BC. Green leafy vegetables such as spinach, broccoli, sprouts and cabbage are moderately high in BC, but the predominant carotenoids in these vegetables are the oxygenated carotenoids (xanthophylls).

Lutein is the major oxygenated carotenoid and is found predominantly in green vegetables such as kale, spinach and parsley. BC, alpha-carotene and lycopene are hydrocarbon carotenoids.

Lycopene is found in tomatoes and red palm oil, but is scarce in other common vegetables. Lutein and lycopene are the carotenoids which are found at the highest levels in human plasma (Steinmetz and Potter 1991b). Zeigler (1989) pointed out that BC represents less than 30% of all the carotenoids in human plasma. Other carotenoids present in human plasma as well as lutein, lycopene and BC are cryptoxanthin, zeaxanthin, epoxycarotenoids, violaxanthin and neoxanthin (Zeigler 1989).

3 CHEMICAL STRUCTURE OF BC AND SOME OTHER CAROTENOIDS

The chemical structures of BC, α-carotene, cryptoxanthin (three provitamin A carotenoids) and two carotenoids lacking vitamin A activity (lutein and canthaxanthin) are shown in Figure 2.2. Other common carotenoids lacking vitamin A activity, but not shown in Figure 2.2, include phytoene, crocetin and fucoxanthin (Krinsky 1992).

BC is a 40-carbon molecule that can be metabolised in animals to form 20-carbon retinol (vitamin A), by a split in the molecule through the central double bond (Greenberg et al. 1985; Pitt 1985). About 50 other carotenoids can also be metabolised to vitamin A, including α-carotene and cryptoxanthin (Moore 1957). Of all 50 provitamin A carotenoids, BC is the most common (Moore 1957).
AN OVERVIEW OF THE EPIDEMIOLOGICAL EVIDENCE SUGGESTING A PROTECTIVE ROLE FOR BC IN THE DEVELOPMENT OF HUMAN CANCER

There have been many epidemiological studies of the association between dietary BC and various human cancers. Retrospective studies and prospective studies, involving dietary questionnaire or the determination of blood levels of BC, have been used to study this association. These have been reviewed by many authors including Peto et al. (1981), Wolf (1982), Ong and Chytil 1983, Mathews-Roth (1985), Pitt (1985), Basu et al. (1988), Zeigler (1989), Steinmetz and Potter (1991a), Block et al. (1992), Willett (1994b), and Michels and Willett (1994).

There have been several studies demonstrating a protective effect of a high dietary intake of green and yellow vegetables for cancer of all sites (Shekelle et al. 1981; Colditz et al. 1985; Shibata et al. 1992a). However, it was not possible to identify one particular nutrient responsible in studies which quantified nutrient intake (Michels and Willett 1994). The BC arm of the US Physician's intervention study, a placebo-controlled randomised clinical trial, is attempting to assess the value of BC supplements for the prevention of cancer at all sites in men (Hennekens and Eberlain 1985).

There has been one blood-based prospective cohort study and a number of nested case-control prospective studies in which the level of BC was determined prior to cancer occurrence. In the Basel study, no relationship was found between serum BC and cancer mortality after 3.7 years of follow-up (Stahelin et al. 1984). However, when cancer mortality was assessed again 7 years after measurement of blood levels, significantly lower mean BC levels were found for the 102 individuals who had died from cancer compared with the survivors (Stahelin et al. 1984). This inverse correlation was confirmed in the 12-year follow-up when 204 cancer deaths and 2421 survivors were compared (Stahelin et al. 1991). In the study of Willett et al. (1984), there were 111 individuals who were free of cancer at the time of blood collection but were later diagnosed with cancer during the subsequent five years. When these cases were compared with 210 matched controls, mean values for total carotenoids were found to be similar for both groups. A similar observation was made for men of Japanese ancestry in Hawaii (Nomura et al. 1985), where the 284 cancer cases and the 302 controls had comparable BC levels.

In contrast to the reports of Willett et al. (1984) and Nomura et al. (1985), significantly lower BC levels were found in the BUPA study (Wald et al. 1988), in 271 males who were subsequently diagnosed with cancer compared with 533 matched controls. In a nested cases-control study in
Finland (Knekt et al. 1990), the mean BC level was found to be higher in the 1419 controls than in the 766 cases, although this difference was only significant in men.

There is strong evidence for an inverse relationship between intake of fruits and vegetables and lung cancer (reviewed by Peto et al. 1981; Steinmetz and Potter 1991a; Block et al. 1992), which has led to the suggestion that BC might be a protective factor. This hypothesis has been supported by inverse associations between intake of BC-containing foods, such as carrots, and lung cancer in multiple studies (reviewed by Michels and Willett 1994). However, according to Willett (1994a) and Michels and Willett (1994), most studies have not been sufficiently comprehensive to distinguish with confidence an effect of BC from that of other nutrients. Furthermore, it is almost impossible to control completely for the confounding effects of smoking and high alcohol intake, both of which can lower the levels of BC in plasma and, therefore, could lead to false estimates of increased cancer risk with decreased blood BC levels (Stryker et al. 1988; Michels and Willett 1994). Notwithstanding these difficulties, the strongest epidemiological evidence for a protective role of BC in cancer, has been found for lung cancer. Several prospective studies have examined this association, including those of Shekelle et al. (1981), Knekt et al. (1991b), and Shibata et al. (1992a and b). After adjustment for smoking, a significant inverse association between BC and lung cancer was reported for men followed-up for 19 years in the Western Electric Study (Shekelle et al. 1981). In the 20 years of follow-up of 4538 initially cancer-free men in the Finnish Mobile Clinic Health Survey (Knekt et al. 1991b), 117 cases of lung cancer were found; in this study an inverse gradient was observed between estimated intake of carotenoids and the incidence of lung cancer among non-smokers, but not among smokers. In the Leisure World Study, no protective effect was found for high BC intake in men, although a weak protective relation was observed for women (Shibata et al. 1992a and b). Several retrospective studies, such as that of Kolonel et al. (1985), have demonstrated inverse relationships between estimated BC intake and lung cancer, or an increased risk of lung cancer from low BC intake (Zeigler et al. 1989).

The findings of blood-based studies in relation to BC and lung cancer have been largely in agreement with those of the above dietary studies (Michels and Willett 1994). Thus, there is strong epidemiological evidence suggesting that BC, as the major provitamin A carotenoid in fruits and vegetables, has a protective role in the development of human lung cancer. It was, with great surprise, therefore, that the scientific community learnt of the results from a Finnish intervention trial in male smokers that reported an increased incidence of lung cancer in individuals receiving supplemental BC (Nowak 1994). The study in question, reported by Heinonen and Albanes (1994), on behalf of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, was a
randomised, double-blind, placebo-controlled trial, the best possible design for a medical intervention study. The outcome of the Finnish study clearly contradicts the large body of epidemiological evidence indicative of an inverse association between higher BC intake and a lower lung cancer incidence. Furthermore, the Finnish results also contradict those of the only other large-scale clinical trial completed so far. This second study was conducted in the Linxian area of China and found that a combination of BC, vitamin E and selenium reduced the number of deaths from stomach cancer by 21% among 15000 participants, compared with individuals who did not take the supplements (Blot et al. 1993). As the Finnish data are contrary to those of so many other studies, it is possible that the findings of Heinonen and Albanes (1994) may have been due to chance, a fact conceded by the authors themselves.

Although the majority of epidemiological studies of the relationship between BC and cancer have focused on lung cancer, a smaller number of studies have examined the relationship, if any, of BC to neoplasms at other sites including the breast, cervix, ovary, endometrium, colorectum, stomach, oesophagus, pharynx, larynx, mouth, pancreas, prostate, urinary bladder and skin (Michels and Willett 1994). In prospective studies, breast cancer appears to be weakly although not definitively associated with BC intake (Shibata et al. 1992a; Hunter et al. 1993; Michels and Willett 1994). However, conflicting results have been obtained from the large number of retrospective investigations that have examined BC intake and this form of cancer (Michels and Willett 1994). Retrospective studies relating BC to cervical cancer and/or cervical dysplasia, or to ovarian cancer, have produced inconsistent results (Michels and Willett 1994), with some investigations demonstrating inverse relations between BC intake and cancer, while others have found no such association. For cancers of the stomach, colon and rectum, the data from dietary studies and from blood-based investigations are only marginally more convincing than those for cervical or ovarian cancer. These results indicate that there are possibly weak inverse relationships between estimated BC intake and these gastro-intestinal cancers (Michels and Willett 1994). Similarly, no consistent results have been obtained in investigations of the relationships between oral, pharyngeal and laryngeal cancers, and intake of BC. No significant inverse associations have been reported for BC and oesophageal cancer. Retrospective dietary studies have demonstrated some evidence of a protective effect for BC in pancreatic cancer. However, this effect has not been confirmed in prospective blood-based studies (Michels and Willett 1994).

No significant association between BC and urinary bladder cancer in men was observed in the Leisure World Study (Shibata et al. 1992). Of all the retrospective dietary studies which have investigated BC intake and urinary bladder cancer (Kolonel et al. 1985; Claude et al. 1986; Risch
et al. 1988; La Vecchia et al. 1989; Nomura et al. 1991; Riboli et al. 1991), only that of La Vecchia (1989) reported a significant inverse relationship between risk of bladder cancer and estimated carotenoid intake. In this analysis, however, total energy intake or other micronutrients were not taken into account (Michels and Willett 1994). Claude et al. (1986) reported that frequent consumption of fruits and vegetables was associated with a reduced risk of bladder cancer, but these workers did not relate these findings to BC or any other nutrients. Retrospective studies have shown no significant difference in pre-diagnostic blood levels of BC between cases and controls (Nomura et al. 1985; Wald et al. 1988, Helzlsouer et al. 1989; Comstock et al. 1991; Knekt et al. 1991a).

In the earliest epidemiological studies of the late 1970s, and continuing into the late 1980's, investigations of the relation between fruit and vegetable intake and cancer risk were heavily biased towards provitamin A carotenoids (Zeigler 1989; Malone 1991). In particular, BC, as the most common provitamin A carotenoid, was generally attributed, but not proven, to be the probable protective agent responsible for many observed inverse associations between intake of these foods and cancer risk. More recently, there has been a growing awareness, highlighted by the reviews of Steinmetz and Potter (1991a) and of Block et al. (1992), that high intake of fruits and vegetables may be beneficial as a result of a constituent(s) other than BC. If this were the case, the presence of BC in certain foods could merely serve as a non-specific marker for beneficial diets or lifestyles (Heinonen and Albanes 1994). It would follow that the potential benefits of high BC intake have been greatly overstated.

There are now more than 200 reports of case-control or cohort studies in which individuals consuming higher than average amounts of fruits and vegetables, or people exhibiting higher than average levels of carotenoids in their blood, were less likely to develop various cancers (Willett 1994a). Apart from BC, other constituents of fruits and vegetables that have been suggested as candidate protective agents within these foods include, for example, other carotenoids, particularly those lacking in vitamin A activity (Zeigler 1989). Intake of vegetables and fruits has also been related to lower risk of stomach cancer in many case-control studies (Block et al. 1992); both the epidemiological data and mechanistic studies have implicated vitamin C as the protective factor (Willett 1994a). Similarly, vegetable and fruit consumption has also been inversely related to risk of colon cancer. This association has been attributed to intake of dietary fibre, but recent evidence has suggested that folic acid might also account for the reduced risk (Willett 1994a). In addition to BC, other carotenoids, vitamin C and folic acid, plants contain numerous other non-nutritional factors that could have potential anti-cancer activity (Wattenberg 1978; Fiala et al. 1985), among
which are aromatic isothiocyanates, flavonoids (such as quercetin), indoles, phenolic acids (such as ellagic acid) and protease inhibitors.

5 STUDIES IN EXPERIMENTAL ANIMALS SUGGESTING A PROTECTIVE ROLE FOR BC IN THE DEVELOPMENT OF CANCER

There have been relatively few animal experiments examining the potential anti-cancer activity of dietary BC (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994). Several reports (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994) have stated that this lack of experimentation has been due to the inability of rodent species to absorb sufficient BC into their bodies without converting it into retinol (vitamin A). However, Mathews-Roth et al. (1977) fed a diet high in BC to mice and guinea pigs. These workers found that unconverted BC accumulated in the skin and in several organs and tissues, including liver, ovary, spleen, adrenal, kidney and body fat. They concluded that the difference between 'yellow-fat' species (such as man) that are good accumulators of carotenoid, and those which do not readily store carotenoids ('white-fat' species, such as mice, guinea pigs and rats) is a quantitative rather than a qualitative one. Furthermore, Mathews-Roth et al. (1977) stated that if 'white-fat' species are given a diet high in carotenoids for a significant period of time, they will eventually accumulate carotenoid in skin and other organs. Kornhauser et al. (1986) reported BC accumulation in rats and mice fed a carotene-fortified diet for extended periods. Furthermore, Kornhauser et al. (1986) reported that the levels of BC in the blood and skin of rats fed such a diet established this species as a useful animal model for studying the protective effects of dietary BC. Hicks et al. (1984) fed a high level (5 mM) of BC to B6D2F1 mice pre-treated with the carcinogen BBN, to examine the effect of dietary BC upon the incidence of experimentally-induced urinary bladder carcinomas. In this study, these workers found high plasma levels of unconverted BC in mice given the carotenoid in the diet (Turton 1995).

Furthermore, at post-mortem the body fat and certain internal organs of these mice, but not of the controls, appeared yellow/orange in colour, indicating that BC had been absorbed into the tissues. Thus, it has been shown that appreciable amounts of unconverted BC can be absorbed by the common laboratory rodents provided they are given a sufficiently high level of BC in the diet.

All the available reports of laboratory animal investigations into the potential anti-cancer activity of BC have been reviewed by Peto et al. (1981), Greenberg et al. (1985), Mathews-Roth (1985), Rogers and Longnecker (1988), Moon (1989), and Krinsky (1992).
A small number of experimental studies have been reported in the literature which indicate that dietary carotenoid supplements can inhibit the process of cancer induction in animals (Moon 1989; Krinsky 1992). These studies have included carotenoids, such as BC, which exhibit provitamin A activity, and others, such as canthaxanthin, which are not metabolised to vitamin A.

Early carotenoid experimentation involved the prevention of tumours induced in rats and mice by either UV alone (Epstein 1977; Mathews-Roth 1983; Mathews-Roth and Krinsky 1987), a combination of UV and chemical carcinogens such as DMBA (Mathews-Roth 1982), BP (Santamaria et al. 1983), or MNNG (Santamaria et al. 1985). Other groups have used dietary or environmental carcinogens in experimental animals supplemented with carotenoids (Krinsky 1992). Under these experimental conditions, BC or canthaxanthin have protected against either DMBA-induced salivary tumours in mice (Alam et al. 1984; Alam and Alam 1987; Alam et al. 1988), or dimethylhydrazine (DMH)-induced colon tumours in mice (Temple and Basu 1987; Basu et al. 1988). Topical administration of BC not only inhibited (Suda et al. 1986) but also reversed (Schwartz et al. 1986) squamous cell carcinoma produced in the hamster buccal pouch by treatment with topical DMBA. Similar results were obtained with oral administration of either BC or canthaxanthin (Schwartz et al. 1988).

BC was reported by Lambert et al. (1990) to protect against two-stage skin carcinogenesis (DMBA/TPA) in Skh mice, although under the same conditions no significant protection was obtained in SENCAR mice. In similar experiments, Steinel and Baker (1990) reported that BC induced a significant inhibition of skin papillomas, but not of the ultimate development of malignant tumours, in Skh mice treated with DMBA and TPA. These results led these workers to conclude that BC was active during the TPA promotion phase of carcinogenesis.

Rettura et al. (1984) reported that dietary BC inhibited the development of rat mammary tumours induced by DMBA. Canthaxanthin, a carotenoid lacking vitamin A activity, was reported by Grubbs et al. (1991) to induce a 65% reduction in the incidence of mammary tumours in rats treated with DMBA, when fed for 3 weeks prior to administration of the carcinogen. Furthermore, these authors showed that the results obtained with canthaxanthin were no different to the reduction by VAA in mammary tumour incidence in rats treated with MNU. In this instance, the experimentalists concluded that the carotenoid was not inhibiting promotion (Krinsky 1992).

Moon (1989) considered the reports of Reider et al. (1983) and Dorogokulpa et al. (1973) to, perhaps, most simulate human carotenoid exposure in relation to carcinogenesis. These studies
involved feeding experimental animals a diet which consisted primarily or entirely of carrots. Reider et al. (1983) reported that feeding carrots alone for 4 or 5 days each week significantly delayed hepatoma-related mortality in rats treated with diethylnitrosamine (DEN). It is probable that during this study intake of protein, fat and most micronutrients was inadequate, although, surprisingly, body weights of carrot-fed and control animals were reported to be similar. Dorogokupla et al. (1973) reported that feeding unlimited amounts of carrots inhibited sarcoma development and prolonged tumour latency in mice given DMBA.

In addition to the lack of protection afforded by BC against DMBA/TPA treatment in SENCAR mice reported by Lambert et al. (1990), several other negative results have been reported in trials of the anti-cancer activity of carotenoids. Imaida et al. (1990), found only weak organ-specific effects of BC against carcinogenesis induced in F344 rats by DMH followed by MNU. In another report (Mathews-Roth et al. 1991), BC or canthaxanthin was fed for 5 weeks before, and 26 weeks after treatment with BBN, and only the mice receiving the supplemental BC showed significant protection against the development of bladder tumours. In contrast to the protective effect of BC against the development of bladder tumours reported by Mathews-Roth et al. (1991), Hicks et al. (1984) demonstrated no effect of BC on urinary bladder carcinomas, when the carotenoid was given to B6D2F1 mice after the administration of BBN. These results are supported by unpublished data from Moon's laboratory (cited by Moon 1989), who found no effect of BC, given by intraperitoneal injection, on BBN-induced bladder tumours in mice. Unpublished data from other animal tumour models cited by Moon (1989) demonstrated no effect of BC in the MNU rat mammary tumour model, and no effect on lung tumours induced in hamsters by either MNU or DEN. However, when retinol was given in the diet, in addition to the dietary BC supplement, inhibition of DEN-induced hamster lung tumours was observed (Moon 1989).

6 POSSIBLE MECHANISMS OF THE ANTI-CANCER ACTIVITY OF BC AND OTHER CAROTENOIDS
Kelloff (1994) stated that the rationale behind the decision to set up clinical trials to examine whether BC protects against the development of human cancer was based on three criteria. These criteria are: 1) the strong epidemiological evidence indicating that BC protects against lung cancer, 2) the possession of a chemical structure indicative of the ability to scavenge free radicals and, 3) the potential for conversion to vitamin A.
The epidemiological evidence linking BC with lung cancer, and cancer at other sites, has already been reviewed above. Free radicals, such as singlet oxygen, are reactive molecular species capable of reacting with cellular macromolecules, including DNA (Rousseau et al. 1992). These reactive moieties, therefore, possess the potential to cause initiating changes in the genetic material which could ultimately lead to the development of cancer (Peto et al. 1981; Palozza and Krinsky 1991; Rousseau et al. 1992). Excited oxygen molecules (singlet oxygen) that are generated as by-products of normal cellular metabolism (Steinmetz and Potter 1991b). Many carotenoids including those with provitamin A activity, such as BC, and those lacking such activity, such as lycopene, are extremely efficient at deactivating singlet oxygen and trapping free radicals including singlet oxygen (Rousseau et al. 1992). BC is one of the most efficient quenchers of singlet oxygen (Rousseau et al. 1992), although other common carotenoids, such as lycopene, are even more effective than BC (Steinmetz and Potter 1991b; Rousseau et al. 1992). These antioxidant effects may protect cells against oxidative DNA damage. Thus, BC could exhibit anti-carcinogenic activity through the ability to prevent free radical-induced DNA damage. In this case, BC would be acting at the early initiation stage of carcinogenesis. Rousseau et al. (1992) have written an excellent review detailing the carcinogenic actions of free radicals and the prevention of free radical damage by BC.

Perhaps the most likely means by which BC could exert an anti-carcinogenic effect is through conversion to vitamin A. If this is true, BC would probably exert its anti-carcinogenic activity during the later promotional stages of carcinogenesis, in a similar manner to vitamin A and the synthetic retinoids. However, Krinsky (1992) in a recent review, pointed out that experimental evidence has been growing that carotenoids without vitamin A activity, such as canthaxanthin, crocetin, fucoxanthin, and lycopene, have similar anti-cancer activities to provitamin A carotenoids, such as BC. It would seem, therefore, that these particular results are attributable to properties of the intact carotenoid molecule, and not necessarily to metabolites such as retinol, retinal or RA. It follows from this suggestion that the anti-cancer activities that have been reported for carotenoids lacking provitamin A activity may be related to the known biological and chemical properties of carotenoids, which include, among others, photoprotection, quenching of free radicals, and antioxidant behaviour (Krinsky 1992). However, if these properties are involved in the anti-carcinogenic actions of carotenoids, the mechanisms remain to be elucidated (Krinsky 1989; Krinsky 1992).

If mechanisms such as free radical quenching and antioxidant behaviour are involved in the anti-cancer activity of carotenoids such as canthaxanthin, it is unclear whether these same
mechanisms are involved in the reported anti-cancer activity of provitamin A carotenoids. Thus, while the anticarcinogenic efficacy of some carotenoids, such as BC, may be related to vitamin A, the available evidence suggests that the chemopreventative activity of other members of the carotenoid family are likely to be unrelated to vitamin A (Moon 1989).

7 INTRODUCTION TO THE EXPERIMENTAL WORK DESCRIBED IN THIS THESIS

The experimental work described in this thesis was conducted in response to the report by Peto et al. (1981). These authors, in reviewing the relatively scant literature describing animal studies investigating the anti-cancer activity of BC, suggested that further experimental work was needed to define more clearly the role of BC in cancer. In particular, (Peto et al. 1981) suggested that experiments were needed that examined the activity of BC against cancers induced by moderate doses of chemical carcinogens.

The main aim of the experimental work described in this thesis, therefore, was to investigate the effect, if any, of a high dietary level of BC on the incidence of urinary bladder TCC induced in rats by a moderate dose of the specific bladder carcinogen BBN. In addition, if BC was found to be active against experimental bladder cancer, it was hoped that some light could be thrown on the possible mechanism of that activity. The rat BBN model has been used extensively in studies of the chemopreventative activity of natural and synthetic retinoids when given after carcinogen treatment (Becci et al. 1979a; Hicks et al. 1982b; Hicks et al. 1985; Moon et al. 1992). The present experiment to evaluate the anti-cancer activity of BC (Trial VIII) was conducted using F344 rats, an inbred strain used extensively for carcinogenicity studies and also in investigations of retinoid activity against bladder cancer. Hicks et al. (1984) have shown in mice that administration of a diet containing BC (6 mM/kg) after BBN treatment had no effect on the final incidence of TCC. Thus, in Trial VIII, dietary BC was administered prior to carcinogen dosing, to investigate any effect of the carotenoid on the early initiation stages of BBN-induced carcinogenesis. Trial VIII is described in Chapter 7 of the thesis.

At the time the design of Trial VIII was conceived, the most plausible mechanism by which BC was thought likely to exert anti-cancer activity was through prior conversion to vitamin A. Thus, a further element of the design of Trial VIII involved the administration of dietary BC (3 mM/kg) to rats fed a diet containing a normal level of vitamin A, or a diet deficient in vitamin A. This experimental design was chosen to investigate whether any anti-cancer activity shown by BC was mediated through conversion to vitamin A, or by some other mechanism.
Before Trial VIII could be attempted, however, an effective commercially-available vitamin A-deficient was required. Initial studies (Trial I) evaluating diets manufactured by our usual supplier failed to demonstrate evidence of vitamin A deficiency within an adequate time period. Two further experiments (Trials II and III) were required before a suitable commercial diet (SSD(ii)) was obtained. Trials I, II and III are described in Chapter 5 of this thesis. Once a suitable vitamin A-deficient diet was available, further experimentation was required to determine a method of maintaining vitamin A-deficient rats in an otherwise healthy condition for long periods. Therefore an experiment (Trial IV) was conducted to determine the appropriate level of vitamin A supplementation required to maintain vitamin A-deficient rats for long periods.

Administration of a low level of water-miscible form of VAP in the rat drinking water was chosen as the method of vitamin A supplementation. As the level of VAP supplementation determined in Trial IV was higher than expected, two small in vitro studies (Trial V(a)) were carried out to investigate the stability of VAP in the drinking water. These in vitro experiments confirmed that there was considerable breakdown of VAP over the 3-4 day period between each replenishment with fresh drinking water. In another small in vivo study (Trial Vb) examined the effect, if any, of falling VAP consumption during a 3-4 day watering period on the plasma retinol levels of groups of rats killed each day during this time. Finally, it was necessary to confirm that high dietary BC would reverse vitamin A deficiency in female F344 rats fed the SSD (ii) diet, and also to demonstrate that female F344 rats could accumulate appreciable levels of BC in their bodies without converting it to vitamin A. Trials IV, V(a), V(b) and VI are described in Chapter 6.

Finally, before BBN was administered to the large number of rats required for Trial VIII, a small pilot experiment (Trial VII) was conducted to examine the survival of vitamin A-deficient rats after repeated oral dosing with BBN. Trial VII is described in Chapter 7, immediately prior to Trial VIII.
CHAPTER 4

GENERAL MATERIALS AND METHODS

1 INTRODUCTION

The experimental work described in this thesis consists of eight trials performed in three separate chronological phases. These phases are described in Chapters 5, 6 and 7. Trials I, II and III (Chapter 5) evaluated several diets in the search for an effective, commercial, vitamin A-deficient diet. When a suitable diet had been chosen, Trials IV to VII (Chapter 6) were carried out to establish baseline parameters in animals fed the deficient diet. Finally, Trial VIII (Chapter 7) evaluated the anti-carcinogenic potential of beta-carotene against experimental urinary bladder cancer in the rat.

To avoid repetition, general methodology common to the eight trials is described in the present chapter. Any variations, and the specific details of each experimental protocol, are described in the experimental design of each trial.

2 MATERIALS

A Animals

Specific Pathogen Free (SPF) category *** weanling, inbred female F344 (Fischer) rats were supplied by Harlan Olac Ltd. (Bicester, Oxfordshire OX6 0TP). Animals were housed in groups of 5 to 8 in polypropylene cages (North Kent Plastics Ltd., Dartford, Kent DA8 2AN) with stainless steel grid bottoms and sawdust covered trays. Temperatures were maintained at 20-21°C. Relative humidity was maintained at 50 - 60% and there was a 12:12h light:darkness cycle.

Animals were observed daily for signs of ill health or evidence of vitamin A deficiency. Those that became moribund or were in extremis were killed and a post-mortem examination carried out.

B Diets

Four commercially-available diets (SDS Ltd., Witham, Essex CM8 3AB) and one laboratory-prepared diet were used. The name, composition, and vitamin A content of each diet is given in Appendix 1.
All diets and drinking water were available ad libitum, the former from stainless steel food pots designed to reduce wastage, and the latter from the mains supply in 560 ml serum bottles.

C Dietary supplements
Where necessary, the vitamin A-deficient diets were supplemented with vitamin A and/or BC in the form of beadlet preparations. Stocks of beadlets were stored at 4°C under oxygen-free nitrogen (B.O.C. Special Gases Ltd., London SW19 3UF) in foil-covered, hermetically sealed containers.

(i) Vitamin A
VAA was added to the diet as stabilised beadlets (Dry Vitamin A Acetate, Type 500, Roche Products Ltd., Welwyn Garden City, Herts AL7 3AY). VAA beadlets contain retinyl acetate dispersed in a matrix of gelatin and sucrose; butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are included as antioxidants. VAA Type 500 beadlets contain about 150,000 pgRE of VAA and approximately 10 mg of BHA and 30 mg of BHT per g.

(ii) BC
BC was added to the diet as beadlets of Rovimix Beta-Carotene 10% (Roche Products Ltd.). Beadlets contain BC (100 mg/g) dispersed in a matrix of gelatin and carbohydrates, with ethoxyquin (ethoxy-trimethyl-quinolin, 23 mg/g) and ascorbyl palmitate (11.5 mg/g) added as antioxidants.

(iii) Water-miscible vitamin A
In some trials VAP was administered in the drinking water as Vitamin A Water-Miscible, Type 100 (Roche Products Ltd.). This is an opalescent, aqueous emulsion of VAP (30,000 µgRE per ml) containing an emulsifier, glycerol, sodium benzoate, citric acid and disodium hydrogen phosphate; BHA and BHT are added as antioxidants. To avoid breakdown, the stock VAP was divided into 1.1 ml aliquots in Eppendorf vials (Sarstedt Ltd, Leicester, Leics LE4 1AW) and stored under oxygen-free nitrogen at 4°C in foiled containers.

3 METHODS
A Body weights, and food and water consumptions
In experiments lasting less than three months, rats were usually weighed weekly. However, with the onset of vitamin A deficiency, weighing was performed twice weekly. In most experiments, continuous food and water consumptions were carried out, with the amount of diet consumed being calculated every 5 to 7 days and water every 3 or 4 days.
B  Diet mixing

BC beadlets were mixed directly into 6 kg batches of diet using an industrial food mixer (Model A2000, Hobart Manufacturing Co. Ltd., London N14 6RT). A premix of VAA in diet was prepared and 60g samples added to 4 kg batches of diet. Each diet was mixed at constant speed for 15 min and stored at 4°C until used.

C  Preparation of VAP-supplemented drinking water

In some experiments vitamin A deficiency was maintained in animals previously made deficient by the administration of low-level VAP in the drinking water. Unless otherwise stated, a concentration of 0.1875 μgRE/ml drinking water was prepared such that a 200g rat drinking 16 ml water each day would receive 3.0 μgRE per day. Preparatory procedures were carried out under non-actinic yellow light. VAP-supplemented drinking water was administered in foil-wrapped water bottles in an animal room with reduced light intensity. Animals received fresh VAP-supplemented drinking water every 3 or 4 days. Continuous water consumptions were performed to estimate vitamin A intake.

D  Post-mortem procedures

(i)  Euthanasia

Rats for scheduled post-mortems, and moribund or in extremis animals were killed by CO₂ overdose and exsanguination by cardiac puncture after laparotomy and thoracotomy.

(ii)  Plasma collection and subsequent analysis

Blood was collected by puncture of the right ventricle using sterile, disposable, plastic syringes and 21 g x 1.5" needles (Gillette UK Ltd., Isleworth, Middlesex TW7 5NP). Aliquots were added to tubes containing lithium heparin (Sterilin Ltd., Hounslow, Middlesex TW3 4EE), gently mixed and spun at 3500 rpm for 10 min. Plasma was pipetted into Eppendorf vials, gassed with oxygen-free nitrogen and stored at -20°C.

Analyses for vitamins A and E, and BC, were carried out on 0.6 ml plasma samples by the courtesy of Dr G. M. Weber (Department of Vitamin and Nutrition Research, F. Hoffmann-La Roche and Co. Ltd., CH-4002, Basle, Switzerland) using the simultaneous HPLC method of Vuilleumier et al. (1983).
(iii) **Excision and fixation of urinary bladder**

The urinary bladder was exposed by laparotomy, emptied by gentle pressure, the urethral opening clamped and the bladder inflated by injecting 0.5 ml of 10% phosphate-buffered formalin (pH 7.4) through the dome via a 25 g x 1.5" needle. The serosal surface of the bladder was bathed with fixative for 1 minute and dissected free. The bladder was reved by cutting across the urethra below the sphincter and placed in formalin fixative.

(iv) **Excision of other tissues**

After removing the bladder, the liver was excised and weighed. In some experiments, samples of the left and median liver lobes were placed in formalin fixative and the remainder weighed and placed in a sealed container and stored at -20°C for analysis of vitamins A and E and BC. In the majority of trials the kidneys and all abnormal tissues were taken for histology.

E **Processing of tissues for paraffin histology**

Urinary bladders were fixed for 48 hours, cut sagitally, processed, embedded in fibrowax, sectioned at 4 mm and stained with haematoxylin and eosin (H&E) using a standard protocol. Other tissues were fixed and processed in a similar manner. In Trials VII and VIII, some kidneys were treated with 'De-cal' (Histological Decalcifying Fluid, National Diagnostics, Somerville, New Jersey, USA) before processing to remove calcium deposits.

F **Photomicrography**

Histopathological assessment of tissues was carried out using a Nikon Type 102 microscope. An Olympus BH-2 photomicroscope was used for photomicrography with 35 mm Technical Pan (Kodak, Hemel Hempstead, Herts HP2 7EH) black and white film developed with Acutol (diluted 1:20, 10 min at 20°C, Paterson Products Ltd., Dagenham, Essex).

G **Classification of urothelial lesions**

Urinary bladder tumours were assessed, classified and graded according to the criteria of Hicks et al. (1982a). The depth of invasion was staged according to the WHO classification of human bladder tumours (Pugh 1973; Mostofi et al. 1973; Hicks et al. 1982a).

H **Statistical analyses**

Statistical analyses were performed only where such analyses was considered essential. Therefore, statistical analyses were limited to all the data of the main trial investigating the anti-cancer activity of BC (Trial VIII). In Trial VIII, the data for body weights, plasma levels of retinol and
BC (but not α-tocopherol), relative bladder weights and tumour volumes were analysed by analysis of variance (ANOVA). Incidences of transitional cell carcinoma (TCC) were analysed using the Fisher exact test (Siegel, 1956).
CHAPTER FIVE

IN SEARCH OF A PROVEN, COMMERCIALLY AVAILABLE VITAMIN A-DEFICIENT DIET

1 GENERAL INTRODUCTION

To evaluate the potential of BC against urinary bladder cancer in vitamin A-deficient rats and vitamin A-sufficient rats (Trial VIII), an effective vitamin A-deficient diet was required which could be supplemented with vitamin A and/or BC. The duration and size of Trial VIII necessitated the use of a commercially-supplied diet. In 1984, Altromin Tier-Labor-Service (Lage, West Germany) manufactured the only commercially-available vitamin A-deficient diet in Europe, but the company could not provide data on the vitamin A content of the formulation. In America, ICN Pharmaceuticals Inc., Life Sciences Group (Cleveland, Ohio, USA) produced a proven diet (Capurro, 1960; Hicks 1968; Cohen et al. 1976; Narisawa et al. 1976) but the transport of multiple batches from America was considered to be too costly and potentially problematic for use in Trial VIII.

SDS Ltd. agreed to develop and manufacture 2 diets, a low level vitamin A diet (LVAD) from natural products, calculated to contain 60 - 120 μgRE/kg vitamin A, and a semi-synthetic vitamin A-deficient (SSD(i)), designed to be virtually free of vitamin A. (For details of the composition of all experimental diets, please refer to Appendix 1).

This chapter describes the evaluation of the efficacy of LVAD and SSD(i) as vitamin A-deficient diets (Trial I), the investigations carried out to determine why these diets were not suitable (Trial II) and finally, the evaluation of a third, modified, semi-synthetic vitamin-A deficient diet SSD(ii) which was manufactured to an exact formulation (Trial III).

2 TRIAL I: EVALUATION OF THE EFFECTIVENESS OF THE LVAD DIET FOR LONG-TERM EXPERIMENTS

A Introduction

The LVAD diet was formulated from natural products to contain a low level of vitamin A and a high fibre content. It was considered that such a diet would, in contrast to a semi-synthetic diet, maintain normal gut physiology necessary for sustaining the health of the animals in the long-term carcinogenicity experiment (Trial VIII), which was to be carried out for a period in excess of 12 -
15 months. Trial I was carried out to examine the time of onset, progress and reversibility of vitamin A deficiency in Female F344 rats fed the LVAD diet. The trial depended heavily upon depression of body weight gain and other clinical signs as indicators of vitamin A status (Moore 1957; Lamb et al. 1974; Underwood et al. 1979). Animals fed the LVAD diet were expected to show signs of vitamin A deficiency after about 12 weeks. As a positive control, groups of animals in Trial I were to be fed the semi-synthetic vitamin A-deficient diet SSD(i), and from reports on similar diets (Moore 1957; Lamb et al. 1974; Shields and Jeffrey 1987), it was anticipated that these animals would become deficient after 5 to 7 weeks.

An additional objective of Trial I, once animals had developed deficiency, was to determine the levels of VAA and BC supplementation which were needed to maintain plasma retinol levels of deficient animals at 30% - 50% of normal values. This was to be investigated by supplementing the LVAD diet with a series of 4 levels of either VAA or BC.

Subsequent analysis of the LVAD and SSD(i) diets by the supplier demonstrated that the LVAD diet contained 38 µgRE/kg vitamin A and the SSD(i) diet contained less than 15 µgRE/kg vitamin A, the threshold limit of detection (Rickett 1987).

B Methods

(i) Experimental Design

A trial involving 204 female F344 rats was set up to study the efficacy of LVAD as a vitamin A deficient diet with the SSD(i) diet as a positive control, and also the levels of VAA or BC supplementation of vitamin A-deficient rats fed LVAD needed to retain the plasma retinol levels of these animals at 30% - 50% of normal values. However, neither the LVAD diet nor the SSD(i) diet was effective in producing vitamin A deficiency and so after 12 weeks the trial was modified. The design of this modified trial (Trial I) is now described.

72 female F344 rats were weaned at 18 days onto the LVAD diet to reduce hepatic vitamin A stores. At 21 - 28 days of age, rats were randomised into 6 groups (Table 5.1). Group 1 (12 rats) received a maintenance diet ("Maintenance" diet, see Appendix 1) containing on average about 1216 µgRE/kg vitamin A. Group 2 (12 rats) were fed the SSD(i) diet and Group 3 (6 rats) were fed the SSD(i) diet supplemented with 2064 µgRE/kg VAA. Group 4 (18 rats) were fed the LVAD diet, Group 5 (12 rats) were fed the LVAD diet supplemented with 2064 µgRE/kg VAA
and Group 6 (12 rats) were fed the LVAD diet supplemented with 4 μM/kg BC. This amount of BC was calculated to provide a similar amount of vitamin A/kg of diet as that provided by 2064 μgRE/kg VAA.

The times and numbers of post-mortems are also shown in Table 5.1. To provide baseline plasma retinol data six rats from Group 4 were killed at the start of the trial (week 0). Six rats from every group except Group 3 were killed at week 7, as those fed the SSD(i) diet were anticipated to become vitamin-A deficient by this time. The trial was terminated and terminal kills (6 rats per group) carried out at week 20.

All rats were weighed and examined in detail for clinical signs of vitamin A deficiency twice weekly. Diet consumptions were determined continuously on rats in all groups every 5 - 6 days. Water consumptions were determined for periods of 2 - 3 days each week.

At post-mortem, the urinary bladder and samples of the trachea were taken for histology. Plasma was prepared for retrospective analysis of vitamins A and E and for BC.

(Animals in Group 1 were fed Maintenance diet to provide data on the normal growth rate of Female 344 rats fed a conventional laboratory animal diet. Such data were important as the trial depended heavily upon depression of body weight gain as well as other clinical signs to indicate the onset and progress of vitamin A deficiency. Analysis of plasma vitamin A was carried retrospectively to substantiate the clinical findings.)

C Results

(i) Body Weights
Summarised body weight increases for rats in Groups 1 - 6 are presented in Fig. 5.1. (For details of the mean body weight data, please refer to Table 5.2). At the start of the trial mean body weight (g ± sd.) of animals in all groups was 49.5 ± 5.5. At 7 weeks (43 days) group mean body weights (± sd.) were: Group 1 (Maintenance), 166.0 ± 7.6; Group 2 (SSD(i)), 166.5 ± 8.6; Group 3 (SSD(i) + VAA), 171.0 ± 8.3; Group 4 (LVAD), 157.0 ± 7.8; Group 5 (LVAD +VAA), 163.6 ± 5.8; Group 6 (LVAD + BC), 163.1 ± 9.3. Therefore there was no evidence of a reduction in body weight at 7 weeks in rats fed LVAD or SSD(i) compared to groups fed the Maintenance diet or LVAD, SSD(i) or diets supplemented with VAA or BC. At 20 weeks (142 days) group mean body weights were: Group 1, 217.3 ± 14.8; Group 2, 211.0 ± 9.0; Group 3, 220.5 ± 9.8; Group 4, 201.7 ± 14.3; Group 5, 203.0 ± 9.6 and Group 6, 215.5 ± 12.9. Thus, at 20 weeks there was
again no evidence of any marked reductions in body weight of rats fed either LVAD or SSD(i) compared to animals fed the Maintenance diet or VAA- or BC- supplemented diets.

(ii) Diet Consumptions
Table 5.2 also shows the mean daily consumption of each diet for Groups 1-6 during the 20 week trial. Rats fed Maintenance diet (Group 1) generally ate more than animals fed other diets. Over the last five weeks of the trial, the average mean daily consumptions (g/rat/day) of each diet (average mean body weight [g] in parentheses) were Maintenance, 15.1 (210.4); SSD(i), 13.9 (209.1); SSD(i) plus VAA, 12.6 (214.7); LVAD, 11.8 (198.1); LVAD plus VAA, 12.5 (201.7) and LVAD plus BC, 12.2 (209.5). Thus over this final 5 week period, Maintenance-fed rats (Group 1) consumed the most and LVAD fed rats (Group 4) consumed the least diet. The group fed LVAD (Group 4) ate only marginally less than the groups fed LVAD diet plus VAA (Group 5) or BC (Group 6). Despite consuming most diet, Maintenance fed rats (Group 1) were lighter than the group fed SSD(i) plus VAA (the heaviest group) during the last five weeks of the trial.

(iii) Water Consumptions
Mean daily water consumptions of rats fed each diet are shown in Table 5.3. For weeks 15 to 20 of the trial (mean of 4 consumptions), the average mean daily water consumption (ml/rat/day) of rats fed each diet were: - Maintenance, 15.9; SSD(i), 15.8; SSD(i) plus VAA, 15.4; LVAD, 13.4; LVAD plus VAA, 15.3 and LVAD plus BC, 14.5. Thus rats fed the LVAD diet drank least water and those fed the Maintenance diet drank the most water during this period.

(iv) Clinical signs of Vitamin A Deficiency
Throughout the trial there were no clinical signs of vitamin A deficiency such as depressed body weight, periocular porphyrin or xerophthalmia (Moore 1957) in any dietary group.

(v) Retrospective Plasma Analysis
About one year after the completion of Trial I, plasma samples, which had been stored at 20°C, were analysed for vitamin A (retinol), vitamin E (α-tocopherol) and BC. The results for the 6 groups are shown in Table 5.4.

a Retinol
Rats weaned onto LVAD (Group 4) and killed at the start of the trial (week 0) had mean ± (sd) plasma retinol of 36.1 ± 2.9 μg/dl. By week 7, plasma retinol in LVAD fed rats had fallen to 20.0 ± 3.3 μg/dl, 58% of that of Group 5 fed LVAD plus VAA. Animals fed LVAD plus BC (Group
6) showed a similar plasma retinol level to animals fed LVAD plus VAA (Group 5), 32.4 ± 2.69
and 34.15 ± 3.40 respectively. At week 7, plasma retinol in SSD(i) fed rats (Group 2) was 22.9 ± 
2.0 µg/dl. By week 20, plasma retinol in rats fed SSD(i) had fallen to 3.9 ± 1.2 µg/dl, 15% of that 
of rats fed SSD(i) plus VAA (Group 3). Plasma retinol in LVAD-fed rats had fallen to 11.2 ± 
6.21, 48% of that of rats fed LVAD plus VAA (Group 5) and 43% of that of rats fed LVAD plus 
BC (Group 6). At 20 weeks, rats fed the 4 vitamin A-sufficient diets (Maintenance, SSD(i) plus 
VAA, LVAD plus VAA and LVAD plus BC) showed similar plasma retinol levels, 28.50 ± 1.57, 
25.73 ± 2.14, 23.12 ± 2.00 and 25.75 ± 1.16 respectively. These levels were generally much 
reduced compared with the corresponding values of these Groups at 7 weeks.

b α-tocopherol
At the beginning of the study (week 0), plasma α-tocopherol levels in the 6 rats in Group 4 fed 
LVAD diet ranged from 0.40 - 1.21 mg/dl (data not shown), with a mean value of 0.87 ± 0.28 
mg/dl. After 7 weeks, in all 6 dietary groups, group mean plasma α-tocopherol levels ranged from 
the lowest value of 0.42 ± 0.04 mg/dl in LVAD fed rats (Group 4) to the highest value of 0.59 ± 
0.16 mg/dl in animals fed LVAD plus VAA (Group 5). Values ranged from 0.25 to 0.81 mg/dl 
(data not shown) for individual rats in the 6 groups. After 20 weeks, group mean plasma 
α-tocopherol levels ranged from the lowest value of 0.40 ± 0.20 mg/dl in rats receiving LVAD 
plus BC (Group 6) to the highest value of 0.80 ± 0.09 mg/dl in rats fed LVAD plus VAA (Group 
5). Thus no diet-related effects were clearly evident in plasma α-tocopherol levels except that 
animals fed LVAD plus VAA demonstrated the highest values at both 7 and 20 weeks.

c BC
Analysis for BC in plasma samples 1 year after the completion of the trial demonstrated no BC in 
any rats of any dietary group at 0, 7 and 20 weeks respectively. This included rats in Group 6 fed 
LVAD plus 4 mM/kg BC.

(vi) Histology
No histological evidence of squamous metaplasia indicative of vitamin A deficiency (Wolbach and 
Howe 1925; Hicks 1968; Pitt 1985) was detected in sections of trachea or bladder from animals 
fed either LVAD (Group 4) or SSD(i) (Group 2) diets.
D Conclusions

Neither the SSD(i) diet nor the LVAD diet induced a significant depression in body weight gain after feeding for 20 weeks (Fig. 5.1). No other clinical signs of vitamin A deficiency were seen in animals fed these diets during the 20 week trial. As judged by this evidence, therefore, neither diet was effective in producing vitamin A deficiency. Retrospective analysis subsequently demonstrated a reduction in plasma retinol in animals fed both the SSD(i) and LVAD diets (Table 5.4). However with both these diets, the time period that would be needed to induce a cessation of body weight gain and other clinical signs of deficiency (i.e. >20 weeks) rendered neither diet of any use in Trial VIII which was designed to evaluate BC against urinary bladder cancer.

There were no important differences in diet consumptions (Table 5.2) or water consumptions (Table 5.3) of animals fed the Maintenance, LVAD or SSD(i) diets, but rats fed the Maintenance diet generally ate more than animals fed the other diets, while rats fed the LVAD diet ate and drank the least.

None of the diets tested had any effect upon plasma α-tocopherol levels (Table 5.4). No BC was detected in the plasma of any rat in any group including rats of Group 6 fed LVAD plus 4μM/kg BC. This result demonstrated that rats fed a relatively low dietary level of BC did not absorb any unconverted BC, but rather converted it all to vitamin A (Moore 1957; Pitt 1985). Plasma retinol levels of rats fed LVAD plus BC (Group 6) were similar to those of rats in Group 5 (LVAD plus VAA) and Group 3 (SSD(i) plus VAA) and were not reduced, as in animals of Group 4 fed LVAD alone. This indicates that BC had acted as provitamin A in animals fed LVAD plus BC (Group 6).

3 TRIAL II: EVALUATION OF LVAD DIET AND SSD(i) DIET AGAINST A PROVEN LABORATORY-PREPARED VITAMIN A-DEFICIENT DIET ('BROMPTON' DIET)

A Introduction

Vitamin A-deficient diets have been reported to induce depression of body weight gain and other clinical signs of vitamin A deficiency in weanling rats after 4 - 8 weeks (Moore 1957; Lamb 1974; Shields and Jeffrey 1987). It was both unexpected and surprising that in Trial I, after 20 weeks, rats fed either the LVAD diet or the SSD(i) diet showed no reduction in body weight gain or clinical signs of deficiency. This lack of effect was partly surprising as subsequent analyses
demonstrated that both diets contained less than the threshold limit of detection of vitamin A (i.e. <15 μgRE/kg).

To investigate the ineffectiveness of the LVAD and SSD(i) diets, a small quantity of a proven, effective, laboratory-prepared, semi-synthetic diet (the 'Brompton' diet) was obtained from Dr P. Jeffrey and Dr P. Shields (The Brompton Hospital, London) and its efficacy was evaluated in parallel with the LVAD and SSD(i) diets in weanling female F344 rats. The composition of the Brompton diet was based on a nutritionally complete semi-synthetic formulation reported by Wise (1982). This formulation was subsequently modified by Dr Wise (1983) as a vitamin A-deficient diet, by omitting the supplemental VAP and adding rice starch, safflower oil and essentially vitamin-free casein, to replace the maize starch, maize oil and unrefined casein in the original diet. It is noteworthy that Wise, in his deficient diet, used rice starch to avoid trace amounts of vitamin A. Maize starch, however, a commonly used source of carbohydrate in semi-synthetic vitamin A-deficient diets (Moore 1957; Rogers et al. 1974; Takahashi et al 1975) was an ingredient in the SSD(i) diet supplied by SDS Ltd.

Shields and Jeffrey (1987) used the Brompton diet in a study of the effects of vitamin A deficiency and cigarette smoke on rat tracheal epithelium. In these studies the Brompton diet routinely produced a weight plateau in weanling (30 - 40 g) male F344 rats after 6 - 7 weeks; other clinical signs also developed rapidly at this time with the animals becoming moribund about 14 days later.

Male rats are reported to develop vitamin A deficiency before females (Moore 1957). As Shields and Jeffrey had used male F344 rats and the experiments described in this thesis used females, it was necessary to examine the effect of sex difference on the induction of vitamin A deficiency in the F344 rat.

Trial II compared the effectiveness of the Brompton, LVAD and SSD(i) diets in female and male F344 rats. There are no reports in the literature of the induction of vitamin A deficiency in inbred female F344 rats, most workers having used outbred Sprague-Dawley or Wistar rats (Hicks 1968 and 1969; Cohen et al. 1976; Underwood et al. 1979). Therefore, the susceptibility of female F344 rats to vitamin A deficiency was compared in this experiment by feeding the LVAD and SSD(i) diets to outbred animals of the Sprague-Dawley and Wistar stocks.
B Experimental Design

144 weanling rats (47 female F344, 36 male F344, 30 female Wistar and 31 female Sprague-Dawley rats) aged 21 - 28 days old, were divided into four groups according to strain and sex (Table 5.5). Each group consisted of 5 or 6 subgroups of 4, 5, 6, 7 or 18 rats fed the following 5 diets: Maintenance diet, LVAD diet and SSD(i) and LVAD diets supplemented with VAA (2064 μgRE/kg). Both Group 1 (female F344 rats) and Group 2 (male F344 rats) also included 4 animals fed the Brompton diet. Group 3 (female Wistar rats) and group 4 (female Sprague-Dawley rats) were not fed the Brompton diet as supplies were limited.

The times of post-mortems are shown in Table 5.5. 12 female F344 rats fed Maintenance diet were killed at week 0 to provide baseline plasma analysis data. Both male and female F344 rats fed the Brompton diet were killed by week 7. Apart from sick or moribund animals, which were killed in extremis, the remaining rats in all 4 groups were killed at week 13.

All rats were weighed weekly and examined in detail for clinical signs of developing vitamin A deficiency twice weekly. At post-mortem, plasma samples were prepared for retrospective analysis for vitamins A and E and for BC.

Animals in all 4 groups were fed Maintenance diet to provide data on the normal growth rate of male and female F344 rats, female Wistar and female Sprague-Dawley rats fed the conventional laboratory animal diet. These results were important because Trial II, like Trial I, depended greatly upon the depression of body weight gain and other clinical signs to indicate the onset and progress of deficiency. Analysis of plasma vitamin A was carried out retrospectively to substantiate the clinical findings.

C Results

(i) Body Weights

a Group 1: Female F344 rats

Body weights of female F344 rats fed the various diets are presented in Table 5.6 and Figure 5.2. At the start of the trial (day 0) the mean body weight (g ± sd.) of all female F344 rats in every dietary subgroup was 50.3 ± 7.0. However at this time, the mean body weights of female F344 rats fed SSD(i) and LVAD were lower than those of rats fed SSD(i) and LVAD supplemented with VAA respectively. At 7 weeks (day 45), the mean body weights of rats fed each diet were: Maintenance, 156.5 ± 6.5; SSD(i), 143.7 ± 6.4; SSD(i) plus VAA, 153.3 ± 7.1; LVAD, 145.7 ± 12.2; LVAD plus VAA, 158.3 ± 7.5 and Brompton, 114.5 ± 10.6. Therefore, at 7 weeks, female
F344 rats fed the Brompton diet demonstrated significantly reduced mean body weight compared to female F344 rats fed each of the other 5 diets. However, at this time, there was no evidence of major differences in mean body weight of female F344 rats fed the SSD(i) diet or the LVAD diet, compared with animals fed these diets supplemented with VAA or those fed the Maintenance diet.

At 13 weeks, mean body weights of female F344 rats fed the various diets were: Maintenance, 190.5 ± 7.6; SSD(i), 167.5 ± 6.5; SSD(i) + VAA, 187.4 ± 8.1; LVAD, 172.1 ± 11.1 and LVAD plus VAA, 190.7 ± 8.4. Thus, at the termination of Trial II (week 13) there was some evidence of a slight reduction in body weight in female F344 rats fed the SSD(i) diet compared with animals fed SSD(i) plus VAA diet. However, there was no marked difference in body weight between female F344 rats fed the LVAD diet and those fed the LVAD plus VAA diet. All female F344 rats fed the Brompton diet were killed in extremis between week 6 and week 7.

**b Group 2: Male F344 rats**

Body weights of male rats fed the 6 experimental diets are presented in Table 5.7 and Figure 5.3. At the beginning of the trial (day 0), the mean body weight (g ± sd) of all male F344 rats in Group 2 was 50.7 ± 9.3. At 6 weeks, the mean body weights of male F344 rats fed each diet were:

- Maintenance 209.4 ± 27.2
- SSD(i), 172.9 ± 16.5
- SSD(i) plus VAA, 207.2 ± 12.3
- LVAD, 194.9 ± 16.5
- LVAD plus VAA, 203 ± 20.3
- Brompton, 111.8 ± 34.7

Thus, at 6 weeks, male F344 rats fed the Brompton diet demonstrated significantly reduced mean body weight compared to rats fed each of the other 5 diets. In addition, male F344 rats fed the SSD(i) diet showed a reduced mean body weight compared to male F344 rats fed the SSD(i) diet plus VAA, but this reduction was less marked than that observed in rats fed the Brompton diet. There was no evidence at 6 weeks of any significant reduction in mean body weight in male F344 rats fed the LVAD diet compared to animals fed the LVAD plus VAA diet.

All male F344 rats fed the Brompton diet had been killed in extremis by week 7. At week 13 mean body weight (g ± sd) of male F344 rats fed the other 5 diets were: Maintenance, 313.5 ± 25.6; SSD(i), 189.3 ± 13.4; SSD(i) plus VAA, 312.9 ± 13.6; LVAD, 278.1 ± 15.5 and LVAD plus VAA, 305.6 ± 27.3. Thus, when Trial II was terminated (week 13) there was clear evidence of a marked reduction in mean body weight in male F344 rats fed the SSD(i) diet (189.3 ± 13.4) compared with rats fed the SSD(i) plus VAA diet (312.9 ± 13.6). However, there was no evidence at 13 weeks of a reduction in body weight of male F344 rats fed the LVAD diet compared with rats fed the LVAD plus VAA diet.
c Group 3: Female Wistar rats

Body weights of female Wistar rats fed the experimental diets are presented in Table 5.8 and Figure 5.4. At the start of the trial (day 0) the mean body weight (g ± sd) of all female Wistar rats in Group 3 was 41.2 ± 5.3. At 7 weeks (day 45) the mean body weights of rats fed each diet were: Maintenance, 164.2 ± 7.9; SSD(i), 168.5 ± 10.3; SSD(i) plus VAA, 180.5 ± 10.1; LVAD, 162.8 ± 16.6 and LVAD plus VAA, 166.8 ± 12.3. Thus, at 7 weeks there was no evidence of significant differences in mean body weights of female Wistar rats fed each diet.

At the end of the trial (week 13) mean body weights of female Wistar rats fed each diet were: Maintenance, 208.9 ± 10.5; SSD(i), 203.8 ± 9.4; SSD(i) plus VAA, 223.8 ± 14.7; LVAD, 189.3 ± 23.8 and LVAD plus VAA, 204.3 ± 16.8. Thus at 13 weeks there was no evidence of any significant differences in mean body weight in female Wistar rats fed LVAD or SSD(i) compared with rats fed LVAD or SSD(i) supplemented with VAA or those fed the Maintenance diet.

d Group 4: Female Sprague-Dawley rats

Body weights of female Sprague-Dawley rats fed the experimental diets are shown in Table 5.9 and Figure 5.5. At the beginning of the trial, the mean body weight (g ± sd) of all female Sprague-Dawley rats in Group 4 was 57.0 ± 12.3. At 7 weeks the mean body weights of rats fed each diet were: Maintenance, 216.2 ± 20.3; SSD(i), 225.0 ± 14.4; SSD(i) plus VAA, 240.3 ± 11.3; LVAD, 214.9 ± 18.3 and LVAD plus VAA, 208.0 ± 15.0. Thus, at 7 weeks there was no evidence of significant differences in mean body weights of female Sprague-Dawley rats fed each diet.

At the end of the trial (week 13) mean body weights of female Sprague-Dawley rats fed each diet were: Maintenance, 266.4 ± 22.1; SSD(i), 272.8 ± 13.1; SSD(i) plus VAA, 287.3 ± 16.4; LVAD, 263.1 ± 20.4 and LVAD plus VAA, 256.5 ± 13.3. Thus, at 13 weeks there was no evidence of any significant differences in mean body weight in female Sprague-Dawley rats fed SSD(i) or LVAD compared with rats fed SSD(i) or LVAD supplemented with VAA or those fed the Maintenance diet.

(ii) Clinical signs of Vitamin A deficiency

a Male and Female F344 rats fed the Brompton diet

4 male and 4 female F344 rats fed the Brompton diet demonstrated a progression of clinical signs indicative of vitamin A deficiency (Moore 1957). These clinical signs, presented in a summarised form in Table 5.10 began with retardation of growth (body weight plateau), increased in severity
with time on the diet and ultimately ended in rapid deterioration in condition and subsequent mortality about 2 weeks after the appearance of the first signs. The progression from mild to severe clinical signs occurred in 3 general stages. Male F344 rats, for example, demonstrated a body weight plateau and the animals were thin with untidy coats after feeding on the Brompton diet for 24 - 33 days. These early mild signs progressed, after 33 - 38 days, to include eye lesions (crusty red exudate - periocular porphyrin - and hair loss around the eye socket), breathing difficulties and muscular weakness (paresis). Finally, after 38 - 45 days, animals became moribund and showed xerophthalmia, emaciation and a hunched posture with a swollen abdomen. At post-mortem the intestines were distended with gas and there was no body fat.

Male F344 rats demonstrated clinical signs of vitamin A deficiency earlier than female F344 rats. For example, male rats showed early clinical signs after 24 - 33 days, while females showed the same signs after 36 - 39 days. Thus, 2 of 4 male F344 rats fed the Brompton diet rapidly lost condition and were killed in extremis after 38 and 45 days, the other 2 animals were killed when moribund, showing xerophthalmia and hunched posture at days 39 and 45. In contrast, of 4 female F344 rats fed the Brompton diet, only 1 was moribund by day 45, while the other 3 only showed hair loss and red exudate around the eyes when killed at 39 days (1) and 46 days (2) respectively.

In general, the time at which clinical signs first appeared in rats of the same sex appeared to be related to the initial body weight of the animals, with smaller (lighter) rats showing clinical signs before larger (heavier) rats (Lamb et al. 1974).

In most cases, not all the clinical signs presented in Table 5.10 were demonstrated by each individual rat, breathing difficulties and paresis, for example, were only seen in a few animals.

b Female F344 rats fed the LVAD, SSD(i), Maintenance, SSD(i) plus VAA and LVAD plus VAA diets

Throughout the trial, female F344 rats fed the LVAD diet showed no clinical signs of vitamin A deficiency. Apart from one animal at the end of the trial (day 92) which was thin and had an untidy pylo-erect coat, no female F344 rats fed the SSD(i) diet showed any clinical signs of deficiency. All female F344 rats fed the SSD(i) plus VAA diet, LVAD plus VAA diet and Maintenance diet were normal throughout the trial.
Male F344 rats fed the LVAD, SSD(i), LVAD plus VAA, SSD(i) plus VAA and Maintenance diets

No clinical signs of vitamin A deficiency were demonstrated by male F344 rats fed the LVAD diet. However, male F344 rats fed the SSD(i) diet did demonstrate clinical signs of deficiency. Of the 6 male F344 rats fed the SSD(i) diet, 2 animals died, at days 80 and 82, having demonstrated a progression of clinical signs similar to that described for F344 rats fed the Brompton diet (Table 5.10). Of the other 4 male F344 rats fed the SSD(i) diet, 2 demonstrated paresis and were hunched with slightly swollen abdomens when killed. The other 2 were slightly hunched.

Although male F344 rats fed the SSD(i) diet demonstrated similar clinical signs to those observed in rats fed the Brompton diet, the former appeared to be more prone to respiratory infection as indicated by apparent breathing difficulties and crusty yellow/red deposits around the nostrils of some of these animals. Furthermore, hair loss around the eye sockets and xerophthalmia were not seen in male F344 rats fed the SSD(i) diet, although the eyeballs of these rats were dull and sunken into the sockets.

Male F344 rats fed the LVAD plus VAA diet, the SSD(i) plus VAA diet and the Maintenance diet demonstrated no clinical signs of vitamin A deficiency. At day 87, one rat fed the SSD(i) plus VAA diet was observed to be wheezing. This may have been due to a focal respiratory infection independent of vitamin A deficiency.

d Female Wistar rats fed the LVAD, SSD(i), LVAD plus VAA, SSD(i) plus VAA and Maintenance diets

No clinical signs of vitamin A deficiency were demonstrated by female Wistar rats fed the LVAD, SSD(i), LVAD plus VAA, SSD(i) plus VAA and Maintenance diets. From day 87 it was noted that 2 female Wistar rats fed the LVAD diet showed wheeziness, but this was probably due to a respiratory infection independent of treatment, as it was also observed in one male F344 rat fed the SSD(i) diet supplemented with vitamin A.

e Female Sprague-Dawley rats fed the LVAD, SSD(i), LVAD plus VAA, SSD(i) plus VAA and Maintenance diets

No clinical signs of vitamin A deficiency were demonstrated by female Sprague-Dawley rats fed the LVAD, SSD(i), LVAD plus VAA, SSD(i) plus VAA and Maintenance diets.
(iii) Retrospective Plasma Analysis

About one year after the completion of Trial II, plasma samples which had been stored at -20°C were analysed for retinol, α-tocopherol and BC. The results for the four groups are presented in Tables 5.11 to 5.14.

a Group 1: Female F344 rats

The mean plasma levels of retinol (µg/dl), α-tocopherol (mg/dl) and BC (µg/dl) of female F344 rats are shown in Table 5.11.

Retinol

Weanling female F344 rats fed the Maintenance diet and killed at the start of the trial (week 0) demonstrated a mean (± sd.) plasma retinol level of 40.48 ± 4.01. At the end of the trial (week 13), plasma retinol in rats fed the Maintenance diet had fallen to 26.62 ± 1.70. This was similar to the plasma retinol values in rats fed the SSD(i) plus VAA diet (28.3 ± 2.12) and the LVAD plus VAA diet (28.84 ± 0.31). In contrast at 13 weeks plasma retinol had fallen to 5.44 ± 0.87 in rats fed SSD(i) alone, or 19.3% of that of rats fed the SSD(i) plus VAA diet. Similarly, in rats fed the LVAD diet at 13 weeks, plasma retinol levels fell to 12.50 ± 3.21, or 50.3% of that of animals fed the LVAD plus VAA diet. In rats fed the Brompton diet, at 7 weeks, plasma retinol levels had fallen to 2.90 ± 0.18.

α-tocopherol

Weanling female F344 rats fed the Maintenance diet and killed at the start of the trial (week 0) demonstrated a mean plasma α-tocopherol level (mg/dl) of 0.76 ± 0.14. At the end of the trial (week 13) plasma α-tocopherol levels in rats fed the various diets were: Maintenance, 0.46 ± 0.30; SSD(i), 0.33 ± 0.07; SSD(i) plus VAA, 0.72 ± 0.31; LVAD, 0.40 ± 0.04 and LVAD plus VAA, 0.65 ± 0.12. Thus, female F344 rats fed the LVAD diet demonstrated a reduction in plasma α-tocopherol compared to rats fed the LVAD plus VAA diet. There was also a significant difference between α-tocopherol levels in rats fed the SSD(i) diet and the SSD(i) plus VAA diet. By week 7 plasma α-tocopherol in rats fed the Brompton diet had fallen to 0.21 ± 0.08 a value markedly different from that of rats fed the Maintenance diet and killed at week 0.

BC

No BC was detected in plasma samples from female F344 rats.
b  Group 2: Male F344 rats

The mean plasma levels of retinol, α-tocopherol and BC of male F344 rats are shown in Table 5.12.

\( \)  Retinol

Plasma samples for analysis were not taken at the beginning of the experiment (see Table 5.5). At 13 weeks, plasma retinol levels (μg/ml) in rats fed the various diets were: Maintenance, 53.63 ± 7.73; SSD(i), 4.68 ± 1.16; SSD(i) plus VAA, 56.2 ± 5.39; LVAD, 9.90 ± 4.56 and LVAD plus VAA, 51.50 ± 2.60. Male F344 rats fed the Maintenance diet and the SSD(i) and LVAD diets supplemented with VAA all demonstrated similar plasma retinol levels at 13 weeks. In contrast, male F344 rats fed the SSD(i) diet showed a 92% reduction in plasma retinol compared with rats fed the corresponding VAA-supplemented diet. Similarly, male F344 rats fed the LVAD diet showed a significant reduction (81%) in plasma retinol compared with rats fed the LVAD plus VAA diet. Male F344 rats fed the Brompton diet demonstrated a mean plasma retinol level of 4.50 ± 1.41 at 7 weeks.

\( \)  α-tocopherol

After 13 weeks, plasma α-tocopherol levels of male F344 rats fed the various diets were:
Maintenance, 0.30 ± 0.09; SSD(i), 0.16 ± 0.13; SSD(i) plus VAA, 0.56 ± 0.08; LVAD, 0.32 ± 0.07 and LVAD plus VAA, 0.43 ± 0.06. Thus, at the end of the trial there was a significant difference in plasma α-tocopherol levels between male F344 rats fed the SSD(i) diet and the SSD(i) plus VAA diet. But the reduction in α-tocopherol levels in rats fed the LVAD diet was not as large in comparison with animals fed the LVAD plus VAA diet. Male F344 rats fed the Brompton diet demonstrated a mean (SD) plasma α-tocopherol level (mg/dl) of 0.04 ± 0.06 after 7 weeks.

\( \)  BC

No BC was detectable in any plasma samples from male F344 rats.

c  Group 3: Female Wistar rats

The mean plasma levels, at 13 weeks, of retinol, α-tocopherol and BC, of female Wistar rats fed the different diets are shown in Table 5.13.
© Retinol

At 13 weeks, plasma retinol levels in female Wistar rats fed the various diets were (µg/dl):
- Maintenance, 17.33 ± 1.59
- SSD(i), 5.27 ± 1.30
- SSD(i) plus VAA, 19.50 ± 3.39
- LVAD, 9.33 ± 1.75
- LVAD plus VAA, 19.72 ± 2.71

Thus, plasma retinol levels in female Wistar rats fed the SSD(i) diet and the LVAD diet were significantly reduced compared to rats fed SSD(i) plus VAA and LVAD plus VAA. Plasma retinol levels in rats fed the Maintenance diet, the SSD(i) plus VAA diet and the LVAD plus VAA diet were all similar.

© α-tocopherol

At the end of the trial (13 weeks) plasma α-tocopherol levels in female Wistar rats fed the various diets were (µg/dl):
- Maintenance, 0.57 ± 0.13
- SSD(i), 0.48 ± 0.12
- SSD(i) plus VAA, 1.08 ± 0.26
- LVAD, 0.67 ± 0.34
- LVAD plus VAA, 0.98 ± 0.18

Thus, female Wistar rats fed the SSD(i) diet demonstrated a significantly lower plasma α-tocopherol level than rats fed the SSD(i) plus VAA diet.

© BC

No BC was detected in any plasma samples from female Wistar rats.

d Group 4: Female Sprague-Dawley rats

Plasma samples were not taken at the beginning of the experiment. The mean plasma levels of retinol α-tocopherol and BC, at 13 weeks, of female Sprague-Dawley rats fed the different diets are shown in Table 5.14.

© Retinol

At 13 weeks, plasma retinol levels in female Sprague-Dawley rats fed the various diets were (µg/dl):
- Maintenance, 22.74 ± 3.09
- SSD(i), 4.27 ± 0.91
- SSD(i) plus VAA, 22.10 ± 4.23
- LVAD, 5.96 ± 0.70
- LVAD plus VAA, 21.41 ± 1.33

Thus, female Sprague-Dawley rats fed the SSD(i) diet and the LVAD diet demonstrated significantly reduced plasma levels compared to rats fed the SSD(i) plus VAA diet and the LVAD plus VAA diet. Very similar plasma retinol levels were detected in female Sprague-Dawley rats fed the Maintenance, SSD(i) plus VAA and LVAD plus VAA diets.
α-tocopherol

At the end of the trial (13 weeks) plasma α-tocopherol levels in female Sprague-Dawley rats fed the various diets were (µg/dl): Maintenance, 0.86 ± 0.20; SSD(i), 0.65 ± 0.21; SSD(i) plus VAA, 1.17 ± 0.18; LVAD, 0.78 ± 0.19 and LVAD plus VAA, 0.98 ± 0.31. Thus, female Sprague-Dawley rats fed the SSD(i) diet demonstrated a reduced plasma α-tocopherol level compared to rats fed the SSD(i) plus VAA diet.

BC

No BC was detected in any plasma samples from female Sprague-Dawley rats.

(iv) Conclusions

a Male and female F344 rats fed the Brompton diet

The Brompton diet, after 7 weeks, induced a marked difference in mean body weight in female F344 rats (114.5 ± 10.6; Figure 5.2) compared to female F344 rats fed the Maintenance diet (156.5 ± 6.5). Similarly, after 6 weeks, the Brompton diet induced a marked difference in mean body weight in male F344 rats (111.8 ± 34.7; Figure 5.3) compared to male F344 rats fed the Maintenance diet (209.4 ± 25.4).

Subsequent to the depression of body weight gain induced in male and female F344 rats by the Brompton diet, these animals demonstrated a progression of clinical signs of vitamin A deficiency which increased in severity with time and ultimately ended in a rapid deterioration in condition and subsequent mortality at about 2 weeks after the appearance of the first signs (Table 5.10).

The observation of reduced body weight gain and the development of clinical signs of vitamin A deficiency in animals fed the Brompton diet suggested that this diet was effective in producing vitamin A deficiency in male and female F344 rats within 6 - 7 weeks. Shields and Jeffrey (1987) reported similar results in male F344 rats fed the same diet.

Retrospective plasma analysis subsequently demonstrated a marked difference in the mean plasma retinol level (µg/dl ± sd) of female F344 rats fed the Brompton diet (2.90 ± 0.18) and killed at 6 - 7 weeks, compared with the mean plasma retinol levels of female F344 rats fed the Maintenance diet and killed either at week 0 (40.48 ± 4.01), or week 13 (26.62 ± 1.70). Similarly male F344 rats fed the Brompton diet also demonstrated a markedly different mean plasma retinol level (4.50
± 1.41) at 6 - 7 weeks, compared with the mean plasma retinol level of male F344 rats fed the Maintenance diet (53.63 ± 7.73) and killed after 13 weeks.

Male F344 rats demonstrated clinical signs of vitamin A deficiency after 4-5 weeks while female F344 rats showed similar results after 6 weeks (Table 5.10). At 13 weeks, male F344 rats fed the Maintenance diet demonstrated a mean plasma retinol level of 53.63 µg/dl, twice that of female F344 rats (26.62 µg/dl) fed the same diet. These findings are consistent with the literature in which male rats are reported to have higher plasma retinol levels and become deficient before females (Moore 1957).

b The SSD(i) diet

At the end of the trial (13 weeks) female F344 rats fed the SSD(i) diet (Figure 5.2) demonstrated a small reduction in mean body weight (167.5 ± 6.5) compared to female F344 rats fed the SSD(i) plus VAA diet (187.4 ± 8.1). However, no other clinical signs of vitamin A deficiency were observed in female F344 rats fed the SSD(i) diet. Retrospective plasma analysis subsequently demonstrated that the mean plasma retinol level in female F344 rats fed the SSD(i) diet (5.44 ± 0.87 µg/dl) was reduced compared with rats fed the SSD(i) diet supplemented with VAA (28.23 ± 2.12).

After 13 weeks male F344 rats fed the SSD(i) diet (Figure 5.3) demonstrated a large reduction in mean body weight (189.3 ± 13.4) compared to male F344 rats fed the SSD(i) diet plus VAA (312.9 ± 13.6). In addition, a progression of clinical signs of vitamin A deficiency similar to that seen in rats fed the Brompton diet was observed in male F344 rats fed the SSD(i) diet, while male F344 rats fed the SSD(i) diet supplemented with VAA were free of clinical signs. Retrospective plasma retinol analysis (Table 5.12) subsequently confirmed that the mean plasma retinol level of male F344 rats fed the SSD(i) diet (4.68 ± 1.16 µg/dl) was markedly reduced compared to that of male F344 rats fed the SSD(i) plus VAA diet (56.2 ± 5.39).

At the end of the trial there was no evidence of any reduction in body weight of female Wistar rats fed the SSD(i) diet (Figure 5.4). Neither did female Wistar rats fed the SSD(i) diet demonstrate any other clinical signs of vitamin A deficiency. However, retrospective plasma retinol analysis (Table 5.13) subsequently demonstrated that female Wistar rats fed the SSD(i) diet had a reduced mean plasma retinol level (5.27 ± 1.30 µg/dl) compared with that of female Wistar rats fed the SSD(i) plus VAA diet (19.5 ± 3.39).
At the end of the trial there was no evidence of any depression of body weight in female Sprague-Dawley rats fed the SSD(i) diet (Figure 5.5). Neither were any other clinical signs of vitamin A deficiency observed in female Sprague-Dawley rats fed the SSD(i) diet. However, marked differences in plasma retinol levels (Table 5.13) were subsequently detected between female Sprague-Dawley rats fed the SSD(i) diet (4.27 ± 0.91 μg/dl) and female Sprague-Dawley rats fed the SSD(i) plus VAA diet (22.10 ± 4.23).

Thus, as judged by body weight depression and clinical signs, the SSD(i) diet was effective in inducing vitamin A deficiency in male F344 rats after 13 weeks. These results contrast with the Brompton diet which induced similar results after 6 - 8 weeks. This finding was confirmed by retrospective plasma retinol analysis. After the same time period female F344 rats fed the SSD(i) diet had demonstrated a reduced body weight gain but no other clinical signs of vitamin A deficiency. However, these rats did demonstrate reduced plasma retinol levels. Neither female Wistar rats nor female Sprague-Dawley rats demonstrated significant reductions in body weight or any other clinical signs of deficiency after 13 weeks. However, each of these rat stocks subsequently showed significantly reduced plasma retinol values.

c The LVAD diet

At the end of the trial (13 weeks), female F344 rats fed the LVAD diet (Figure 5.2) did not demonstrate any marked reduction in body weight gain compared with female F344 rats fed the LVAD plus VAA diet. Neither did female F344 rats fed the LVAD diet demonstrate any clinical signs of vitamin A deficiency. However, plasma retinol values of female F344 rats fed the LVAD diet were significantly reduced compared with female F344 rats fed the LVAD plus VAA diet, as detected by retrospective plasma analysis.

The LVAD diet produced no marked reductions in body weight gain, nor any clinical signs of vitamin A deficiency in male F344 rats, female Wistar rats or female Sprague-Dawley rats. However, retrospective plasma analysis subsequently demonstrated significant reduction in plasma retinol values of each of the rat groups fed the LVAD diet. Thus, the LVAD diet, as judged by reduction in body weight gain and other clinical signs after 13 weeks, was not effective in inducing vitamin A deficiency in any type of rat examined.

d Differential effectiveness of different batches of the SSD(i) diet

In Trial I, female F344 rats fed the SSD(i) diet for 20 weeks demonstrated no body weight changes or clinical signs indicative of vitamin A deficiency, though retrospective plasma analysis
demonstrated reduced plasma retinol levels suggesting that clinical signs of vitamin A deficiency would have been seen had the trial progressed beyond 20 weeks. However, after only 13 weeks, female F344 rats fed a different batch (manufactured at a different time) of the SSD(i) diet in Trial II did demonstrate a marked reduction in mean body weight compared to animals fed the SSD(i) plus VAA diet. Though no clinical signs of vitamin A deficiency were seen in these female F344 rats, clear clinical evidence of vitamin A deficiency was observed at 13 weeks in a parallel group of male F344 rats fed the SSD(i) diet and, had Trial II proceeded beyond 13 weeks, it is likely that the female F344 rats fed the second batch of the SSD(i) diet would have also demonstrated clinical signs before 20 weeks. Thus, female F344 rats fed the second batch of the SSD(i) diet in Trial II demonstrated reductions in body weight indicative of developing vitamin A deficiency at 13 weeks while female F344 rats fed the first batch of the SSD(i) diet in Trial I had failed to demonstrate body weight reductions or clinical signs by 20 weeks. These varying results in rats fed 2 different batches of the same diet may be due to seasonal variations in the level of trace amounts of contaminants with vitamin A activity (Rogers et al. 1974) associated with natural diet ingredients such as maize starch.

In formulating the Brompton diet, Wise (1983) used rice starch rather than maize starch in order to avoid the trace amounts of vitamin A present in the latter. As the Brompton diet was an extremely effective formulation, the use of vitamin A-deficient diets incorporating rice starch as the carbohydrate source may be more suitable than those based on maize starch. It is noteworthy that both batches of the SSD(i) diet were analysed and found to contain less than 15 µgRE/kg vitamin A, the threshold limit of detection. Thus, any trace amounts of vitamin A that were responsible for the difference in efficacy of the two batches were both very small and undetectable. These results suggest that present analytical techniques are not able to distinguish between a very good vitamin A-deficient diet, such as the Brompton diet, and a poor diet, such as the SSD(i) diet, where the difference in efficacy is the result of significant but undetectable trace amounts of vitamin A below the threshold limit of detection.

e Rat Strain and stock differences in response to the LVAD diet and the SSD(i) diet

With regard to the comparative effectiveness of the SSD(i) and LVAD diets to induce vitamin A deficiency in the outbred Wistar and Sprague-Dawley rat stocks and the inbred F344 rat strain, no clinical signs were observed with either diet in female rats of any of these 3 groups. Only female F344 rats fed the SSD(i) diet demonstrated significant body weight reduction compared to female F344 rats fed the SSD(i) plus VAA diet. Retrospective plasma analysis demonstrated reductions in mean plasma retinol levels at the end of the trial (13 weeks) in female F344 rats, female Wistar
rats and female Sprague-Dawley rats fed the SSD(i) diet or the LVAD diet. These reductions, expressed as percentage reductions from the mean plasma retinol levels in the corresponding rats fed the SSD(i) plus VAA diet or the LVAD plus VAA diet were: female F344 rats, 80.7% (SSD(i)) and 49.7% (LVAD); female Wistar rats, 73% (SSD(i)) and 52.7% (LVAD); female Sprague-Dawley rats, 80.7% (SSD(i)) and 72.2% (LVAD).

Thus, as judged by body weight changes, the smaller, inbred female F344 rats appeared to become vitamin-A deficient before the larger, outbred rat stocks. Plasma retinol levels were reduced by a similar extent in female F344 rats, female Wistar rats and female Sprague-Dawley rats fed the SSD(i) diet for 13 weeks. Although female F344 rats and female Sprague-Dawley rats both demonstrated 80% reduction in plasma retinol at this time, only female F344 rats showed any significant body weight changes.

Female Wistar rats fed the maintenance diet for 13 weeks demonstrated a slightly lower, normal mean plasma retinol level (17.3 µg/dl ± 1.59) compared with similarly treated female F344 rats (26.62 ± 1.70) and female Sprague-Dawley rats (22.74 ± 3.09).

f Plasma α-tocopherol levels

Interpretation of the plasma α-tocopherol levels observed in Trial II is difficult as such plasma measurements reflect the balance of influx and efflux in the pools of α-tocopherol in the gastro-intestinal tract and the various tissues (Diplock 1985). However, the data in Trial II from female F344 rats (Table 5.11), male F344 rats (Table 5.12), female F344 rats (Table 5.13) and female Sprague-Dawley rats (Table 5.14) demonstrate that animals from each group showing a very low mean plasma retinol level tend to show lower plasma α-tocopherol levels compared to animals with normal retinol levels. This tendency was observed irrespective of whether rats were fed the Brompton diet, the SSD(i) diet or the LVAD diet. Rather than being indicative of vitamin E deficiency, these low plasma α-tocopherol levels may be the result of reduced α-tocopherol intake as a consequence of reduced diet consumption in vitamin A-deficient animals. Other physiological consequences of vitamin A deficiency such as secondary infection with subsequent increases in the production of free radicals by neutrophils and macrophages may also reduce α-tocopherol levels (Diplock 1985).

The observed ranges in all 4 rat groups demonstrate quite large variations in plasma α-tocopherol values between individual rats within each group and fed the same diet.

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It is noteworthy that unlike retinol, there appears to be no sex difference with respect to plasma α-tocopherol levels as demonstrated by male and female F344 rats fed the various diets in Trial II (Tables 5.11 and 5.12). Furthermore, female Sprague Dawley rats (Table 5.14) fed the Maintenance diet or the LVAD or the SSD(i) diets supplemented with VAA demonstrated plasma α-tocopherol levels which were almost twice as large as those for the other 3 types of rats (Tables 5.1, 5.12 and 5.13) fed the same diets.

g  Plasma BC levels
No BC was detectable in the plasma of any rats fed any diet at any time point. Any trace amounts of BC which may have been present in any of the diets used in Trial II would probably have been present in amounts too small to avoid intestinal conversion to retinol. Thus where such trace amounts of BC were present in these diets, none would be absorbed unchanged into the plasma as BC.

4  TRIAL III: EVALUATION OF A NEW COMMERCIALLY-AVAILABLE SEMI-SYNTHETIC VITAMIN A-DEFICIENT DIET, SSD(ii)
A  Introduction
In Trial II, the laboratory-prepared Brompton diet induced vitamin A deficiency in male and female F344 rats in 5 - 7 weeks. In the same experiment a second batch of the commercially-prepared SSD(i) diet demonstrated evidence of developing vitamin A deficiency in F344 rats after 13 weeks. However, despite being more effective than the initial batch of the SSD(i) diet used in Trial I, this second batch of the SSD(i) diet was clearly not as effective as the Brompton diet. A single batch of the commercially-prepared LVAD diet fed to rats in Trial I and Trial II also proved ineffective in producing vitamin A deficiency within an appropriately short time period. These two experiments demonstrated that neither the SSD(i) nor the LVAD diet were suitable as vitamin-A deficient diets for use in the main trial (Trial VIII) to evaluate the anti-cancer potential of BC. For Trial VIII, a commercially-available vitamin-A deficient diet of similar efficacy to the Brompton diet was required. Consequently SDS Ltd agreed to manufacture a new semi-synthetic vitamin-A deficient diet, (SSD(ii)). Specific instructions were given that the SSD(ii) diet was to be produced to the exact specifications outlined by Wise for the Brompton diet (Wise, 1983), and this included defining the suppliers of individual diet constituents. In this way, it was hoped to avoid variations in the level of contaminating traces of vitamin A associated with individual diet constituents, whether from seasonal variations or from changes in the commercial
suppliers of such constituents. Analysis demonstrated the SSD(ii) diet, like the SSD(i) and the LVAD diets, contained less than 15 µgRE/kg vitamin A, the threshold limit of detection.

The primary objective of Trial III was to evaluate the efficacy of the SSD(ii) diet in female F344 rats, in parallel with the Brompton diet, the SSD(i) diet and the LVAD diet. The SSD(ii) diet was expected to induce body weight reduction and other clinical signs indicative of vitamin A deficiency in 6-7 weeks, as the Brompton diet had done in Trial II.

There were two additional objectives of Trial III. Firstly, the examination of the effect in female F344 rats of mixing increasing proportions (25%, 50% and 75%) of the LVAD diet into the SSD(ii) diet. It was thought that the addition of the LVAD diet to the SSD(ii) diet might improve the performance of the latter in long-term experiments by increasing the dietary fibre content. The second additional objective was the evaluation of the responses of male F344 rats, female Wistar rats and female Sprague-Dawley rats to the SSD(ii) diet. This would yield information on sex and strain variation in the development of vitamin A deficiency. These results would complement similar data obtained in Trial II for the LVAD and the SSD(i) diets.

B Experimental Design

92 weanling rats (60 female F344, 11 male F344, 10 female Wistar and 11 female Sprague-Dawley), 21-28 days old, were divided into 4 groups according to sex and strain (Table 5.15). Group 1 (female F344 rats) consisted of 9 sub-groups of 5, 6 or 18 animals fed the following 9 diets: Maintenance diet, Brompton diet, SSD(ii) diet, SSD(ii) diet plus VAA (2064 µgRE/kg), SSD(ii) diet plus 25% LVAD diet, SSD(ii) diet plus 50% LVAD diet, SSD(ii) diet plus 75% LVAD diet, LVAD diet and SSD(i) diet. Group 2 (male F344 rats), Group 3 (female Wistar rats) and Group 4 (female Sprague-Dawley rats) each consisted of 2 sub-groups of 5 or 6 rats fed either the Maintenance diet or the SSD(ii) diet.

The times of post-mortems are also shown in Table 5.15. Twelve female F344 rats fed the Maintenance diet were killed at week 0 to provide baseline plasma analysis data. Female F344 rats fed the Brompton diet and demonstrating clinical signs of fully developed vitamin A deficiency were killed at week 6. Similarly, female F344 rats, male F344 rats, female Wistar rats and female Sprague-Dawley rats fed the SSD(ii) diet were killed at week 8, week 7, week 8 and week 10 respectively. Sick or moribund animals were killed in extremis. All other animals were killed at week 13. At post-mortem samples of plasma were prepared for later analysis of vitamins A and E and BC.

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All rats were weighed weekly and examined in detail for clinical signs of developing vitamin A deficiency at least twice weekly. Continuous food consumptions were carried out throughout the trial. Animals in all 4 groups were fed the Maintenance diet to define the normal growth rate. Trial III, like Trials I and II, depended greatly upon the depression of body weight gain and other clinical signs to indicate the onset and progress of deficiency. Analysis of plasma vitamin A was performed retrospectively to substantiate the clinical findings.

C Results

(i) Body Weights

a Group 1: Female F344 rats

Body Weights of all female F344 rats fed the various diets are presented in Table 5.16 and graphically (excluding rats fed the SSD(ii) diet supplemented with proportions of LVAD) in Figure 5.6. At 6 weeks the sub-group fed the Brompton diet had reached a body weight plateau and the mean body weight (81.9 g ± 12.5) was reduced compared with those fed the Maintenance diet (131.6 g ± 8.2). At 7 weeks the mean body weights of female F344 rats fed all the other diets were: Maintenance, 149 g ± 6.8; SSD(i), 122.1 g ± 22.6; LVAD, 131.3 g ± 11.3; SSD(ii) plus VAA, 143.8 g ± 7.5; SSD(ii), 132.7 g ± 10.1; SSD(ii) plus 25% LVAD, 131.2 g ± 12.6; SSD(ii) plus 50% LVAD, 127.2 g ± 11.0 and SSD(ii) plus 75% LVAD, 124.7 g ± 11.5. Thus, at 7 weeks there was no evidence of any marked differences in body weight between the test diets other than the Brompton diet and the control diets (Maintenance and SSD(ii) diet plus VAA). At 8 weeks there was a reduction in mean body weight between the rats fed the SSD(ii) diet (134.9 g ± 13.7) and those fed the SSD(ii) plus VAA diet (153.7 g ± 6.7). At the end of the trial (13 weeks) the mean body weights of all remaining rats were: Maintenance, 191.2 g ± 6.2; SSD(i), 163.0 g ± 7.8; LVAD, 139.3 g ± 16.8; SSD(ii) plus VAA, 181.2 g ± 6.4; SSD(ii) plus 25% LVAD, 162.8 g ± 11.1; SSD(ii) plus 50% LVAD, 152.0 g ± 19.8 and SSD(ii) plus 75% LVAD, 142.4 g ± 17.2. Thus, at the termination of the experiment there were significant reductions in body weight, in comparison with female F344 rats fed either the Maintenance diet or the SSD(ii) diet plus VAA diet, of rats fed the remaining 5 test diets.

b Group 2: Male F344 rats

Body weights of male F344 rats fed the SSD(ii) diet or the Maintenance diet are presented in Figure 5.7 and Table 5.17. At 7 weeks there was a large reduction in body weight between the rats fed the SSD(ii) diet (139.0 g ± 23.7) and those fed the Maintenance diet (211.7 g ± 13.4).
Group 3: Female Wistar rats

Body weights of female Wistar rats fed the SSD(ii) diet or the Maintenance diet are presented in Figure 5.8 and Table 5.18. At 7 weeks the mean body weights of female Wistar rats fed each diet were: Maintenance, 155.2 g ± 14.3 and SSD(ii), 138.8 g ± 18.6. Thus at 7 weeks there was no evidence of any meaningful differences in body weight between rats fed either diet. The female Wistar rats fed the SSD(ii) diet were killed at week 8 when there was evidence of a slight reduction in body weight between rats fed the SSD(ii) diet (142.9 g ± 28.4) and the Maintenance diet (163.5 g ± 14.4).

d Group 4: Female Sprague-Dawley rats

Body weights of female Sprague-Dawley rats fed either the SSD(ii) diet or the Maintenance diet are presented in Figure 5.9 and Table 5.19. At 7 weeks body weights of rats fed each diet were: Maintenance, 217.0 g ± 18.6 and SSD(ii), 214.8 g ± 11.6. Thus there was no difference in body weight after 7 weeks. After 10 weeks when the rats fed the SSD(ii) diet were killed there was evidence of a slight reduction in body weight between the rats fed the SSD(ii) diet (230.2 g ± 10.1) and those fed the Maintenance diet (247.7 g ± 18.8).

(ii) Diet consumptions

Absolute and relative consumptions of all diets by all rats in Trial III are tabulated in Tables 5.20 - 5.27 and summarised in Table 5.28.

a Female F344 rats

Mean consumption of all diets fed to female F344 rats are presented as absolute values (g/rat/day) in Table 5.20 and as relative values (g/kg rat body weight/day) in Table 5.21. The average mean absolute consumption of rats fed the Brompton diet (over the first 5 weeks of Trial III) was 10.1 g/rat/day and that of rats fed the SSD(ii) diet (over the first 7 weeks of the trial) was 12.4 g/rat/day. Over the 13 weeks of Trial III the average mean absolute consumptions (g/rat/day) of the remaining diets were: Maintenance diet, 14.2; SSD(i) diet, 15.3; LVAD diet, 12.4; SSD(ii) plus VAA diet, 12.9; SSD(ii) plus 25% LVAD diet, 11.5; SSD(ii) plus 50% LVAD diet, 11.6 and SSD(ii) plus 75% LVAD diet, 11.9. Thus rats weighing 163.0 g at 13 weeks, fed the SSD(i) diet, consumed the most diet (15.3 g/rat/day), exceeding the consumption of the heaviest group, weighing 191.2g at 13 weeks, fed the Maintenance diet (14.2 g/rat/day). The least diet (10.1 g/rat/day) was consumed by rats fed the Brompton diet.
The mean relative consumption (g/kg rat body weight/day) of all the diets fed to female F344 rats fell by 43% - 63% as body weights increased between week 1 and week 13. The slight difference in absolute diet consumption already noted at 13 weeks between female F344 rats fed the Maintenance diet (14.2 g/rat/day) and those fed the SSD(i) diet (15.3 g/rat/day) was greatly magnified, in terms of the relative consumptions, (the Maintenance diet, 78.7 g/kg rat body weight/day and the SSD(i) diet, 124.5 g/kg rat body weight/day) by the large difference in body weight between these two groups at 13 weeks.

From the relative consumption of the SSD(ii) plus VAA diet (Group 5), the relative consumption of VAA (µgRE/kg rat body weight/day) throughout the 13 week trial was calculated. This was as follows: week 1, 1455; week 2, 1028; week 3, 1140; week 4, 1020; week 5, 859; week 6, 936; week 7, 780; week 8, 730; week 9, 701; week 10, 643; week 11, 643; week 12, 562 and week 13, 659. Thus between week 1 and week 13 the relative consumption of VAA per kilogram of body weight fell by over 50%.

b Male F344 rats

Mean consumptions of the SSD(ii) diet and the Maintenance diet fed to male F344 rats are presented as absolute values (g/rat/day) in Table 5.22 and as relative values (g/kg rat body weight/day) in Table 5.23. The average mean absolute consumption of the SSD(ii) diet over the first 7 weeks of the trial was 11.9 g/rat/day, while that of the Maintenance diet over the whole 13 weeks of the trial was 17.4 g/rat/day. In the first week (week 1) animals fed the Maintenance diet had a mean body weight of 56 g, almost 4 g less than that of rats fed the SSD(ii) diet (59.8 g). Despite this, rats fed the Maintenance diet, in week 1, consumed almost 25% more diet than those concurrently fed the SSD(ii) diet. The consumption of the SSD(ii) diet by male F344 rats dropped dramatically in week 7. This low consumption was probably due to the poor health of these rats, most of which were demonstrating advanced vitamin A deficiency at this time (Pitt 1985).

As body weight increased from week 1 to week 13, the relative consumption of the SSD(ii) diet and the Maintenance diet fell by 67.5% (week 1 - week 7) and 70.7% (week 1 - week 13) respectively.

c Female Wistar rats

Mean consumptions of the SSD(ii) diet and the Maintenance diet fed to female Wistar rats are presented as absolute values (g/rat/day) in Table 5.24 and as relative values (g/kg rat body weight/day) in Table 5.25. The average mean absolute consumption of the SSD(ii) diet over the first 7
weeks of the trial was 16.8 g/rat/day, while that of the Maintenance diet over the 13 weeks of Trial III was 14.7 g/rat/day.

As body weights increased from week 1 to week 13, the relative consumption of the SSD(ii) diet and the Maintenance diet fell by 42.9% (week 1 - week 7) and 57.9% (week 1 - week 13 respectively).

d Female Sprague-Dawley rats
Mean consumptions of the SSD(ii) diet and the Maintenance diet fed to female Sprague-Dawley rats are presented as absolute values (g/rat/day) in Table 5.26 and as relative values (g/kg rat body weight/day) in Table 5.27. The average mean absolute consumption of the SSD(ii) diet over the first 10 weeks of the trial was 17.9 g/rat/day, while that of the Maintenance diet over the whole 13 weeks of the trial was 20.1 g/rat/day.

As body weights increased from week 1 to week 13, the relative consumption of the SSD(ii) diet and the Maintenance diet fell by 58.5% (week 1 - week 10) and 63.4% (week 1 - week 13 respectively).

(iii) Clinical signs of Vitamin A deficiency
a Female F344 rats fed the Brompton diet
In Trial III female F344 rats fed the Brompton diet demonstrated similar clinical signs to those described for the same diet in Trial II (see Table 5.10). In Trial III, however, all these clinical signs appeared earlier than in the preceding experiment. For example, in Trial III rats ceased to gain weight producing a body weight plateau after 29 days of being fed the Brompton diet whereas, this effect was not observed in Trial II until after 36 - 39 days. In Trial III all rats in this group were killed on day 39.

b Female F344 rats fed the SSD(ii) diet
The SSD(ii) diet also induced clinical signs of vitamin A deficiency. As with the Brompton diet, female F344 rats fed the SSD(ii) diet demonstrated a body weight plateau, untidy coats, thin appearance, crusty red exudate around the eye sockets (periocular porphyrin deposits) and/or hair loss around the eye sockets, breathing difficulties and hunched posture. However, the first of these signs to appear, periocular porphyrin, was not seen until day 44. Only 2 out of the 5 female F344 rats fed the SSD(ii) diet appeared very sick when all this group were killed after 53 days.
c  Female F344 rats fed the LVAD diet

No abnormalities were observed amongst the 5 female F344 rats fed the LVAD diet in Trial III until day 57 when 2 rats had developed signs of respiratory disease; one of these was killed in extremis while the other was separated and took no further part in the experiment.

In contrast to the Brompton and SSD(ii) diets, the LVAD diet did not induce any clinical signs of vitamin A deficiency. However, 1 rat did show periocular hair loss after 85 days. Nevertheless all 3 remaining animals appeared hunched and unwell when killed after 13 weeks; this was probably due to incidental respiratory disease.

d  Female F344 rats fed the SSD(i) diet

After the thirteen weeks of Trial III, female F344 rats fed the SSD(i) diet failed to demonstrate any clinical signs characteristic of vitamin A deficiency. All the rats appeared unwell with pylo-erect, untidy coats when they were killed after 13 weeks.

One rat in this group was found dead after 50 days; it had been thin from day 38 and by day 47 it was showing great difficulty in breathing. This was probably due to respiratory disease and not related to the development of vitamin A deficiency.

e  Female F344 rats fed the SSD(ii) diet mixed with 25%, 50% or 75% LVAD diet

In contrast to the clinical findings in female F344 rats fed 100% SSD(ii) diet, no characteristic clinical signs of vitamin A deficiency were observed in female F344 rats fed the SSD(ii) diet mixed with various proportions (25%, 50% or 100%) of the LVAD diet. At the end of the 13 week trial, however, most of the rats in each of these 3 dietary groups demonstrated a thin appearance associated with depressed body weight gain, with some animals losing weight. Furthermore, several individual rats appeared sick showing a hunched posture, an untidy (pylo-erect) coat and obvious breathing difficulties. Three of these sick animals (2 fed diet containing 50% LVAD and the other fed 75% LVAD) showed hair loss around the orbit of the eye, but the eyeballs of these animals were normal and showed no evidence of periocular periocular porphyrin deposits or xerophthalmia. It is suggested that these animals were sick as a result of respiratory infection and not due to developing vitamin A deficiency. This view is supported by the comparatively healthy clinical status of the rats fed the SSD(ii) plus 25% LVAD diet. Of the 3 groups fed diets containing varying proportions of the LVAD diet, that fed 25% LVAD was the most likely group to develop vitamin A deficiency.
Clinical signs of vitamin A deficiency in male F344 rats, female Wistar rats and female Sprague-Dawley rats fed the SSD(ii) diet

The SSD(ii) diet induced clinical signs of vitamin A deficiency when fed to male F344 rats, female Wistar rats and female Sprague-Dawley rats. All 3 of these groups demonstrated reduced body weight gain and were thin in comparison to parallel groups fed the Maintenance diet. Most of the rats in each group fed the SSD(ii) diet demonstrated periocular porphyrin deposits and dull, slightly sunken eyeballs. (A small amount of porphyrin was also seen around the eyes of 1 female Sprague-Dawley rat fed the Maintenance diet.) In all 3 groups, periocular hair loss usually followed the appearance of porphyrin deposits.

Periocular porphyrin was the first ocular clinical sign to be observed. This was demonstrated by all 3 groups simultaneously (day 36). However, the development of vitamin A deficiency, as manifested by the progression of clinical signs, differed between these 3 groups. Both male F344 rats and female Wistar rats demonstrated untidy coats with several animals showing a hunched posture and/or breathing difficulties. Male F344 rats, however, progressed more quickly, becoming moribund 6 - 7 weeks after the start of the trial. No female Wistar rats had yet become moribund when they were killed 7 - 8 weeks after the start of the trial. In contrast, female Sprague-Dawley rats fed the SSD(ii) diet had demonstrated a body weight plateau (they were also thinner than those fed the Maintenance diet), porphyrin deposits and periocular hair loss (some animals) but otherwise they appeared in a healthy condition when they were killed 10 weeks after the start of the trial.

In each of these 3 groups of rats the appearance of specific clinical signs was generally not synchronous between the individual members of the group. Thus, within the same cage some animals would demonstrate the clinical signs of severe vitamin A deficiency while other rats would appear to be only moderately deficient. Such findings are common in vitamin A-deficient animals reared from weaning on a vitamin A-deficient diet (Lamb et al. 1974)

Rats fed the Maintenance diet

Apart from a single female Sprague-Dawley rat that showed specks of porphyrin around the eye sockets - a common observation amongst normal laboratory animals from time to time - no rats of any type demonstrated any of the clinical signs of characteristic vitamin A deficiency. All were normal and demonstrated normal growth curves.
(iv) **Retrospective Plasma Analysis**

About 9 months after the completion of Trial III, plasma samples which had been stored at -20°C, were analysed for retinol, α-tocopherol and BC. The results are presented in Tables 5.29 to 5.32.

**a  Female F344 rats**

The mean plasma levels of retinol (µg/dl), α-tocopherol (mg/dl) and BC (µg/dl) of female F344 rats fed all 9 experimental diets are shown in Table 5.29.

**Retinol**

Weanling female F344 rats fed the Maintenance diet and killed at the start of the trial (week 0) demonstrated a mean plasma retinol level (µg/dl ± SD) of 32.00 ± 2.56. At the end of the 13 week trial plasma retinol in rats fed the Maintenance diet had fallen to 23.32 µg/dl ± 1.53. Also after 13 weeks, female F344 rats fed the SSD(ii) diet supplemented with VAA demonstrated a mean plasma retinol level of 25.03 µg/dl ± 1.62. In contrast, female F344 rats fed all the other diets demonstrated greatly reduced mean plasma retinol levels. Mean plasma retinol values for rats fed all these diets were: Brompton, 0.65 µg/dl ± 0.79; SSD(ii), 1.37 µg/dl ± 1.27; SSD(i), 2.90 µg/dl ± 1.25; LVAD, 3.77 µg/dl ± 2.72; SSD(ii) plus 25% LVAD, 1.08 µg/dl ± 1.74; SSD(ii) plus 50%, 0.85 µg/dl ± 0.98 and SSD(ii) plus 75% LVAD, 2.00 µg/dl ± 0.60. Rats fed the Brompton diet or the SSD(ii) diet were killed at week 6 or week 8 respectively; all other rats were killed at the end of the trial at week 13.

**α-tocopherol**

Weanling female F344 rats fed the Maintenance diet and killed at the start of the trial (week 0) demonstrated a mean plasma α-tocopherol (± SD) of 0.86 mg/dl ± 0.07. At the end of the trial the mean plasma α-tocopherol level in rats fed the Maintenance diet was 0.75 mg/dl ± 0.14. In contrast to rats fed the Maintenance diet, rats fed all other diets, including the SSD(ii) diet supplemented with VAA, demonstrated reduced plasma α-tocopherol levels. Mean α-tocopherol levels for rats fed all these diets were: Brompton, 0.37 mg/dl ± 0.07; SSD(ii), 0.03 mg/dl ± 0.01, SSD(i), 0.33 mg/dl ± 0.09; LVAD, 0.40 mg/dl ± 0.07; SSD(ii) plus VAA, 0.04 mg/dl ± 0.02; SSD(ii) plus 25% LVAD, 0.16 mg/dl ± 0.04; SSD(ii) plus 50% LVAD, 0.21 mg/dl ± 0.09 and SSD(ii) plus 75% LVAD, 0.25 mg/dl ± 0.06.

**BC**

No BC was detected in plasma from female F344 rats fed any of the diets.
b Male F344 rats

The mean plasma levels of retinol (μg/dl), α-tocopherol (mg/dl) and BC (μg/dl) of male F344 rats fed either the Maintenance diet or the SSD(ii) diet are presented in Table 5.30.

\( \odot \) Retinol

At the end of the 13 week trial the mean plasma retinol level (± SD) of male F344 rats fed the Maintenance diet was 60.14 μg/dl ± 7.23. In contrast, two surviving male F344 rats fed the SSD(ii) diet and killed at 7 weeks demonstrated a greatly reduced mean plasma retinol level of 1.95 μg/dl ± 0.92.

\( \odot \) α-tocopherol

At the end of the 13 week trial the mean plasma α-tocopherol level (± SD) of male F344 rats fed the Maintenance diet was 0.53 mg/dl ± 0.13. In contrast, two surviving male F344 rats fed the SSD(ii) diet and killed at 7 weeks demonstrated a greatly reduced mean plasma α-tocopherol level of 0.04 mg/dl ± 0.01.

\( \odot \) BC

No BC was detected in the plasma of male F344 rats fed either the Maintenance diet or the SSD(ii) diet.

c Female Wistar rats

The mean plasma levels of retinol (μg/dl), α-tocopherol (mg/dl) and BC (μg/dl) of female Wistar rats fed either the Maintenance diet or the SSD(ii) diet are presented in Table 5.31.

\( \odot \) Retinol

At the end of the 13 week trial the mean plasma retinol level (± SD) of female Wistar rats fed the Maintenance diet was 16.28 μg/dl ± 3.29. In contrast, female Wistar rats fed the SSD(ii) diet demonstrated a greatly reduced mean plasma retinol level of 2.04 μg/dl ± 1.22 when they were killed at week 8.

\( \odot \) α-tocopherol

At the end of the 13 week trial the mean plasma α-tocopherol level (± SD) of female Wistar rats fed the Maintenance diet was 0.94 mg/dl ± 0.09. In contrast, female Wistar rats fed the SSD(ii)
diet demonstrated a mean plasma $\alpha$-tocopherol level of 0.09 mg/dl ± 0.04, more than a factor of 10 smaller than that of rats fed the Maintenance diet and killed 5 weeks later.

$\&$  

No BC was detected in the plasma of female Wistar rats fed either the Maintenance diet or the SSD(ii) diet.

d  

Female Sprague-Dawley rats

The mean plasma levels of retinol (µg/dl), $\alpha$-tocopherol (mg/dl) and BC (µg/dl) of female Sprague-Dawley rats fed either the Maintenance diet or the SSD(ii) diet are presented in Table 5.32.

$\&$  

Retinol

At the end of the 13 week trial the mean plasma retinol level (± SD) of female Sprague-Dawley rats fed the Maintenance diet was 15.96 µg/dl ± 1.43. In contrast, female Sprague-Dawley rats fed the SSD(ii) diet demonstrated a greatly reduced mean plasma retinol level of 0.62 µg/dl ± 0.73.

$\&$  

$\alpha$-tocopherol

At the end of the 13 week trial the mean plasma $\alpha$-tocopherol level (± SD) of female Sprague-Dawley rats fed the Maintenance diet was 0.96 mg/dl ± 0.11. In contrast, female Sprague-Dawley rats fed the SSD(ii) diet demonstrated a mean plasma $\alpha$-tocopherol level of 0.06 mg/dl ± 0.02.

$\&$  

BC

No BC was detected in the plasma of female Sprague-Dawley rats fed either the Maintenance diet or the SSD(ii) diet.

D  

Conclusions

(i)  

The evaluation of the SSD(ii) diet against the Brompton diet, SSD(i) diet and the LVAD diet in female F344 rats

Female F344 rats fed the SSD(ii) diet demonstrated depressed body weight gain (Figure 5.6, Table 5.16) and other clinical signs of vitamin A deficiency including sunken eyeballs, periocular porphyrin deposits and hair loss around the eyes in 7 - 8 weeks. Retrospective plasma analysis
confirmed that these rats had greatly reduced plasma retinol levels at post-mortem (Table 5.29). Similar results were obtained with the Brompton diet, in this case, however, the effects were demonstrated after 5 - 6 weeks. Thus the commercially-prepared SSD(ii) diet successfully induced vitamin A deficiency within an appropriately short period of time, although not quite as quickly as the laboratory-prepared Br. Brompton diet.

After 8 weeks neither SSD(i) diet nor the LVAD diet had induced any significant reduction in body weight (Figure 5.6, Table 5.16). As in Trial II, significant reductions in body weight and plasma retinol (Table 5.29) were demonstrated by rats fed either the SSD(i) diet or the LVAD diet after 13 weeks. Despite the low plasma retinol values of these rats, they showed no evidence of periocular porphyrin deposits or hair loss.

(ii) The evaluation of the SSD(ii) diet mixed with different proportions of the LVAD diet in female F344 rats

There were no differences in the responses of female F344 rats fed the SSD(ii) diet mixed with 25%, 50% or 75% LVAD diet. At 13 weeks, animals fed each of these 3 diets demonstrated significant reductions in body weight (Table 5.16) and plasma retinol levels (Table 5.29) in comparison with rats fed the SSD(ii) diet supplemented with VAA. As with rats fed the SSD(i) diet or the LVAD diet alone, no other clinical signs indicative of vitamin A deficiency, such as periocular porphyrin or hair loss, were observed. Thus, when mixed with different proportions of the LVAD diet, the SSD(ii) diet was reduced in efficacy to the level of the LVAD diet alone.

(iii) Sex and strain variations in response to the SSD(ii) diet

Differences between the sexes of various strains of rats in response to vitamin A deficient diets are well reported in the literature (Moore 1957). As judged by depressed body weight gain (Figure 5.7, Table 5.17), the appearance of other clinical signs such as periocular porphyrin deposits, and reduced plasma retinol levels (Table 5.30), male F344 rats developed deficiency after 6 - 7 weeks as compared to 7 - 8 weeks for females.

Similarly, female rats of the 3 strains - F344, Wistar and female Sprague-Dawley - differed in the time taken for the SSD(ii) diet to induce vitamin A deficiency. All these variations are illustrated in Figure 5.10 in which the growth curves of females of the 3 strains are presented with those of male F344 rats. Vitamin A deficiency, shown here by depressed or zero growth rate, developed after 6 - 7 weeks (male F344 rats), 7 - 8 weeks (female F344 rats), 8 weeks (female Wistar rats) or 10 weeks (female Sprague-Dawley rats), respectively.
The clinical signs of vitamin A deficiency induced by the SSD(ii) diet were similar in all the rat strains and sexes examined. The only differences being in the length of time that deficiency took to develop. Once vitamin A deficiency had fully developed all rats fed the SSD(ii) diet demonstrated markedly reduced body weight and plasma retinol levels in comparison to control rats fed either the SSD(ii) diet supplemented with VAA or the Maintenance diet.

In the literature it is well documented that normal male rats have much higher plasma retinol levels than normal female rats (Moore 1957). The data from Trial III add further support to this. At 13 weeks the mean plasma retinol levels (µg/dl ± sd, with the observed range in parentheses) of male and female F344 rats fed the Maintenance diet were: 60.14 ± 7.23, (52.20 - 71.30) and 23.32 ± 1.53, (21.30 - 25.60), respectively. The data from female rats of the 3 strains - F344, Wistar and Sprague-Dawley, although derived from small groups of only 5 animals suggest small strain differences in the normal plasma retinol level. At 13 weeks the mean plasma retinol levels (µg/dl ± sd, observed ranges in parentheses) of these rats fed the Maintenance diet were: female F344 rats, 23.32 ± 1.53 (21.30 - 25.60); female Wistar rats, 16.28 ± 3.29 (12.20 - 20.10) and female Sprague-Dawley rats, 15.96 ± 1.43 (14.60 - 18.10)

5 DISCUSSION
A Resume of Trials I-III
(i) Trial I
In Trial I a commercially-available low vitamin A deficient diet (LVAD) - intended for use in the subsequent experiment to evaluate the activity of BC against urinary bladder cancer (Trial VIII) - was assessed for the capacity to induce vitamin A deficiency in weanling female F344 rats. Depression of body weight gain, the appearance of clinical signs and retrospective plasma retinol were used as indicators of deficiency. The LVAD diet, manufactured from natural products, was evaluated alongside a commercially-produced semi-synthetic vitamin A-free diet (SSD(i)). Analysis revealed that the LVAD diet containing 38 µgRE/kg vitamin A and the SSD(i) diet contained less than 15 µgRE/kg vitamin A, the threshold limit of detection. After 20 weeks, neither diet had induced any significant depression in body weight gain nor any other clinical sign; as judged by this evidence, therefore, neither diet was effective in producing vitamin A deficiency. Retrospective plasma analysis subsequently revealed reduced retinol levels in rats fed both diets. However, the time period required (more than 20 weeks) for full vitamin A deficiency to develop (including depressed body weight gain and other clinical signs) would have rendered both diets unsuitable for the subsequent trial to evaluate BC against urinary bladder cancer.

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(ii) **Trial II**

This experiment was carried out to compare the effectiveness of the LVAD and SSD(i) diets with a proven vitamin A-deficient diet. To this end, a proven effective, laboratory-prepared, semi-synthetic, vitamin A-deficient diet (the Brompton diet) was tested alongside the two commercial vitamin-A deficient diets. The effect of sex and strain differences in response to these diets were also assessed, using male and female rats of the inbred F344 strain and females of the outbred Wistar and Sprague-Dawley stocks. As judged by depressed body weight gain, other clinical signs such as xerophthalmia, and greatly reduced plasma retinol levels, the Brompton diet induced vitamin A deficiency in male and female F344 rats after 6 or 7 weeks, respectively.

In contrast, after 13 weeks, the SSD(i) diet induced depressed body weight gain, other clinical signs such as periocular porphyrin deposits and reduced plasma retinol in male F344 rats, but only depressed body weight gain and reduced retinol in female F344 rats. At the same time, the larger outbred female rats of the Wistar and Sprague-Dawley stocks fed the SSD(i) diet demonstrated reduced plasma retinol but did not show any evidence of depressed body weight gain or any other clinical sign of deficiency.

Similarly, after 13 weeks, the LVAD diet also induced reduction in plasma retinol levels of male and female F344 rats, and female rats of the Wistar and Sprague-Dawley stocks, but none of these animals showed any evidence of depressed body weight or any other clinical signs.

(iii) **Trial III**

In Trial III, another commercially-produced semi-synthetic vitamin A-deficient diet SSD(ii) was evaluated in female F344 rats in parallel with the Brompton, LVAD and SSD(i) diets. Specific instructions had been given that the SSD(i) diet was to be manufactured to the exact specifications outlined by Wise (1983) for the laboratory-prepared Brompton diet. Subsequent analysis showed that the SSD(ii) diet, like the SSD(i) diet, contained less than 15 μgRE/kg vitamin A. In addition to evaluating the effectiveness of the SSD(ii) diet, the effect of increasing the fibre content of this diet was also examined in Trial III. This was achieved by adding LVAD diet to the SSD(ii) diet. Information was also obtained on sex and strain differences in response to the administration of the SSD(ii) diet.

The SSD(ii) diet induced vitamin A deficiency in female F344 rats in 7-8 weeks, as judged by significantly depressed body weight gain, other clinical signs such as periocular hair loss and
porphyrin deposits, and greatly reduced plasma retinol levels. Similar results were obtained with the Brompton diet after only 5-6 weeks.

In contrast, after 13 weeks, no clinical signs of vitamin A deficiency were observed in rats fed either the LVAD or the SSD(i) diets, despite significant reductions in body weight gain and plasma retinol. After 13 weeks, no clinical signs of vitamin A deficiency were observed in rats fed mixtures of the SSD(ii) diet and different proportions of the LVAD diet (25%, 50%, 75%), although all 3 mixtures produced significant reductions in body weight gain and plasma retinol. Thus, when mixed with as low as 25% LVAD diet, the SSD(ii) diet was reduced in efficacy to the level of the LVAD diet alone. When fed the SSD(ii) diet, male F344 rats became deficient 1 week earlier (6-7 weeks) than females (7-8 weeks). Female Wistar and Sprague-Dawley rats became vitamin A-deficient after 8 and 10 weeks, respectively.

**B Discussion**

In this chapter a series of experiments (Trials I - III) are described. These were performed in the search for an effective, commercially-available vitamin A-deficient diet for use in the subsequent experiment (Trial VIII) to evaluate the anti-cancer activity of BC in vitamin A-deficient and vitamin A-sufficient rats.

The development of vitamin A deficiency in rats fed the various vitamin A-deficient diets (LVAD, SSD(i), SSD(ii) and Brompton) was assessed using depression of body weight gain and the appearance of other clinical signs of vitamin A deficiency such as periorcular porphyrin deposits or xerophthalmia. These results were subsequently confirmed by retrospective analysis for plasma retinol. Depression of body weight gain is an early sign of developing vitamin A deficiency (Moore 1957; Underwood *et al.* 1979) and when accompanied by more specific clinical signs, such as periorcular porphyrin deposits or xerophthalmia, depression of body weight gain forms a sensitive and accurate indicator of vitamin A deficiency in the rat (Moore 1957). These criteria for the assessment of vitamin A deficiency were relied upon heavily in Trials I-III as the results of plasma retinol analyses (performed by F. Hoffman-La Roche, Basle, Switzerland) were not available until several months after the end of the experiment. Sometimes body weight gain was depressed in the absence of other clinical signs of vitamin A deficiency. This was seen in female F344 rats fed the SSD(i) diet (Trial II).

It had been thought that the use of a low vitamin A diet (LVAD) manufactured from natural products for the induction and long term maintenance of vitamin A-deficient rats might have been
physiologically more advantageous than the use of a low-fibre semi-synthetic formulation. However, the time required for vitamin A deficiency to develop (13-20 weeks, Trials I-II) precluded the future use of the LVAD diet in Trial VIII. The LVAD diet had been designed to contain between 60 - 120 μgRE/kg vitamin A. However, subsequent diet analysis, performed for SDS Ltd., showed that the LVAD diet contained 38 μgRE/kg and the semi-synthetic "vitamin A-free" (SSD(i)) diet contained less than 15 μgRE/kg vitamin A. The SSD(ii) diet successfully induced vitamin A deficiency in female F344 rats after 6-8 weeks (Trial III). If the results of the diet analyses are correct, the results of Trials I-III suggest that while the SSD(i) diet contained less than 15 μgRE/kg vitamin A, the SSD(ii) diet may contain even less (perhaps, 0 μgRE/kg). Thus, for a given vitamin A-deficient diet to be effective within a suitably short time period (5-8 weeks), it must contain considerably less than 15 μgRE/kg vitamin A. If this is so, analysis of diets for vitamin A content is of questionable value as a level just below the threshold limit of detection (of the most sensitive modern HPLC methods) still renders diets incapable of inducing deficiency within the required time period. It is interesting to note that the addition of only 25% LVAD to the SSD(ii) diet rendered the latter as ineffective as 100% LVAD (Trial III); thus the difference in vitamin A content between an effective diet and an ineffective one must be very small.

In Trial I female F344 rats fed the SSD(i) diet showed no depression of body weight gain nor any other clinical signs of vitamin A deficiency after 20 weeks. A new batch of the same diet was used in Trial II. Although not as effective as the SSD(ii) diet, this second batch of the SSD(i) diet was slightly more effective as a vitamin A-deficient diet than the LVAD diet. After 13 weeks, male F344 rats fed the SSD(i) diet in Trial II demonstrated a series of clinical signs similar to those seen in rats fed the Brompton diet while, in the same experiment, male F344 rats fed the LVAD diet showed no such evidence of vitamin A deficiency. These results suggested that the two batches of the SSD(i) diet differed in vitamin A content, the second batch being a little more effective at inducing vitamin A deficiency than the first batch. Such variation could be due to insufficient cleaning of the industrial plant used in the manufacture of the diet, seasonal variations in the vitamin A content of the natural raw materials in the diet, or by the purchasing of raw materials from different sources by the manufacturer.

It was to keep trace levels of vitamin A to a minimum that Wise designed the Brompton diet, using rice starch rather than the more commonly used maize starch as the carbohydrate source (Wise 1983; Moore 1957; Underwood et al. 1979). The Brompton diet proved extremely effective as a vitamin A deficient diet, inducing full deficiency in male and female F344 rats after 5-6 and 6-7
weeks, respectively. The only significant difference between the Brompton diet and the SSD(i) diet was that rice starch was used in the former while maize starch was used in the latter. It was deduced, therefore, that this was probably the principal reason why the SSD(i) and the LVAD diets proved ineffective within the required time period (5-8 weeks).

With this in mind, SDS Ltd. were requested to manufacture a new commercial diet, the SSD(ii) diet. This was, in fact, a commercial version of the Brompton diet; strict instructions were given to follow Wise's formulation directly, including the purchase of ingredients from the same suppliers as Wise (Wise 1983).

The finding that the SSD(ii) diet induced full vitamin A deficiency two weeks after the Brompton diet (Trial III) was probably due to similar reasons as those causing variations between batches of the SSD(i) diet. Nevertheless, the SSD(ii) diet induced full vitamin A deficiency in female F344 rats in 6-8 weeks and, therefore, was a suitable diet for further experiments.

In agreement with previously published work, male rats always became vitamin A-deficient before females, whether fed the Brompton, SSD(i) or SSD(ii) diets (Moore 1957). Males have much higher circulating plasma vitamin A levels yet have much lower vitamin A stores than females (Moore, 1957). This may explain the finding that males tend to be affected by vitamin A-deficient diets before females.

In the present experiments, 3 rat strains (F344, Wistar, Sprague-Dawley) became deficient at different times, the largest strain (Sprague-Dawley) taking the longest time. As the plasma retinol levels of all 3 strains were very similar, though not identical, the strain differences in response to the SSD(ii) diet may be related to different levels of vitamin A storage.

Retrospective analysis for retinol was used to confirm the clinical assessment of vitamin A deficiency in these experiments. Plasma samples from rats in Trials I, II and III were stored at -20°C for 27, 20 and 10 months respectively, before analysis. Plasma retinol levels were remarkably stable when stored at -20°C for up to 42 months. This is illustrated in Table 5.33 with samples of plasma taken from 7 rats killed at the end of Trial II. These samples were analysed 20 months after the post-mortems. Duplicate samples from the same 7 rats remained frozen for a further 22 months before they were also analysed. Although the number of samples is small, the results demonstrate no significant depletion in retinol levels from 20 to 42 months.
In contrast, further storage had a marked effect upon the levels of \( \alpha \)-tocopherol in the same samples, which were depleted by about 40%; two of the seven samples were reduced by more than 50%. Thus, \( \alpha \)-tocopherol may be performing the function of an antioxidant within the plasma samples stored for long periods and thereby protecting retinol from breakdown.

The frozen plasma samples from Trials I-III were stored under air and not under oxygen-free nitrogen, the latter being the procedure recommended by Arroyave et al. (1983). Under oxygen-free nitrogen, the breakdown of \( \alpha \)-tocopherol in frozen plasma was reduced to much lower levels (data not shown). However, all the assays for plasma retinol for Trials I-III in frozen samples stored for long periods can be treated with confidence, as they would be unaffected by storage conditions (Table 5.33). In contrast, all the plasma \( \alpha \)-tocopherol levels in Trials I-III were probably 40-50% higher in the freshly taken samples.

In these first 3 experiments rats which had shown no clinical evidence of vitamin A deficiency (neither reductions in body weight gain nor eye lesions) were often subsequently demonstrated to have had very low plasma retinol levels. This relationship, in trials I-III between plasma retinol levels and the presence or absence of reductions in body weight and eye lesions is illustrated in Table 5.34. Plasma retinol levels in Trials I-III of the four types of rats fed the SSD(i), SSD(ii), LVAD and Brompton diets are presented as percentages of plasma retinol in appropriately fed control rats (rats fed Maintenance diet or vitamin A-deficient diets supplemented with VAA). These data suggest that, regardless of rat strain and sex, animals fed any of the diets did not demonstrate significant reductions in body weight gain and any other clinical signs of vitamin A deficiency, such as eye lesions, until the plasma retinol level had fallen to about 20% or 10% of control values, respectively. This is in general agreement with reports in the literature (Moore 1957; Pitt 1985; Shields and Jeffrey 1987).

The levels of energy available for metabolism ("calorific value") of the four commercially-supplied diets, in increasing order of energy were: Maintenance, 10.30 MJ/kg; LVAD, 11.09 MJ/kg; SSD(i), 13.38 MJ/kg and SSD(ii), 13.81 MJ/kg. In Trial I, female F344 rats fed the Maintenance diet generally consumed the most diet. This would be expected since this diet contains the least available energy. However, in Trial III, female F344 rats fed the SSD(i) diet consistently consumed the most diet, while there was no pattern to the consumption of the other commercial diets.
In retrospect, the design of Trials I-III would have been improved by the incorporation of additional rats on control diets. This would have allowed more comparisons between groups of rats killed at time points prior to the terminal kills.

The three experiments described in this chapter demonstrate the difficulties that can be encountered when attempting to obtain a commercially-supplied vitamin A-deficient diet. The majority of published work in the field of rodent vitamin A-deficiency has been carried out using laboratory-prepared diets in relatively small quantities. Nevertheless, as a result of the present work, a new, proven, commercially-available, semi-synthetic, vitamin A-deficient diet, with a known vitamin A content, is now available. To my knowledge, this is the first such diet in Europe.
CHAPTER 6

DETERMINATION OF A METHOD FOR THE LONG TERM MAINTENANCE OF VITAMIN A-DEFICIENT RATS AND OTHER INVESTIGATIONS PRIOR TO THE MAIN TRIAL (TRIAL VIII) TO INVESTIGATE THE ANTI-CANCER ACTIVITY OF BC.

1 GENERAL INTRODUCTION

In this chapter several experiments (Trials IV-VI) are described. These were performed to investigate various aspects related to low-level vitamin A supplementation in deficient rats. This information was required to carry out the main experiment (Trial VIII) investigating the anti-cancer activity of BC. All references to 'rat' or 'rats' from Chapter Six onwards refer to female F344 rats, unless otherwise stated.

In the main cancer experiment (Trial VIII) rats were to be made vitamin A-deficient by feeding the SSD(ii) diet. They were then to be maintained for a prolonged period in a deficient, but otherwise healthy condition, with plasma retinol levels of about 20% of control values. It was therefore necessary to determine, a) the time course of the development of vitamin A deficiency, as demonstrated by the fall in plasma retinol level, b) whether animals could be maintained in a deficient but otherwise healthy condition for long periods using low-level vitamin A supplementation, and c) the time point in the development of deficiency at which to start low-level vitamin A supplementation. These three aspects were examined in Trial IV. In this experiment, rats were made vitamin A-deficient and, at the earliest sign of morbidity (week 8, day 56), were supplemented with a range of levels of vitamin A in the drinking water. Plasma retinol levels were monitored in these animals during the development of deficiency, and for up to 16 weeks after the start of vitamin A supplementation. During this trial a grading system based on the clinical signs of vitamin A deficiency was developed, to assess the onset and degree of deficiency in rats fed the SSD(ii) diet.

In the main experiment (Trial VIII), vitamin A-deficient rats were to be maintained by low-level supplementation of the drinking water with water-miscible VAP (Roche Products Ltd., Welwyn Garden City, Herts AL7 3AY). Drinking water was obtained from the mains supply. It was subsequently discovered that mains water in the London area contains a relatively high level of nitrates (Putnam 1985). Despite the presence of the antioxidants BHA and BHT in the VAP
preparation, it was later discovered that the high nitrate level in the mains water could cause oxidative breakdown of the VAP, effectively reducing the concentration of vitamin A in the drinking water to very low levels (Putnam 1985). At a later stage of these experiments, Roche Products Ltd. carried out an experiment to investigate the breakdown of VAP in drinking water over a four day period. A period of four days was investigated as this was the maximum length of time before the replenishment of water bottles with fresh drinking water in the animal holding rooms. The results of this experiment are presented in Trial V(a).

Trial V(a) demonstrated a considerable breakdown of VAP in drinking water over a four day period. It therefore became necessary to examine whether, with the renewal of VAP in the drinking water every 3 or 4 days, the plasma level of retinol in the vitamin A-deficient rats fluctuated in parallel (Trial V(b)).

Two further essential aspects that needed to be examined before the main experiment (Trial VIII) were evaluated in Trial VI. Firstly an investigation of the reversal of vitamin A deficiency in rats fed the SSD(ii) diet by the administration of dietary BC and, secondly, an examination of the level of unconverted BC in the plasma of vitamin A-normal animals fed the SSD(ii) diet supplemented with vitamin A and BC. The rat is an excellent converter of dietary BC to vitamin A (Moore 1957). As a result, unconverted BC does not accumulate in body fat in contrast to other species, such as the cow and man, in which BC may colour the fat yellow-orange. For this reason, Moore (1957) described the rat as a "white fat species". The fact that the rat is an efficient converter of BC to vitamin A has hampered the use of this species for investigations of the anti-cancer activity of BC (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994). However, Mathews-Roth (1977) fed a high BC diet to mice and guinea pigs, 2 other efficient converters of BC, and demonstrated appreciable levels of unconverted BC in several organs of the body. Furthermore, Kornhauser (1986) has shown that rats fed high levels of BC are a good model in which to study the protective effects of BC. In Trial VI, rats were fed the SSD(ii) diet alone or SSD(ii) supplemented with a normal level of vitamin A. At day 54, when animals fed the unsupplemented diet were vitamin A-deficient, rats fed each diet were given supplementary dietary BC (6 mM/kg diet). At day 90, the levels of unconverted BC and retinol in the plasma of rats in each dietary group were analysed.
TRIAL IV: THE DEVELOPMENT AND LONG-TERM MAINTENANCE OF VITAMIN A DEFICIENCY IN FEMALE F344 RATS.

A Introduction

In the main cancer experiment (Trial VIII) it was envisaged that vitamin A-deficient rats would be maintained in a deficient but otherwise healthy condition for the duration of the trial, by low-level dietary supplementation with vitamin A. In this way it was hoped that deficient rats could be maintained with plasma retinol levels of about 20% of control values. To achieve this experimental regimen, the correct level of vitamin A supplementation had to be determined and the time point at which supplementation was to begin had to be defined.

Vitamin A-deficient rats have been maintained by dietary supplementation with RA (Moore 1960; Lamb et al. 1974); low level VAP in the diet (Shields and Jeffrey 1987). In longer-term experiments, vitamin A-deficient rats have been maintained by regular intramuscular injections of VAP (Capurro et al. 1960; Hicks 1968 and 1969), or by periodic administration of VAP by stomach tube (Cohen et al. 1976; Narisawa et al. 1976). None of these methods was appropriate for maintaining vitamin A-deficient rats in the study described in this thesis evaluating the anti-cancer activity of BC.

Regardless of the form of vitamin A, supplementation has usually been started at the early body weight plateau (Anzano et al. 1980; Shields and Jeffrey 1987). However, the time course of the development of deficiency varies, as does the time when body weight begins to plateau, depending on the size of the initial hepatic reserves (Moore 1957). This, in turn, varies according to rat strain and sex and upon the experimental design used (Moore 1957). For example, animals weaned directly on to a vitamin A-deficient diet have much smaller reserves and consequently become deficient much sooner than rats weaned to a deficient diet after a short period on a vitamin A-normal maintenance diet (Moore 1957).

When weanling rats are fed a vitamin A-deficient diet the hepatic vitamin A stores begin to drop immediately, the plasma levels of retinol remaining normal until the liver reserves are effectively exhausted (Moore 1957; Olson 1984; Pitt 1985). The plasma level then falls, followed by a decline in the level at the peripheral tissues, leading to overt clinical evidence of deficiency (Pitt 1985).

Trial IV was carried out to determine the dietary regimen capable of the long term maintenance of vitamin A-deficient rats with plasma retinol levels at 20% of control values. The original intention
had been to supplement the basic SSD(ii) diet of deficient and control animals with the appropriate levels of VAA beadlets. However, to maintain deficient rats in a deficient but healthy condition, each rat would have to consume no more than 0.6 μgRE VAA per day (Cohn 1984). Based on a daily diet consumption of 15 g/rat, the diet would have to contain nor more than 40 μgRE VAA/kg, or 266 μg/kg of beadlets. However, it is not possible to mix such a small quantity of beadlets into a ground diet and ensure uniform mixing, even using techniques of "premixing".

Therefore, it was decided to administer vitamin A to deficient animals as water dispersible VAP in the drinking water, while VAA beadlets would be given in the diet to control animals. VAP could be diluted easily to provide the low level of vitamin A required. VAA beadlets were used for control animals as they were more stable than VAP and had already been used successfully in previous projects as a dietary supplement (Hicks et al. 1983). As each kilo of control diet contained a minimum of 1.376 g of beadlets, uneven mixing was not considered to be a problem. Whether ingested as VAP or VAA, both would yield retinol after hydrolysis in the gut (Pitt 1985; Wolf 1984).

Trial IV could be divided into two parts, vitamin A depletion (week 0 - week 8) and vitamin A supplementation (week 8 - week 24). In the first part the time course of the development of vitamin A deficiency in female F344 rats fed the SSD(ii) diet was determined, with particular regard to the onset of clinical signs and plasma retinol levels. Once deficiency was fully developed, at week 8, a range of low levels of VAP, calculated to give 0.03 - 3.0 μgRE/rat/day (0.1-10.0 iu/rat/day) was given to 6 groups of deficient rats. The effects of each VAP level upon the plasma retinol level and subsequent animal survival was assessed for up to 16 weeks after the start of supplementation.

B Experimental Design

To carry out Trial IV, 235 rats were purchased. Owing to the large number required, these were obtained as 188 aged 18-24 days (Batch 1) and 47 aged 14-17 days (Batch 2).

The older rats (Batch 1) were acclimatised for 3 days. During the first 2 days they were randomised into groups (4 rats per cage) and fed the standard maintenance diet. They were weighed during the third day (day 0 minus 1). To prevent excessive accumulation of hepatic vitamin A reserves, the rats were starved for 18 hours during the third day. The experimental diets were administered on day 0, when these animals were 21-27 days of age. The 47 younger rats (Batch 2) were maintained on high protein pelleted diet with their mothers for 4 days. When they
were 18-21 days old, they were weaned on to the standard maintenance diet which they received for 2 days before being starved for 18 hours on the third day. On the next day, when they were 22-25 days old, they were weighed and assigned to the cages of the other 188 rats, increasing the number of animals in each cage to 5. At this time (day 5 of the experiment), the first 188 rats had already been eating the experimental diets for 5 days. The age-range of all the rats (Batches 1 and 2) when they were first given these diets was 21-27 days.

The design of the trial is shown in Table 6.1. Group 1 (50 rats) were fed the SSD(ii) diet, supplemented with VAA beadlets (2064 μgRE/kg) to provide an adequate (control) level of vitamin A. Group 2 (35 rats) were fed the SSD(ii) diet without added VAA. Each of Groups 3-8 were composed of 25 rats (150 in total); these were also fed the SSD(ii) diet without added VAA until week 8 (day 56) when six supplemental levels of VAP were administered. These levels of VAP were calculated to give intakes ranging from 0.03 to 3.00 μgRE/rat/day, assuming a daily water consumption of 16 ml/rat. The times and numbers of scheduled post-mortems are also shown in Table 6.1. At day 0, 5 rats from Group 1 and 5 animals from Group 2 were killed to provide information about plasma vitamin A levels prior to administration of the experimental diets. At weeks 3, 5, 6 and 8, 5 rats from Group 1 (SSD(ii) diet plus VAA) and 5 rats from Group 2 (SSD(ii) diet alone) were killed to obtain data about the depletion of plasma vitamin A levels during the development of vitamin A deficiency. At 9, 10, 12 and 24 weeks animals from Groups 3-8, as well as from Groups 1-2, were killed to determine the effects of different low-levels of VAP supplementation on the plasma vitamin A values of vitamin A-deficient rats. Some of the smaller rats in Groups 3-8 died (were found dead or killed in extremis) as a result of vitamin A deficiency between weeks 8-12. These unscheduled mortalities are also shown in Table 6.1.

At all post-mortems, the urinary bladder and a sample of trachea were taken for histology. A plasma sample was prepared, frozen and stored under oxygen-free nitrogen at -20°C for retrospective analyses for vitamins A and E and for BC.

All rats were weighed regularly and examined in detail for clinical signs of vitamin A deficiency twice weekly. The development of deficiency was monitored by changes in body weight and the appearance of clinical signs (Moore 1957; Lamb 1974). This clinical evidence was confirmed by retrospective plasma vitamin A analyses.

Fresh VAP solutions were administered in foiled bottles twice weekly to rats kept in darkened rooms. These solutions were prepared by serial dilution of a single aliquot of VAP. Aliquots had
been stored in the dark under oxygen-free nitrogen at 4°C. A daily water consumption of 16 ml per rat was assumed in the calculation of daily VAP intakes.

C Results

(i) Body weight increases

At the start (day 0) of Trial IV, the mean (± sd) body weight of 188 rats (see 'Experimental design') in all 8 groups was 34.0 g ± 8.9. The effects of the vitamin A-deficient diet (SSD(ii)) and the various supplemental levels of VAP upon body weight increases is shown in Figure 6.1. (For the average body weights of each group at weekly intervals throughout the trial, refer to Table 6.2). By week 6, all the rats fed the SSD(ii) diet (Groups 2-8) began to show depressed body weight gain in comparison to controls (Group 1). These differences in body weight gain were even more pronounced at week 8, when the 6 supplemental levels of VAP were administered to Groups 3-8. By week 10, the body weight of the unsupplemented rats in Group 2 showed a plateau, indicative of vitamin A deficiency (Lamb et al. 1974). In contrast, VAP administration between weeks 8-10 increased the rate of body weight gain in Groups 5-8 (0.375-3.0 μgRE/rat/day). In week 10-12 the mean body weights of rats in Groups 3, 4 and 5 (0.03-0.375 μgRE/rat/day) all fell slightly. Indeed, as a result of vitamin A deficiency all rats in Groups 2, 3 and 5 were dead (scheduled and unscheduled deaths) by week 12, while 2 animals in Group 4 survived until week 14. From week 12, only rats in Groups 7 and 8 (1.5-3.0 μgRE/rat/day) continued to increase in body weight. By week 17, rats in Group 8 had almost abolished the deficit in body weight gain which had developed at week 8 as a consequence of vitamin A deficiency. By week 19, only 1 rat remained in Group 7; this animal did not gain any weight until week 22 when it began to lose weight. At 24 weeks, at the end of the experiment, only rats in Group 8 (3.0 μgRE/rat/day) continued to gain weight, and this at a similar rate to that of the control animals in Group 1.

(ii) Clinical signs of vitamin A deficiency

From week 6-7, rats fed the unsupplemented SSD(ii) diet (Groups 2-8) began to demonstrate clinical signs of vitamin A deficiency. As in Trial III (Table 5.10), a progression from mild to severe clinical signs was apparent, with increasing time on the diet. The earliest signs were seen in the eye, where the usually bright slightly bulging eyeball (Figure 6.2) became dull and slightly sunken with one or two specks of porphyrin pigment around the orbit (Figure 6.3). With increasing time on the diet, the eyes became more sunken and further porphyrin was deposited (Figure 6.4). Hair was lost around the eyes which, as they became more sunken, appeared to lose their circular shape, taking on a slit-like appearance (Figures 6.5 and 6.6). In the final stages before death, the animals which did not recover demonstrated a hunched posture, often with brown
staining on the fur around the urino-genital and anal orifices. Although their abdomens appeared swollen, these animals were emaciated. Their eyes were closed (Figure 6.7) and usually there was evidence of xerophthalmia.

There was great variation in the time of onset of the first clinical signs within each group and between different groups. Thus, by week 10, 6 rats in Group 5 (supplemented with 0.375 μgRE/rat/day VAP from week 8) had died and several others were showing clinical signs. In contrast, only 1 rat in Group 2 (given no VAP supplement) showed early clinical signs by week 10, while all the other animals in this group appeared normal. In general, in each group it was the smallest rats which were the first to develop vitamin A deficiency. This finding is supported by similar observations in the literature (Lamb et al. 1974). These results indicate that the initial body weight of the animals at the start of the experiment was related to the variable time of onset of clinical signs; the lighter the rat, the earlier it would develop deficiency.

By week 8, individual rats in Groups 3-8 had shown some clinical signs of vitamin A deficiency; and two rats in Group 5 had been found dead. After the start of VAP administration, in week 8, animals in all the supplemented groups, except Group 8, continued to develop clinical signs of vitamin A deficiency and showed a high mortality. In contrast, rats in Group 8 (given 3.0 μgRE/rat/day VAP) showed clear evidence that VAP supplementation reversed the clinical signs of vitamin A deficiency. By week 11, all rats in this group no longer showed clinical signs, but appeared clinically normal (Figure 6.8).

At post-mortem, animals fed an adequate amount of vitamin A (group 1) showed normal viscera with a normal amount of body fat (Figure 6.9). In contrast, animals which were in the final stages of advanced vitamin A deficiency, or those which had died as a result of deficiency, demonstrated a complete lack of body fat (emaciation). Their intestines were usually devoid of ingesta and often, in rats which had shown swollen abdomens, greatly distended (Figure 6.10). Sometimes these distended intestines appeared to be dotted with many tiny red spots on the mucosal surface, suggesting evidence of intestinal haemorrhage.

(iii) Analysis of plasma retinol and α-tocopherol

The results of the plasma analyses for retinol and α-tocopherol are shown in Table 6.3.
a  Retinol
At the start of the experiment, there was no difference between the retinol levels of pooled plasma samples from Group 1 or Group 2, the retinol values being 27.30 μg/dl and 25.8 μg/dl respectively. Throughout the trial (week 0 - week 24), the mean retinol level of control rats (Group 1) ranged from 27.30 μg/dl to 38.62 μg/dl; the average mean value being 30.16 μg/dl. In contrast, the mean (± sd) retinol level of rats fed the vitamin A-deficient diet (Group 2) fell to 16.80 (± 10.23) μg/dl at week 3, then continued falling to 5.30 (± 5.10) μg/dl at week 5, 4.08 (± 3.12) μg/dl at week 6 and 5.94 (± 6.44) μg/dl at week 8. By week 12, the mean retinol value of the remaining rats in this group had fallen to zero. A similar decline in plasma retinol was observed between weeks 8-12 in the surviving animals supplemented with 0.03-1.5 μRE/rat/day VAP (Groups 3-7). Only rats in Group 8, given 3.0 μRE/rat/day VAP, demonstrated a relatively stable plasma retinol value; between weeks 12-24, the mean (± sd) retinol level ranged from 4.40 (± 0.72) μg/dl to 4.75 (± 1.40) μg/dl. This was about 16% of the retinol level of control rats (Group 1).

b  α-tocopherol
At the start of the trial, the α-tocopherol levels of rats in Group 1 (control diet) and Group 2 (vitamin A-deficient diet) were 0.97 mg/dl and 0.90 mg/dl, respectively. Throughout the trial, rats fed the control diet (Group 1) demonstrated a mean (± sd) plasma α-tocopherol level ranging from 0.79 (± 0.15) mg/dl to 1.14 (± 0.08) mg/dl. In contrast, the α-tocopherol level of rats fed the vitamin A-deficient diet (Group 2) fell in parallel with the fall in plasma retinol such that, by week 8, the mean (± sd) value was 0.40 (± 0.08) mg/dl. The mean (± sd) α-tocopherol level of rats in Group 8 (supplemented with 3.0 μRE/rat/day VAP) ranged from 0.36 (± 0.01) mg/dl to 0.46 (± 0.13) mg/dl. This was about 46% of the plasma α-tocopherol level of control rats (Group 1).

(iv)  Histology of the urothelium and trachea
The histological appearance in normal rats (Group 1) of the bladder urothelium and the tracheal epithelium are shown in Figures 6.11 and 6.12 respectively. In contrast, individual animals from Groups 3-7 fed the vitamin A-deficient diet and maintained for at least 4 weeks by low levels of VAP (ranging from 0.03 to 1.5 μRE/rat/day) demonstrated histological changes in these tissues. The urothelia were greatly thickened, consisting of hyperplastic transitional cells (Figure 6.13) and often containing areas of squamous metaplasia. Squamous metaplasia was also present in the tracheal epithelium of these animals (Figure 6.14). Such changes are consistent with previous
findings reported for vitamin A-deficient rats (Wolbach and Howe 1925 and 1933; Moore, 1957; Hicks 1969). Vitamin A-deficient rats maintained for up to 16 weeks by supplementation with the highest dose of VAP (3.0 μgRE/rat/day, Group 8) showed no histological evidence of vitamin A deficiency; in these animals the epithelial tissues of the bladder and trachea were found to be normal.

D Conclusions
The time course of the development of vitamin A deficiency in rats fed the SSD(ii) diet was followed using, as indicators of deficiency, depressed body weight gain, appearance of clinical signs and, in particular, plasma retinol levels. From week 4, rats fed the SSD(ii) diet (Groups 2-8) demonstrated reduced weight gain compared with control animals (Group 1) fed the same diet supplemented with VAA (Figure 6.1). The earliest clinical signs of vitamin A deficiency - dull eyes and specks of periocular porphyrin pigment - were observed by week 6 (Figure 6.3). These signs increased in severity between weeks 6-8 (Figures 6.4-6.7). Eventually, these animals became moribund and were killed in extremis or were found dead between weeks 8-10. The clinical signs, particularly the eye lesions were similar to those reported by Moore (1957). The sunken eyeballs (enophthalmus) are due to atrophy of the harderian gland, causing the eyeball to sink more deeply into its socket (Moore 1957). The harderian gland of some rodents, including rats, produces and secretes porphyrin (Sakai 1981), which exudes around the eye socket in vitamin A deficiency (Moore 1957).

The smallest rats in each group fed the vitamin A-deficient diet were usually the first in which clinical signs became evident; their larger cage mates often survived several more weeks before they too showed clinical evidence of vitamin A deficiency. This variation caused some blurring of the dose-response relationship between the level of VAP supplementation and subsequent development of clinical signs. For example, up to week 12 rats in Group 2 given no VAP showed fewer clinical signs of vitamin A deficiency than those in Groups 3-7 given 0.03-1.5 μgRE/rat/day VAP. Notwithstanding these effects, it is considered that variation between individual animals did not alter the key outcome of Trial IV; namely that the top dose of VAP alone was effective in maintaining vitamin A-deficient animals in an otherwise healthy condition for up to 16 weeks. However, these observations do emphasise the importance of a narrow starting weight range when setting up experiments in which synchronous vitamin A deficiency is required.

Once present, the progression of clinical signs from mild to severe followed a fairly standard pattern and time course (outlined above). This progression was graded on a five point scale.
(Table 6.4) to allow the latter stages of the development of vitamin A deficiency to be monitored. In general, grade 1-3 clinical signs were found to be reversible by supplemental VAP whereas grades 4-5 were not.

Between week 0 and week 3, the mean plasma retinol value of rats fed the vitamin A-deficient diet alone (Group 2) fell from 25.8 μg/dl to 16.8 μg/dl and continued falling to 5.3 μg/dl by week 5 (Table 6.3). Unfortunately no plasma samples were taken between week 0 and week 3. If this had been done, it might have demonstrated a plateau phase prior to the decline in plasma retinol as reported in the literature (Olson 1984; Pitt 1985). An immediate fall in plasma retinol from week 0 would only be expected if the animals had negligible liver vitamin A stores at the start of the experiment (Olson 1984). This is unlikely, as they were weaned on to normal maintenance diet from mothers which had been fed a similar maintenance diet themselves.

To summarise, the time course for the development of vitamin A deficiency in rats fed the SSD(ii) diet alone (Group 2): plasma retinol began to fall some time between week 0 and week 3. It continued to fall, so that by week 5 it was about 16% of the level in control rats (Group 1), continuing at this level up to the start of VAP supplementation at week 8. In parallel to the effects on plasma retinol, by week 4 a reduction in the rate of body weight gain was also evident. By week 6, the smallest rats began to demonstrate the first clinical signs of vitamin A deficiency and by week 8 onwards they were moribund or dead.

From week 8 when the first mortality from vitamin A deficiency occurred, a range of 6 low levels of VAP (0.03-3.0 μgRE/rat/day) were administered in the drinking water of rats fed the SSD(ii) diet (Table 6.1). Of the various VAP levels administered, only the top dose 3.0 μgRE/rat/day) was effective. This level of VAP promoted the survival of 23/25 rats in Group 8 for 16 weeks. Furthermore, this dose of vitamin A was sufficient to reverse all clinical signs of deficiency and promote a good rate of body weight gain while maintaining the mean plasma retinol value at about 16% of that of the control animals (Group 1). There was no evidence of any histological changes associated with vitamin A deficiency in the trachea or urinary bladder of rats given 3.0 μgRE/rat/day/VAP.

In contrast, none of the lower levels of supplemental VAP were effective in promoting long-term survival of vitamin A-deficient rats (Groups 3-7). When killed, the majority of these animals showed moderate or severe clinical signs, indicating that the latter stages of vitamin A deficiency had not been reversed. Furthermore, where it was possible to collect blood samples, subsequent
analyses demonstrated that plasma retinol values in these animals had fallen to negligible amounts or below detectable limits. Individual animals from the groups given the lower VAP doses (Groups 3-7) and which survived for more than 4 weeks frequently demonstrated histological changes in the urinary bladder (Figures 6.12 and 6.13) and trachea (Figure 6.14) indicative of vitamin A deficiency. These tissues were normal in animals from Group 8 supplemented with 3.0 μgRE/rat/day.

Thus, a dose of 3.0 μgRE/rat/day VAP, when given in 2 changes of drinking water each week, was effective in maintaining vitamin A-deficient rats in an otherwise healthy condition for 16 weeks. This level of VAP was, however, about 5 times higher than which had been expected to be effective before the trial was undertaken. It was suspected, therefore, that VAP was being broken down in the drinking water, despite the presence of the antioxidants BHT and BHA.

In this study only 1 time point for the start of VAP supplementation was examined, namely 8 weeks (day 56) after the start of the experiment. This was chosen as it was when the first mortality occurred. However, in retrospect, as many of the animals were already showing advanced clinical signs of vitamin A deficiency (Grade 3 or more) by this time, it would have been more appropriate to start supplementation earlier than day 56. If this had been done, fewer unscheduled deaths would have occurred. These results were further complicated by the large weight range of the animals, as discussed previously. Experiments involving vitamin A deficiency require animals with as narrow a weight range as possible, when these are purchased as normal weaners from a commercial supplier. Where large experiments put a greater demand on the animal supplier, large weight ranges are often unavoidable. In these circumstances, in particular, it would be better to begin VAP supplementation before the smallest animals have shown grade 2 clinical signs, but after the majority of the animals have begun to be affected. The wider the age-range, the more difficult this fine balance becomes.

To finalise, Trial IV demonstrated that vitamin A-deficient rats can be maintained for up to 16 weeks, with plasma retinol levels about 16% of control values, but in an otherwise healthy condition. This can be achieved by 2 changes of drinking water each week containing freshly prepared VAP giving a dose level of 3.0 μgRE/rat/day.
A Introduction

In Trial IV, it was shown that low-level VAP given to rats in the drinking water from the mains supply was capable of maintaining vitamin A-deficient animals in an otherwise healthy condition for up to 16 weeks. However, of the 6 different dose levels examined, only the highest (3.0 μgRE/rat/day) reversed all clinical signs of deficiency and permitted the survival of animals with plasma retinol levels at about 20% of normal control values. In contrast, the lower dose levels of VAP, ranging from 0.03 to 1.5 μgRE/rat/day, all failed to maintain survival of vitamin A-deficient rats beyond about 8 weeks from the start of VAP administration. These results were unexpected, as the level of vitamin A required to maintain adult rats in a vitamin A-deficient state was reported to be 0.6 μgRE/rat/day (Cohn 1984). The findings, therefore, suggested that the VAP in the drinking water was undergoing considerable degradation during each 3 or 4 day VAP administration period (the time periods between the refilling of water bottles with fresh drinking water).

To investigate this possibility, a series of in vitro experiments were carried at Roche Products Ltd. (Putnam 1985) to examine the stability of VAP in dilute aqueous solution. Two experiments were conducted, both of which were performed over a 72 hour period, equivalent to a 3 day VAP administration period. In the first experiment, the stability of VAP Type 100 in fresh deionised water was determined at starting concentrations of 0.3 μgRE/ml and 3.0 μgRE/ml. The second experiment involved investigating any differences between fresh deionised water and fresh glass distilled water with respect to the stability of VAP in Rovisol Type 100, a commercial mixture of vitamins A, D and E (Roche Products Ltd.). In this experiment the starting concentration of the VAP in the Rovisol Type 100 solutions was 300 μgRE/ml (1000 iu/ml).

B Materials and Methods

(i) Preparation of samples

a VAP Type 100

A 0.1 g sample of VAP Type 100 was added to 1000 ml of freshly prepared deionised water (dilution 1; vitamin A concentration: 3.0 μgRE/ml). 25 ml of dilution 1 was added to 225 ml of fresh deionised water (dilution 2; vitamin A concentration: 0.3 μgRE/ml). A total of 6 aliquots of each dilution was prepared, to provide samples for the determination of VAP concentration at each of 6 time points.
b Rovisol Type 100

100 ml solutions containing 1g of Rovisol were prepared with fresh deionised water or fresh glass distilled water. Each solution contained vitamin A at a concentration of 300 μgRE/ml.

For UV spectrophotometry, 1 ml of each 1% Rovisol solution was diluted to 100 ml with deionised or glass distilled water to give a final concentration of 3.0 μgRE/ml. This 3.0 μgRE/ml solution was freshly prepared before each determination.

(ii) Experimental Design

All solutions were left standing at room temperature (22°C) in non-actinic glassware. The concentration of vitamin A in each solution was determined at time 0, 12, 24, 36 and 48 and 72 hours using UV spectrophotometry.

C Results

The results of the experiment to assess the stability of VAP in dilute solutions of VAP Type 100 in deionised water are presented in Figure 6.15 (for the raw data, please refer to Table 6.5). Both of the two dilutions of VAP Type 100 examined (i.e. VAP at initial concentrations of 3.0 μgRE/ml [dilution 1] and 0.3 μgRE/ml respectively [dilution 2]) demonstrated a fall in the concentration of vitamin A palmitate over the 72 hour test period. In both cases, the fall in concentration was (Figure 6.15) steepest over the first 24 hours and then became more shallow over the next 48 hours. The more dilute solution, dilution 2, demonstrated the greater reduction in concentration falling from 0.28 μgRE/ml at time 0 to 0.17 μgRE/ml after 12 hours, a decrease of 39%. After 72 hours, the concentration of VAP in this dilution had fallen to 0.11 μgRE/ml, a total decrease of 59%. In contrast, the concentration of VAP in the other solution (dilution 1) fell from 3.07 μgRE/ml to 2.26 μgRE/ml over the first 12 hours, a fall of 26.5%, while the concentration after 72 hours was 1.46 μgRE/ml, a total decrease of 52%.

The results of the experiment to assess the effect of deionised water or glass distilled water upon the stability of VAP in solutions of Rovisol Type 100 are shown in Figure 6.16 (for the raw data, please refer to Table 6.6). Over the 72 hour test period, there was no significant difference in the concentration of VAP between the solution made up in deionised water and that in glass distilled water. After 72 hours, the concentration in the solution made with deionised water had fallen from 3.13 μgRE/ml at time 0 to 2.6 μgRE/ml, a total reduction of 17%. Likewise, the concentration in the solution made with glass distilled water had fallen by 16%, from 3.07 μgRE/ml at time 0 to 2.59 μgRE/ml after 72 hours.
D Conclusions

The stability over 72 hours of dilute aqueous solutions of VAP, as VAP Type 100, in deionised water was investigated (Figure 6.15). Additionally, the effect of deionised water or glass distilled water was investigated upon the stability of VAP in solutions of Rovisol Type 100 (Figure 6.16).

VAP was unstable in very dilute solutions of VAP Type 100 in deionised water. After 12 hours, the concentration of the solution initially diluted to give 0.3 µgRE/ml, had fallen by 39%, while the more concentrated solution (diluted to give 3.0 µgRE/ml) had fallen by almost 27%. Furthermore, deterioration in the vitamin A absorbance curve, suggesting a chemical change, was very noticeable (Table 6.5). This was especially noticeable in the 0.3 µgRE/ml dilution, with the UV maximum changing from 328 nm to 314 nm after 12 hours. The higher concentration did not show UV changes until the vitamin A determination at 36 hours.

The water, whether deionised or glass distilled, made little difference to the stability of VAP in solution of Rovisol Type 100 over 72 hours. In contrast to the first experiment involving VAP Type 100, it was noticed that when more concentrated solutions of Rovisol (300 µgRE/ml VAP) were kept and diluted immediately prior to each spectrophotometric determination, the reductions in vitamin A content were much lower. After 12 hours, the reduction was between 3-4% while after 72 hours it was only between 16-17%, with no deterioration in the maximum UV absorbance of vitamin A (UV max.) being detected. This greater stability of VAP in the solutions of Rovisol was probably due to a concentration effect and not to the vitamin E present in Rovisol Type 100. The antioxidant activity of vitamin E is associated with the alcohol form α-tocopherol, and not to the ester, α-tocopherol acetate, the form present in Rovisol Type 100 (Putnam 1985).

Furthermore, a concentration effect was observed in the experiment with VAP Type 100, with the lower concentration being less stable than the higher concentration. The cause of these concentration effects is uncertain, but it may be related to the relative proportionality between palmitate molecules and active oxygen atoms. It is noteworthy that both VAP Type 100 and Rovisol Type 100 contain the antioxidants BHA and BHT, in the absence of which the fall in VAP concentrations would have been, no doubt, even greater.

(i) Relevance of these findings to the maintenance of vitamin A-deficient rats

The findings of this in vitro investigation of the stability of VAP in dilute solution have clear implications for the studies described in this thesis in which low-level VAP supplementation was used for the long-term maintenance of vitamin A-deficient rats. Vitamin A-deficient animals are maintained by the administration of VAP in the drinking water such that, if no VAP degradation
occurred, each rat would consume 3.0 µgRE/ml each day. To do this, VAP type 100 was diluted in deionised water to give a concentration in the water bottles of 0.1875 µgRE/ml. In the light of the present in vitro investigation, and given that water from the mains supply was used, it is clear that the concentration of VAP available in the water bottles for consumption would fall by at least 59% over 72 hours.

Extrapolation of the VAP degradation curve (Figure 6.15, dilution 2) from 72 hours to 96 hours would suggest that over a 4 day period the total fall in the VAP concentration in vitro could be between 59% to 70%.

In the absence of any degradation, rats administered a solution of VAP containing 0.1875 µgRE/ml would consume a theoretical total of 9.0 µgRE in 3 days or 12.0 µgRE in 4 days. In reality, however, the total VAP consumptions are estimated to be, at the most, 5.4 µgRE in 3 days and between 4.8-6.0 µgRE in 4 days. Based on these estimated figures, each rat would consume an average of 1.8 µgRE/day in every 3 day VAP administration period or 1.35 µgRE/day in every 4 day period. The method used to estimate the intake of vitamin A from the amount of water consumed and the VAP breakdown curve (Figure 6.15, dilution 2) is shown in Appendix 2.

The in vitro investigations of VAP stability reported here were performed in closed systems from which no significant withdrawals of each solution were made. In contrast, in the animal holding rooms, rats would be regularly drinking from the water bottles. The volume of water withdrawn during drinking would be replaced with an equal volume of air. This regular intake of air would be expected to increase the level of VAP breakdown still further in comparison to that observed in vitro.

Thus, experiments carried out in vitro to assess the stability of dilute solutions of VAP Type 100 in deionised water have demonstrated that VAP under these conditions is unstable and likely to break down significantly over 3 or 4 days, even in the presence of the antioxidants BHA and BHT and after oxygen has been initially removed from the water, as in the case of fresh glass distilled water. These experiments have explained why such a high level of vitamin A (3.0 µgRE/rat/day) was shown to be required when VAP Type 100 is administered in the drinking water for the long-term maintenance of vitamin A-deficient rats.
TRIAL V(B) AN INVESTIGATION OF THE EFFECT OF FLUCTUATING VAP INTAKE UPON THE PLASMA RETINOL LEVELS OF VITAMIN A-DEFICIENT RATS.

A Introduction

The method developed for vitamin A administration in Trial IV was to prepare VAP Type 100 in dilute solution as a low-level vitamin A supplement. This would allow the maintenance of vitamin A-deficient rats in an otherwise normal condition. This supplement is administered in the drinking water (at a concentration of 0.1875 μgRE/ml when freshly made up) and replenished with each change of fresh water every 3 or 4 days.

The in vitro experiments (Trial V(a)) carried out by Roche Products Ltd. demonstrated that dilute solutions of VAP Type 100 in deionised water (0.3 μgRE and 3.00 μgRE/ml vitamin A respectively) were substantially broken down over a 3-4 day period. The degree of VAP breakdown in the solution initially containing 0.3 μgRE/ml at time 0 was 31% after 24 hours, 49% after 48 hours, 59% after 72 hours and, by extrapolation, 60-70% after 96 hours. A degree of VAP breakdown at least equivalent to those levels would therefore be expected over the 3 or 4 day periods of VAP administration to vitamin A-deficient rats in the animal holding room. The instability of VAP in the drinking water, coupled with the regular replenishment of water bottles with freshly prepared VAP solution, would thus be expected to cause the concentration of vitamin A available for consumption by vitamin A-deficient rats to fluctuate in a cyclical manner.

The experiments described in this thesis rely upon the analysis of retinol levels in plasma samples taken at post-mortem to confirm the vitamin A status of rats fed a vitamin A-deficient diet. In the light of the in vitro studies demonstrating VAP instability in drinking water, Trial V(b) was carried out to investigate whether fluctuating VAP consumption would result in variations in the plasma retinol levels of vitamin A-deficient rats. This involved the daily determination of plasma retinol levels in vitamin A-deficient rats over a period of 7 days (day 57 to day 64 of the experiment), during which fresh VAP was administered in the drinking water on day 57 and day 61, respectively.

B Experimental Design

30 female F344 rats aged 18-21 days old were acclimatised for a period of 2 days, during which they were fed a standard laboratory maintenance diet in pelleted form. After acclimatisation, the rats were starved for 18 hours before being randomised by weight into 2 groups (day 0). The design of the trial is shown in Table 6.7. Group 1 (12 rats) was fed the SSD(ii) diet plus a control
level of VAA (2064 µgRE/kg). Group 2 (18 rats) was fed the SSD(ii) diet alone. From day 47, when demonstrating clinical signs of vitamin A deficiency, the rats in Group 2 were given VAP Type 100 in the drinking water. When freshly prepared, the concentration of vitamin A in this drinking water was 0.1875 µgRE/ml. The water was obtained from the mains supply. Fresh VAP was administered every 3 or 4 days, on days 47, 50, 54, 57 and 61 respectively.

The times of post-mortems are also shown in Table 6. At post-mortem, plasma was prepared for retrospective analysis for vitamins A and E and for BC, and macroscopic findings related to vitamin A deficiency were noted. To examine the effect of fluctuating vitamin A intake (due to VAP breakdown in the drinking water) on plasma retinol levels, 2 or 3 rats from Group 2 (SSD(ii) diet alone) were killed on successive days after the administration of fresh VAP on day 57. At 24 hour intervals, plasma samples were taken from rats killed on days 57, 58, 59, 60 and 61. After the animals were killed on day 61, new drinking water, containing fresh VAP, was administered to the remaining rats, these being killed 2 days later on day 63. Animals fed the control diet (Group 1) were killed concurrently with vitamin A-deficient rats on days 57, 60, 63 and 64, respectively.

All rats were weighed regularly. From week 5, the animals were weighed each week and were examined in detail for clinical signs of vitamin A deficiency every working day. From week 4 until the end of the experiment, water consumption was monitored continuously over periods of 3 or 4 days.

C Results

(i) Body weights

The body weights of rats in Trial V(b) are presented in Figure 6.17. (The full tabulated data are shown in Table 6.8). At the start of the trial (week 0), the group mean body weights (g ± sd) were: Group 1 39.8 ± 3.1 and Group 2 28.4 ± 2.9, respectively. The mean body weight of rats in Group 1 increased steadily over the first 5 weeks of the trial and continued to rise up to week 8, with the rate of gain slowing slightly over the later weeks. Rats in Group 2 gained weight at a similar rate to Group 1 over the first 3 weeks. However, between weeks 3-5 these animals demonstrated an appreciably slower rate of gain compared with those in Group 1, while from weeks 5-8 no further increase in weight was observed in Group 2 rats.

The large difference in mean body weight (11.4 g) between the two groups at the start of the trial was the result of the method used to allocate the individual rats to each group. Initially, a system of random numbers had been used; however, this method produced 2 groups which both
demonstrated very large ranges in body weight. If these groups had been used, individual rats would have become vitamin A-deficient at widely divergent times. Therefore, to induce the animals in Group 2 to become deficient more or less simultaneously, the rats in both groups were redistributed (before going onto their diets on day 0) according to weight. The heaviest rats were placed in Group 1 and the lighter cases (the latter part of the batch) into Group 2. In this way, 2 groups were formed which differed markedly in mean body weight but in which the ranges of individual weights were very narrow, as indicated by the standard deviations of each group mean.

(ii) Clinical signs of Vitamin A deficiency

The macroscopic findings at post-mortem in rats of Trial V(b) are shown in Table 6.9. All the rats (12/12) in Group 1 demonstrated a normal appearance. After laparotomy, all these animals were observed to possess very large abdominal fat depots. The intestines of these rats were normal, containing plenty of intestinal ingesta and showing no evidence of intestinal haemorrhage or swelling due to the build up of intestinal gas.

In contrast, more than half (10/18) of the rats in Group 2 demonstrated a range of clinical signs of vitamin A deficiency similar to that described previously in Trials II, III and IV. These clinical signs (see grading system described in Table 6.4) ranged from grade 1 (3 rats), through grades 2 (3 rats) and 3 (3 rats) to grade 4 (1 rat). After laparotomy, the majority (16/18) of these animals were observed to possess no (3 rats) or negligible (13 rats) abdominal fat depots; the remaining 2 animals showed only moderate fat reserves. The 3 animals with no fat depots (classified as grades 2, 3 and 4 based on external appearance) also demonstrated intestinal gas; 2 of these animals also showed intestinal haemorrhage, the only 2 to do so.

To investigate whether vitamin A deficiency affected relative liver weight, the livers of animals from Groups 1 and 2 were weighed at post-mortem. The absolute and relative liver weights (g liver/kg rat body weight) are shown in Table 6.10. There was some evidence that the absolute liver weights of animals in Group 2 were lighter than those in Group 1. In contrast, livers from vitamin A-deficient rats (Group 2) did not show a reduction in relative liver weight, in comparison with control (Group 1) animals. This was because the body weights of animals in Group 1 were heavier than those of animals in group 2.

(iii) Plasma retinol levels

The group mean plasma retinol levels of rats killed on day 57 to day 64 are shown in Figure 6.18 (For average plasma retinol values in each group on day 57 to day 64, refer to Table 6.11). On
day 57, the mean (± sd) plasma retinol levels (µg/dl) were: Group 1 (fed SSD(ii) plus VAA diet), 25.60 (± 0.62) and Group 2 (fed SSD(ii) diet plus VAP in the drinking water), 1.75 (± 0.21). The administration, on day 57, of fresh VAP to the rats remaining in Group 2 did not result in any marked differences in the mean plasma retinol values obtained on days 58, 59, 60 and 61. Similarly, when fresh VAP was given to the remaining animals on day 61, no significant effects were observed on the plasma retinol values obtained on day 63.

(iv) Water consumption

Group mean water consumptions, calculated as absolute values (g of water consumed/rat/day) and as values relative to body weight (g of water consumed/100g rat body weight/day), from day 22 to the end of the experiment on day 64 are presented in Table 6.12. For the 4 day period of days 22-26, the mean (± sd) absolute water consumptions were: Group 1 (fed the SSD(ii) plus VAA diet), 22.6 (± 3.5) g rat/day and Group 2 (fed the SSD(ii) diet plus VAP in the drinking water) 16.0 (± 0.7) g rat/day. In general, the absolute water consumptions of the two groups remained roughly at these levels until the end of the experiment, with Group 2 animals drinking about 25% less than those in Group 1.

For the 4 day period days 22-26, the mean (± sd) relative water consumptions were: Group 1, 201.7 (± 31.6) g/body weight/day and Group 2 163.8 (± 7.6) g/100g body weight/day. With increasing body weight, the relative water consumption of rats in Group 1 steadily declined from that observed at days 22-26 to 108.1 (± 14.1) g/100g body weight/day by day 64. Until days 44-47, a similar decline in the relative water consumption was observed in rats fed the vitamin A-deficient diet (Group 2). However, from day 47 to the end of the experiment, the period corresponding to the plateau phase of the body weight curve (Figure 6.17) and to the administration of VAP (from day 47), the relative water consumption of rats in Group 2 rose slightly. At day 57-61, these animals drank 135.2 (± 28.6) g of water/kg body weight/day, a value which was 135% of the relative consumption of animals in Group 1.

(v) Estimated VAP intake

The average daily intake of VAP over each 3 or 4 day consumption period for rats in Group 2 is also shown in Table 6.12. (For full details of the method used to estimate VAP intake from the VAP degradation curve (Figure 6.15, dilution 2) and the absolute water consumption, refer to Appendix 2). From day 22-26 to the end of the experiment the estimated average daily VAP intake of rats in Group 2 ranged from 3.69 - 5.81 µgRE/rat/day.
Conclusions

Trial V(b) was carried out to investigate whether fluctuating vitamin A intake (caused by VAP breakdown in the water bottles) would effect the plasma retinol levels of VAP-supplemented vitamin A-deficient rats. The experiment involved the daily determination of plasma retinol in deficient rats over a period of 7 days (days 57-64), during which fresh VAP was administered in the drinking water on days 57 and 61 respectively.

Despite a large difference in mean body weight at the start of the trial, a clear difference in the rate of body weight gain was discernible between control animals (Group 1) and Group 2 rats fed the vitamin A-deficient diet (Figure 6.17). Between weeks 5-8 the mean body weight of rats in Group 2 remained constant, showing the plateau characteristic of vitamin A deficiency. At week 8 (day 57), the presence of overt clinical signs and depressed plasma retinol values (Table 6.11) confirmed that the rats in Group 2 were vitamin A-deficient.

After the administration of fresh VAP in the drinking water on days 57 and 61, no significant changes were seen in the mean plasma retinol value of rats in Group 2. Throughout the experimental period of day 57 - day 63, the mean plasma retinol value in these animals ranged between about 6-15% of the control values in Group 1. Thus, the administration of fresh VAP in the drinking water every 3 or 4 days did not significantly affect the plasma retinol levels of vitamin A-deficient rats supplemented in this way, particularly when the standard deviations were taken into account (Figure 6.18).

Throughout the trial, control rats consumed about 23 ml/rat/day of drinking water. In contrast, vitamin A-deficient animals in Group 2 drank only about 16 ml/rat/day, or 25% less than the controls (Table 6.12). Estimated values for the daily VAP consumption of vitamin A-deficient rats ranged from 3.69 - 5.81μgRE/rat/day. These values were calculated from the absolute water consumptions (Table 6.12) and the VAP breakdown curve (Figure 6.15, dilution 2). These estimated daily VAP intakes would provide more vitamin A than the 0.6 μgRE/rat/day which Cohn (1984) recommended as being the minimum required for the maintenance of vitamin A-deficient rats. It is likely, therefore, that compared with the curve of VAP breakdown measured in Trial V(a), a higher degree of degradation took place under the experimental conditions of Trial V(b). A possible cause for this could be the continual displacement of drinking water by volumes of fresh air every time animals took a drink from the water bottles. This would provide additional oxygen and thereby potentiate further breakdown of the remaining VAP present in the drinking water.
TRIAL VI. THE REVERSAL OF VITAMIN A DEFICIENCY AND THE
ABSORPTION OF UNCONVERTED BC IN RATS FED THE SSD(H) DIET AND
A HIGH LEVEL OF DIETARY BC.

A Introduction

In the main study to evaluate the anti-cancer activity of BC (Trial VIII), it was envisaged that
dietary BC would be administered to carcinogen-treated vitamin A-deficient and vitamin A-normal
rats. For Trial VIII, rats previously made vitamin A-deficient had to be restored to normal vitamin
A status with dietary BC as the only vitamin A source. Also, animals fed a diet combining an
adequate level of vitamin A had to accumulate appreciable levels of unconverted BC in their
plasma and tissues.

The rat is an efficient converter of dietary BC to retinol (Moore 1957). In normal physiological
circumstances, little unconverted BC is absorbed by the rat, so the concentration in the plasma is
low (Pitt 1985; Wolf 1984). This has hampered laboratory investigation into the activity of BC
against experimentally-induced cancer (Rogers and Longnecker 1988; Moon et al. 1989; Rogers

Before Trial VIII was undertaken, therefore, a small experiment (Trial VI) was carried out, to
confirm that very high dietary BC (6 mM/kg of diet) could reverse the evidence of vitamin A
deficiency and give rise to the accumulation of appreciable levels of unconverted BC in the plasma
of rats fed the SSD(ii) diet. This involved the administration of BC to 2 groups of rats, one group
being vitamin A-deficient and the other being fed an adequate level of vitamin A. A third group of
rats were also fed a diet adequate in vitamin A but were not given BC. These acted as a control
group. Throughout the experiment body weights and clinical signs of vitamin A deficiency were
monitored. The animals were killed after 6 or 13 weeks of BC administration.

B Experimental Design

30 weanling female F344 rats aged 18-24 days old were acclimatised for 2 days during which they
were fed pelleted maintenance diet. After acclimatisation, the animals were randomised into 3
groups. These groups, each of 10 animals, were starved for 18 hours before the experimental diets
were administered on day 0.

The experimental design is shown in Table 6.13. Animals in Group 1 were fed the SSD(ii) diet
plus VAA (2064 μgRE/kg) until day 54 when, in addition, they were given BC (6 mM/kg). Group
2 (control rats) were fed the SSD(ii) plus VAA diet but were not given BC. Rats in Group 3 were

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fed the SSD(ii) diet alone until day 54 when they received BC (6mM/kg) mixed into the basal diet. It had been intended that BC was to be administered to Groups 1 and 3 when rats in Group 3 were showing overt clinical signs of vitamin A deficiency. However, due to unforeseen circumstances, BC was administered earlier than anticipated, on day 54, when rats in Group 3 were showing markedly reduced growth rate, but no other clinical signs of deficiency. Furthermore, no plasma samples were prepared at the time of BC administration.

The times of post-mortems are also shown in Table 6.13. After 6½ weeks of BC administration, 5 rats from Group 2 and 5 rats from Group 3 were killed on day 90. All 10 animals in Group 1 and the remaining 5 in each of Groups 2 and 3 were killed on day 140. At post-mortem, plasma samples were prepared for retrospective analysis of retinol, BC and α-tocopherol.

All rats were weighed regularly. From these data, the development and reversal of vitamin A deficiency in Group 3 rats was monitored, by comparison with the growth curve of rats in Group 2 (fed a control diet).

C Results

(i) Body weights

The body weights of rats in Trial VI are presented in Figure 6.19. (For average body weights of each group at weekly intervals throughout the trial, refer to Table 6.14). The mean (± sd) body weight (g) of all the rats in Trial VI at the start of the experiment (day 0 minus 1) was: 49.1 (± 7.4). Animals in Groups 1 and 2 gained weight at a similar rate throughout the experiment; there was no evidence that a diet containing 6mM/kg BC had any adverse effects on body weight gain. From week 3 to week 8, rats in Group 3 (fed the vitamin A-deficient diet alone) demonstrated a lower mean body weight than animals in Groups 1 and 2.

After the administration in week 8 of BC to animals in Group 3, these rats showed a dramatic increase in the rate of body weight gain, such that by 10 weeks this group demonstrated a higher mean body weight than either Group 1 or Group 2. From week 10 to the end of the experiment, all 3 groups were of similar mean body weights.

(ii) Analyses for plasma retinol and BC

The results of the analyses for plasma retinol, BC and α-tocopherol are shown in Table 6.15. There were no differences in the plasma retinol levels of rats in all 3 groups, either at day 90 or day 140. Thus, at day 90, animals in Group 3 were not vitamin A-deficient. Similarly, no
significant differences were evident in plasma BC levels, although there was often considerable variation between individual values within the same group. All the animals fed BC (Groups 1 and 3) demonstrated high levels of unconverted BC present in their plasma. The values for α-tocopherol were remarkably similar and showed little variation throughout all the groups at both time points.

D Conclusions

Although rats in Group 3 (fed the vitamin A-deficient diet) had shown markedly reduced growth rate (Figure 6.19) between weeks 3-8, these animals had not demonstrated periorcular pigment or xerophthalmia indicative of fully developed vitamin A deficiency. However, following the administration of BC at week 8, the growth rate of Group 3 animals dramatically increased, such that by week 10 these rats were heavier than those of either Group 1 or Group 2. Such a dramatic change in the growth rate of rats in Group 3 suggests a growth-promoting effect for BC. As no similar effects were seen on the body weight of rats in Group 1 (fed a diet containing an adequate 'control' level of vitamin A until the administration of BC), the observation of a 'growth promoting effect' of BC in Group 3 appears to be good indirect evidence that the rats in this group were at least partially vitamin A-deficient at week 8. It is unfortunate that no plasma samples were available from these rats at this time point.

Plasma samples were prepared from animals in Groups 2 and 3 at day 90, 6½ weeks after BC was first administered to Group 3. These results (Table 6.15) clearly demonstrated no difference in the plasma retinol levels of rats in these 2 groups; if Group 3 had been vitamin A-deficient at week 8, this deficiency had been reversed by week 13. Furthermore, the plasma data also demonstrated that substantial amounts of unconverted BC were absorbed into the plasma of rats fed 6 mM/kg BC in the diet. This was true even of rats in Group 1 which had been fed a diet adequate in vitamin A. At post-mortem, the plasma of rats fed BC was orange/yellow in colour, as were the liver, kidneys, adrenals and body fat. The normal appearance of the viscera and the excised liver from a control rat (group 2) are shown in Figures 6.20 and 6.21 respectively. In contrast, the appearance of the viscera and the excised liver from a rat fed BC (6 mM/kg) are shown in Figures 6.22 and 6.23, respectively. The urinary bladders of rats fed 6 mM/kg BC were more yellow in colour than those from rats in Group 2. Subsequent HPLC analysis of homogenised liver samples from these animals revealed high levels of BC (data not shown).

Under normal dietary circumstances the rat is regarded as a 'white fat species', being a good converter of dietary BC to retinol. However, Trial VI demonstrated that when fed in sufficiently
high concentrations, unconverted BC is absorbed into the plasma. Qualitative evidence from this experiment suggested that BC accumulated in certain tissues and the body fat of the rat, colouring these orange/yellow. Under these conditions the rat is a suitable model in which to investigate the anti-cancer activity of BC (Kornhauser et al. 1986).

6 DISCUSSION OF TRIALS IV - VI

Before the main experiment (Trial VIII) to evaluate the anti-cancer activity of dietary BC could be started, it was necessary to obtain certain preliminary data. This information was determined in a series of experiments (Trials IV-VI).

Trial IV was undertaken to investigate: 1) the time course for the development of vitamin A deficiency in rats fed the SSD(ii) diet and, 2) to find a suitable level of supplemental VAP which would maintain vitamin A-deficient rats in an otherwise healthy condition, but with plasma retinol levels at about 20% of control values.

Depending on initial body weight, individual rats became fully vitamin A-deficient in 8-10 weeks, as judged by body weight changes, reduced plasma retinol values and clinical signs such as xerophthalmia. This was consistent with results from Trial III, as well as those reported for various other vitamin A-deficient diets (Lamb et al. 1974; Cohen et al. 1976). A fall in plasma retinol level compared with control animals was the first change to occur, probably between weeks 0-3. This was followed by a depression in the rate of body weight gain at week 4. At 6 weeks, the first clinical signs of vitamin A deficiency became apparent. Without supplementation with vitamin A the smallest rats, which were affected before their larger cagemates, would begin to die from week 8.

One of the problems of experimenting with vitamin A-deficient animals is the time required to exhaust the liver stores of vitamin A before full deficiency develops (Moore 1960). RA supports all the normal physiological functions of vitamin A except vision and reproduction (Moore 1960; Pitt 1985); rats exposed to RA for several months go blind and lose the capacity to successfully reproduce (Lamb et al. 1974; Pitt 1985). In 1960, Moore reported that dietary supplementation with RA (5 µg/g of diet) could be used to maintain vitamin A-deficient rats until they were required for experimental purposes. As RA is not stored in the body, the clinical signs of vitamin A deficiency rapidly reappear when the RA supplemented diet is withdrawn (Moore 1960; Lamb et al. 1974). A procedure involving cyclical feeding of vitamin A-deficient with a diet supplemented with and then lacking in RA was used by Lamb et al. (1974) to produce batches of
synchronous vitamin A-deficient animals. The technique of RA supplementation was designed to avoid the time taken for weanling rats to develop vitamin A deficiency thereby rapidly producing batches of deficient animals for short term experiments. It was not designed as a method for the long-term maintenance of vitamin A-deficient rats. Thus, supplementation with RA was inappropriate for the long-term maintenance of vitamin A-deficient rats in the experiment reported in this thesis (Trial VIII) evaluating the anti-cancer activity of BC in vitamin A-deficient rats.

In short term studies carcinogenicity studies involving vitamin A deficiency, such as the biochemical investigation of initiation, it is often possible to carry out the experiments before the animals die from deficiency and without the necessity of vitamin A supplementation (Dogra et al. 1985). However, for longer-term investigations, some form of vitamin A supplementation is essential. Capurro et al. (1960) reported that a weekly intramuscular injection of 22.5 μgRE VAP (equivalent to 3.2 μgRE/rat/day) maintained vitamin A-deficient rats without allowing vitamin A storage in the liver. This regimen was used by Hicks (1968 and 1969) to maintain rats for up to 17 weeks in an investigation of the response of the urinary bladder to vitamin A deficiency. Both Capurro et al. (1960) and Hicks (1968 and 1969) required small numbers of vitamin A-deficient rats for investigations of moderate duration (17-40 weeks). The experiment described in this thesis to investigate the anti-cancer activity of BC in vitamin A-deficient rats (Trial VIII) was originally designed to involve at least 30 vitamin A-deficient rats in a study lasting about 60 weeks. Weekly injections would have been an inappropriate system of vitamin A supplementation to use in this case. Periodic administration of vitamin A by stomach tube at specific time points within a study has been used by some workers to maintain vitamin A-deficient rats (Cohen et al. 1976; Narisawa et al. 1976). This system was used by Cohen et al. (1976) who administered 30 μgRE/rat during week 9 and week 16 of a 22 week carcinogenicity experiment. Similarly, in a 45 week experiment, Narisawa et al. (1976) gave 3.0 μgRE/rat/ each week from week 35 to the end of the study. As Trial VIII was originally intended to last for 60 weeks or more, regular administration of vitamin A by stomach tube to maintain vitamin A-deficient animals would have involved too much animal handling during the study. This would have been particularly troublesome if, for scientific completeness, all the other experimental groups were given vitamin A-vehicle concomitantly.

Shields and Jeffrey (1987) reported that supplementation of a basal vitamin A-deficient diet with low levels of VAP (15-120 μgRE/kg of diet) maintained vitamin A-deficient male F344 rats for at least 2 weeks after full deficiency had developed. These workers did not report whether a similar
dietary regimen was capable of maintaining vitamin A-deficient rats for longer periods. Vitamin A is administered in the diet as beadlets that must be uniformly mixed into the diet. Cohn (1984) commented that a level of only 0.6 μgRE/day vitamin A was required to maintain vitamin A-deficient rats in an otherwise healthy condition for long periods. In the light of this, for the studies described in this thesis, it was felt that it was not possible to prepare diets that would provide rats with this low dietary intake of vitamin A. Therefore, the decision was taken to supplement vitamin A in the drinking water.

To determine a suitable level of supplemental VAP capable of maintaining vitamin A-deficient rats for long periods, a series of 6 low doses of VAP were administered twice weekly in the drinking water of such animals. The 6 levels of VAP were chosen to give daily intakes of vitamin A ranging from 0.03 to 3.0 μgRE/rat, assuming a daily water consumption of 16 ml/rat. Before Trial IV was carried out, it had been expected that a daily VAP intake of about 0.6 μgRE/rat (Cohn 1984) would adequately promote the long-term survival of vitamin A-deficient animals in an otherwise healthy condition. However, in Trial IV only the highest VAP dose, equivalent to a daily vitamin A intake of 3.0 μgRE/rat, was effective. This level of supplemental VAP maintained rats for 16 weeks with plasma retinol values about 20% of control values. In addition, rats given this level of supplementation were free from clinical signs of vitamin A deficiency and gained weight at a reasonable rate. All the lower VAP levels were ineffective in maintaining long-term survival of vitamin A-deficient rats. As the only effective VAP level (3.0 μgRE/rat/day) was about 5 times greater than the expected level (about 0.6 μgRE/rat/day), these results suggested that some degree of VAP breakdown must have occurred within the system.

The stability of low concentrations of VAP in aqueous solution was examined in Trial V(a). In 2 in vitro experiments, VAP in dilute solution was confirmed to be highly unstable, even in the presence of the antioxidants BHT and BHA. Over a 72 hour (3 day) period a solution containing 0.3 μgRE/ml was found to break down by 59%. Over a 4 day period, this degree of degradation would be likely to increase to about 70%. In rat drinking water, the degree of VAP breakdown would be expected to be at least equivalent to that found in Trial V(a). Indeed, a higher proportion of VAP might be degraded in the open system afforded by the water bottle; as each rat takes a drink, further air containing fresh oxygen would be drawn into the drinking bottles giving rise to further oxidation.
In Trial V(b), the effect, if any, of fluctuating vitamin A intake - caused by VAP breakdown and replenishment every 3 or 4 days - upon the plasma retinol level of vitamin A-deficient rats was examined. No significant effects were detected.

Thus, a system capable of producing and maintaining vitamin A-deficient rats for use in future carcinogenicity studies was developed and investigated in Trial IV and Trial V(a) and V(b). Despite a high degree of breakdown, twice-weekly administrations of freshly prepared VAP at an initial concentration of 0.1875 μgRE/ml were effective in maintaining vitamin A-deficient rats for at least 16 weeks with plasma retinol values 20% of control levels. In addition, Trial IV demonstrated the importance of the initial body weight range at the start of the experiments which involve the production of vitamin A-deficient rats. Animals should be obtained with as narrow as possible body weight range. This avoids problems arising from the asynchronous development A deficiency (Lamb et al. 1974). Furthermore, the clinical signs of vitamin A deficiency described in Trial IV were used to develop a monitoring system to follow the later stages of deficiency. This grading system had a 5 point scale (grade 1-5). Grades 1-3 were generally reversible with the highest dose of VAP (3.0 μgRE/rat/day). Rats showing grade 4-5 clinical signs were usually not reversible and became moribund or died. The chronology and nature of clinical signs in this system were similar to those described by Moore (1957).

In Trial VI, a high dietary level of BC (6mM/kg of diet) was shown to be effective in reversing the evidence of vitamin A deficiency. This was not surprising as BC is a major precursor of vitamin A and the rat is a good converter of carotenoids to retinol (Moore 1957; Pitt 1985; Moon et al. 1989). High levels of unconverted BC were also demonstrated in the plasma of these rats, which was often orangey/red in colour. Similar colouration was detected in the liver, body fat and other organs and tissues, including the urinary bladder, suggesting that unconverted BC was absorbed and stored at these sites. These results confirmed the report of Kornhauser et al. (1986) that rats supplemented with high levels of dietary constitute a valid model in which to investigate the anti-cancer activity of BC.
CHAPTER 7

THE EVALUATION OF THE ACTIVITY OF DIETARY BC AGAINST CARCINOGEN-INDUCED URINARY BLADDER CANCER IN NORMAL AND VITAMIN A-DEFICIENT RATS

1 GENERAL INTRODUCTION

Chapter 7 describes 2 experiments: a small pilot study (Trial VII), and the main trial (Trial VIII) to evaluate the anti-cancer potential of dietary BC against carcinogen-induced urinary bladder cancer in normal and vitamin A-deficient rats. As Trial VIII involved a large number of animals studied over a long period of time, it was necessary, before starting the experiment, to assess the ability of vitamin A-deficient rats to survive the stress of repeated administrations of carcinogen required for the induction of urinary bladder tumours. This assessment was carried out in Trial VII. However, due to inappropriate storage of the stock VAP solution, used to maintain the vitamin A-deficient rats in an otherwise healthy condition, Trial VII did not go according to plan. Although almost all control animals (i.e. rats fed an adequate amount of vitamin A and dosed with carcinogen) survived beyond the dosing period, only 1 vitamin A-deficient rat survived after carcinogen treatment. Consequently, as Trial VII did not achieve all its objectives, it has not been reported in full and only the important findings from the experiment are included.

2 TRIAL VII: AN INVESTIGATION OF THE SURVIVAL OF VITAMIN A-DEFICIENT RATS AFTER REPEATED ORAL EXPOSURE TO CARCINOGEN

A Introduction

Urinary bladder tumours can be induced in rodents by treatment with the carcinogen BBN (Druckrey et al. 1964; Ito et al. 1969; Fukushima et al. 1976; Hicks et al. 1985; Ohtani et al. 1986). BBN is a specific bladder carcinogen, that is it gives rise only to tumours of the urinary bladder when administered orally (Druckrey et al. 1964). However, several repeated treatments are required for tumour induction, the frequency of exposure being more important than the cumulative dose in determining the carcinogenic response. For technical reasons it is sometimes found that repeated treatment of normal rats may result in some fatalities during or immediately after the dosing procedure, particularly when the experiments involve large numbers of animals. These deaths are generally due to 'miss dosing' or 'dosing accidents' and the stress of anaesthesia...
and carcinogen administration. Vitamin A deficiency might further compromise the ability of rats to survive the dosing procedures, leading to an increased number of mortalities. Therefore, before embarking upon Trial VIII, a small preliminary experiment (Trial VII) was set up, to assess the survival of vitamin A-deficient animals following repeated oral doses of BBN.

An additional aim of Trial VII was to evaluate the use of urine analysis as a non-invasive technique for assessing tumour development after carcinogen treatment. Analysis of urine samples, collected at different times after carcinogen treatment, involved the detection of small amounts of blood in the samples (haematuria). Assuming that only large, well developed bladder tumours would become haemorrhagic, and so give rise to haematuria, this technique seemed a potentially important non-invasive means by which tumour development could be assessed. If effective in this trial, urine analysis was to be used in the main trial (Trial VIII), in parallel with interim kills, to provide an accurate assessment of tumour development and, thereby, help to determine the time of the terminal kills.

B Methods

(i) Treatment groups

There were 2 treatment groups. Group 1, control animals, (10 rats) were fed the SSD(ii) diet supplemented with an adequate level of vitamin A in the form of VAA (2064.12 μgRE/kg). Group 2, vitamin A-deficient animals, (7 rats) were fed the SSD(ii) diet alone; from week 7, this group was supplemented twice weekly with freshly prepared VAP (0.1875 μgRE/ml) in the drinking water, to maintain the animals in a vitamin A-deficient but otherwise healthy condition. Both groups were given weekly aliquots of BBN dissolved in 30% ethanol. Doses of 50 mg or 100 mg BBN were administered by gavage each week for up to 7 weeks. Thus, rats received cumulative BBN doses of up to 350 mg or 700 mg, respectively. No animals were treated with the ethanol vehicle alone. After dosing, the animals were maintained on their experimental diets until the terminal kills 42-50 weeks later.

(ii) Carcinogen dosing procedures

a Preparation of dosing solution

The BBN was a generous gift from the laboratory of Dr. R. Moon (Life Science Division, IIT Research Institute, Chicago, Illinois, USA), where it was synthesised. Assuming 1 ml of BBN weighed 1 g, a solution of the carcinogen dissolved in 30% ethanol was prepared by gentle mixing on a magnetic stirrer. The concentration of this solution was such that the required amounts of BBN could be given in dosage volumes of 0.5 ml or 1.0 ml, respectively. The solution was
prepared about 60-90 minutes before use, kept in a hermetically sealed container and protected from exposure to direct light.

b  Administration of carcinogen

Animals were gently anaesthetised by exposure to an atmosphere of CO$_2$ in a perspex box, which had been specifically designed for the purpose. Anaesthetised rats were gagged and given the appropriate dose of BBN, using a gavage needle attached to a 1 ml disposable syringe. Dosed animals were placed in cages consisting of stainless steel tops and disposable plastic bottoms. Each cage contained some high grade absorbent sawdust, a glass food pot filled with the appropriate diet and a water bottle; vitamin A-deficient rats were given water supplemented with freshly prepared VAP. Once in these cages, the dosed rats were transferred to a 'walk-in fume cupboard'. This arrangement comprised a set of shelves with fold-down doors, connected to the air extraction system used during dosing. The animals were kept under these conditions for 48 hours, to allow all the carcinogen to be metabolised and excreted into the absorbent sawdust. During this holding period, all the animals were checked regularly and any showing signs of illness were removed and humanely killed. After 48 hours, the rats were transferred back to their normal cages in the animal holding room, care being taken to leave all contaminated sawdust in the disposable cages for incineration. (Details of the safety procedures used during carcinogen dosing are shown in Appendix 3).

(iii)  Urine analysis

a  Collection of urine samples

Urine samples were collected by placing animals in metabolism cages (metaboles) overnight. These metaboles were purchased from Techmate Ltd, 10 Bridgetown Avenue, Old Wolverton, Milton Keynes, MK12 5QL. A total of 8 metaboles were used. Each one was numbered and housed a single rat. While in the metaboles, the animals were allowed free access to drinking water (with or without VAP) but, to avoid contamination of the urine by food particles, they were denied diet. Urine was collected in a tube at the base of each metabole. The weights of each urine tube and water bottle were recorded before and after each collection period, allowing estimation of the weight of urine excreted and water consumed by each rat. Prior to analysis, the urine samples were stored at 4°C.

In Trial VII, urine samples were collected at 2 different time points after the end of carcinogen dosing; namely, 32-35 weeks and 41 weeks, respectively. During the first period (32-35 weeks after dosing), samples were collected from the same rats, once each week for 4 weeks. On each
occasion, rats were placed in different metaboles to those which they had previously occupied. Between each use, the metaboles were dismantled, thoroughly washed in hot water and left to dry. The second set of urine samples (gathered 41 weeks after dosing) were collected from the same rats that had been used previously but, this time, the animals were metaboled only once.

b Analysis of urine samples

Urine samples were analysed using 'Clinitest Labstix' testing strips (Ames Laboratories Ltd, Hemel Hempstead, Herts.). These strips gave accurate readings for urinary pH as well as semi-quantitative determinations of haematuria. In addition, semi-quantitative estimations of urinary protein, glucose, ketones and nitrite were carried out. Using a refractometer, the specific gravity of each sample was determined. Only the results for haematuria are reported.

(iv) Examination of urinary bladders

a Macroscopic examination

Nine of 10 Group 1 rats (controls; vitamin A-normal, BBN dosed) and 1 of 7 Group 2 rats (vitamin A-deficient, BBN dosed) survived to the time of the necropsies. The majority of the mortalities in Group 2 were attributable to a severe vitamin A deficiency accidentally induced by a deterioration in the stock solution of VAP (used to allow the survival of vitamin A-deficient animals), rather than to the effects of the carcinogen. At 42 weeks after dosing, 4 rats fed control diet and the sole surviving vitamin A-deficient animal were killed. The remaining 5 control animals were killed 50 weeks after dosing. At post-mortem, the urinary bladder was removed to formalin and a plasma sample was prepared for retrospective retinol analysis. After fixation, each bladder was bisected longitudinally into 2 halves. Then, using a low-power dissecting microscope (x10 magnification) and keeping the tissue moist with fresh formalin to avoid damage through desiccation, the bladder was examined for macroscopic abnormalities including opaque areas, thickened areas and solid masses. Masses ranged in size and shape from tiny raised areas and 'bleb-like' lesions, to larger ones extending greatly into the bladder lumen. The approximate position and shape of each lesion were recorded on a map of each bladder. These 'bladder maps' formed a record of the macroscopic findings and helped also to ensure that all lesions were sampled for microscopic examination (for an example of a bladder map, please refer to Figure 7.1).

b Microscopic examination

After the macroscopic examination, the bladders were processed for paraffin histology. In some cases, where multiple tumours were present at different levels of the same bladder, further
longitudinal sections of the bladder were prepared with a razor blade prior to processing. This produced several pieces of bladder wall with associated tumours for embedding at roughly the same level. In this way, more of the bladder wall could be examined microscopically than would have been possible using standard step sections, while the amount of microtomy required was reduced. Multiple sections (4 μm thick) were cut from each block, care being taken to sample every macroscopic lesion and to section through the stalk of papillary tumours. Although multiple bladder tumours were found in all but 2 of the 8 rats examined, the histological result recorded for each bladder was the most severe lesion observed.

(v) Classification system for diagnosis of urothelial tumours

Only tumours of the bladder epithelium were found in the 10 rats examined. The classification system used to describe these different tumours was essentially that of Hicks et al. (1982a). In this system, shown in Table 7.1, lesions are classified as hyperplasias or carcinomas. Hyperplasias range in form and severity from simple, flat hyperplasias (subdivided into mild, moderate or marked, depending on the thickness - number of cell layers - of the epithelium) to papillary hyperplasias and/or nodular hyperplasias. Invasive carcinomas are classified according to the pattern of growth (papillary and/or nodular, adenomatous, solid or disseminated), the predominant cell type (transitional, squamous, mucous or undifferentiated), cytological grade of malignancy (low-grade, well differentiated, to high-grade, poorly or undifferentiated) and depth of invasion (pathological stage of the tumour). These 4 criteria for the classification of invasive carcinomas follow the WHO system for urinary bladder tumours in man as described by Mostofi (1973). The pathological stage of a carcinoma is defined as the deepest point in the bladder wall to which it has spread in continuity (Pugh 1973). In the WHO system, there are 6 stages including a stage for non-invasive carcinoma. As outlined by Pugh (1973), these 6 stages are: carcinoma in-situ or non-invasive (PIN); invasion of the stromal core of the stalk of a papillary lesion, but not extending to the lamina propria between the epithelial and muscular layers of the bladder wall (P1a); invasion into the lamina propria of the bladder wall (P1b); superficial bladder muscle invasion (P2); deep bladder muscle invasion extending to the peritoneal surface (P3); and infiltration of adjacent or distant organs by local or metastatic spread (P4). The classification system used to diagnose the rat bladder tumours found in Trials VII and VIII was similar to that of the WHO system for human lesions. Although the degree of differentiation (grade) of each tumour was taken into account for the purpose of diagnosis, the grades of each rat tumour are not presented as part of the results. This is because, unlike human bladder lesions, there are no established objective criteria for grading rat bladder lesions (Squire 1986).
Some other tumour classification systems used in experimental animals distinguish an extra class of urothelial lesion known as a papilloma (Ito and Fukushima 1986; Ito and Shirai 1986; Squire 1986). These lesions are regarded usually as benign neoplasms, being neither reversible, like hyperplasia, nor malignant (e.g. carcinoma). Squire (1986) describes papillomas as being discrete large, exophytic, papillary lesions which exhibit complex branching of papillary processes. These masses have pedunculated or sessile connections to the bladder wall and may have fibrovascular cores extending into the individual papillary processes. Papillomas exhibit none or only slight cellular atypia. Occasionally, mitoses may be observed, often in a suprabasal position.

Furthermore, papillomas do not exhibit the malignant characteristics of invasive growth or metastasis. Squire (1986) distinguished papillomas from papillary hyperplasias based on the morphological pattern of growth. Papillomas were masses that did not appear to be part of a diffuse or multifocal hyperplastic change. The classification system of Hicks et al. (1982a), used to diagnose tumours in Trials VII and VIII, does not recognise papillomas as a discrete class of tumour. Thus, in Trial VIII, lesions with the morphological appearance of 'papillomas', as outlined by Squire (1986), were distinguished from malignant tumours, but were included with the hyperplasias in the final histological results. This is because the emphasis of Trial VIII was the prevention of carcinomas by dietary BC. Although not reported as such in the final results, for the purpose of comparison with the results of other experimentalists, all the tumours categorised as papillary hyperplasias were subdivided into 2 groups, according to their size and complexity. Early lesions consisting of a single papillary fold of the urothelium extending into the bladder lumen and larger, more complex lesions comprising many unbranched papillary processes were classified as papillary hyperplasia I (Pap. Hyp. I). The second group of lesions, classified as papillary hyperplasia II (Pap. Hyp. II), were discrete, exophytic lesions exhibiting complex branching of papillary processes, often with fibrovascular cores extending into the individual papillary processes. This second group of 'papillary hyperplasias', as diagnosed under the classification system of Hicks et al., (1982), corresponded morphologically to the tumours described by some classification systems as 'papillomas' (e.g. that of Squire, 1986).

C Results

(i) Number of animals surviving after BBN treatment

Immediately after the last dose of BBN, 9 out of 10 Group 1 rats fed control diet were alive, while only 1 out of 7 Group 2 rats fed the vitamin A-deficient diet had survived. However, the majority of deaths in the deficient group were attributable to a severe vitamin A deficiency accidentally induced by a deterioration in the stock solution of VAP, rather than to the effects of the carcinogen. For this reason, when 5 of the 7 deficient rats had died (after 3 BBN doses), the
concentration of VAP was increased by a factor of 3. Immediately, the condition of the remaining 2 rats improved. One of these 2 rats was later found dead after the seventh BBN dose, but this death was probably related to a dosing accident.

\(\text{(ii) Urine analysis}\)

Table 7.2. shows the results of the clinical assessment for haematuria in urine samples collected 32-35 weeks and 41 weeks after BBN dosing. The results obtained 32-35 weeks post-dosing were inconclusive, with positive and negative readings from 7/8 rats on different days. All 7 rats examined 41 weeks after dosing gave positive readings for haematuria, the most noteworthy of these values being those for rat 5, 6 and 7 in Group 1.

\(\text{(iii) Examination of urinary bladders}\)

The urinary bladders from the 8 rats were examined for the presence of tumours. The purpose of this examination was an attempt to correlate haematuria, found by urine analysis, with the presence of urinary bladder tumours. The results of this examination are given alongside the readings for haematuria in Table 7.2. Macroscopically, all of the 4 bladders removed at 42 weeks after BBN dosing showed multiple abnormalities. These bladders (from Group 1, rats 1-3; Group 2, rat 1) each contained between 1 to 3 small tumours. Microscopically, in each of these bladders, the most severe lesion was diagnosed as papillary hyperplasia (Pap. Hyp. I). No differences were observed, in either macroscopic or microscopic findings, between the single vitamin A-deficient rat (Group 2, rat 1) and the animals fed control diet (Group 1, rats 1-3).

Thus, in this limited study, there was no evidence that vitamin A deficiency was likely to induce a more severe lesion than seen in rats fed an adequate level of vitamin A.

Rats fed control diet and killed 50 weeks after exposure to a total dose of 700 mg BBN (Group 1, rats 4-7) showed greater numbers of macroscopic lesions, many of them larger in size, compared with rats from the same group and killed 42 weeks after treatment with 350 mg BBN. The bladders of the animals killed at the later time showed the following macroscopic findings: rat 4, 16 masses (1 large, 6 small and 9 tiny bleb-like lesions); rat 5, 10 masses (3 medium, 2 small and 5 tiny blebs); rat 6, 11 masses (2 large, 3 medium, 4 small and 2 blebs) and rat 7, 2 small masses. The bladder map for rat 6 is reproduced in Figure 7.1. Under the dissecting microscope, tumours in some of these bladders were observed to be haemorrhagic. Histologically, the most severe lesions in each of these 4 rats were: rat 4, papillary hyperplasia (Pap. Hyp. II); rat 5, TCC stage 1a; rat 6, TCC 1b; rat 7, papillary hyperplasia (Pap. Hyp. II).
(iv) **Retrospective plasma retinol analysis**

A mean plasma retinol level ($\mu$g/dl ± sd) of 24.93 ± 1.16 was determined for the 3 rats fed control diet and killed 42 weeks after BBN treatment. This contrasted with the value of 3.90 $\mu$g/dl obtained for the single surviving vitamin A-deficient animal killed at the same time point. The mean plasma retinol value for the 4 animals fed control diet and killed after 50 weeks is not reported.

**D Conclusions**

Although 6 of the 7 vitamin A-deficient rats died during or immediately following the period of carcinogen dosing, these mortalities were attributed to breakdown of the VAP used as a survival-promoting source of vitamin A. Administration of BBN to vitamin A-deficient rats did not appear to greatly increase the incidence of deaths, during or immediately following the dosing period.

Oral gavage of rats with BBN was an effective treatment for inducing bladder tumours (Table 7.2); 100% of the surviving animals were found to have bladder tumours at post-mortem (42-50 weeks after carcinogen treatment). However, none of the rats killed after 42 weeks were found to have urinary bladder carcinomas, while carcinomas were seen in only 2 out of 4 animals killed at the later time point (50 weeks). Thus, to induce an incidence of approximately 100% carcinomas, a waiting period longer than 50 weeks would be required after treatment with a total dose of up to 700 mg BBN, given in 5 weekly aliquots.

No evidence was found to suggest that vitamin A deficiency induced more severe urinary bladder lesions than seen in rats fed an adequate level of vitamin A. However, this conclusion was drawn from an extremely limited trial, with only 1 rat in the vitamin A-deficient group surviving to the terminal kill. Furthermore, different dosages of BBN had been given to control and vitamin A-deficient rats, making comparison between these groups impossible.

The results for the determination of haematuria in urine samples collected between weeks 32-35 indicate that the presence and degree of haematuria in such samples is transient (Table 7.2). This is illustrated by the variable results obtained for the same rats week by week. At week 41, when rats were metaboled only once, varying degrees of haematuria were found in urine samples from 4 animals (3 from Group 1, and 1 from Group 2) which, when they were killed 1 week later (at 42 weeks), all demonstrated the presence of bladder tumours. The remaining 4 metaboled rats showed substantial levels of haematuria at 41 weeks. However, these animals were not killed until
9 weeks after their urine samples had been analysed, making any correlation with histological findings difficult. Nevertheless, taken as a whole, the results for haematuria indicated that analysis of urine samples might be an effective non-invasive method of determining the presence of haemorrhagic urinary bladder tumours in BBN-treated rats. This conclusion was supported by the observation under the dissecting microscope of haemorrhagic areas in some of the bladder tumours at post-mortem. Insufficient data obtained to determine whether urine analysis for the presence of haematuria could distinguish between malignant tumours and hyperplasias; the available data, however, would tend to indicate that it would not do so.

Analysis of plasma from the single vitamin A-deficient rat which survived to the terminal kill 42 weeks after BBN treatment revealed a retinol level of 3.90 µg/dl. This retinol value was 15.6% of the mean retinol level (24.93 ± 3.9 µg/dl) found in the 3 corresponding rats fed control diet, a finding which was consistent with vitamin A deficiency. Although there was only 1 value, this finding was important as it confirmed that a vitamin A-deficient animal could be maintained as such for more than 66 weeks by supplementation of the drinking water with VAP (0.1875 µgRE/ml). Furthermore, animals maintained in this way could survive at least up to 5 repeated doses of 100 mg BBN.

3 TRIAL VIII: EVALUATION OF THE ANTI-CANCER ACTIVITY OF DIETARY BC AGAINST CARCINOGEN-INDUCED URINARY BLADDER CANCER IN THE RAT

A Introduction

In the early 1980s, epidemiological investigations had suggested that lower than average plasma vitamin A levels were associated with higher than average cancer incidences (Wald et al. 1980; Kark et al. 1981). Furthermore, dietary studies had suggested that lower consumption of pigmented fruits and vegetables (sources of provitamin A carotenoids) were also associated with higher than average cancer risk (Bjelke 1975; Shekelle et al. 1981). In 1981 reviewing this data, Peto et al. focused attention on the possibility that provitamin A carotenoids, in particular BC, might have a beneficial effect upon human cancer.

In addition to pointing out the need for human intervention studies to investigate the link between BC and human cancer, Peto et al. (1981) also suggested that there was an urgent need for further laboratory animal investigations into the possible mechanisms whereby BC might exert anti-cancer activity. For example, if BC was active against the carcinogenic process, was this activity mediated through conversion to vitamin A, or was it through some inherent potential of the BC
molecule itself. Peto and associates (1981) pointed out that free radicals, such as singlet oxygen, could induce DNA damage leading to carcinogenic initiation of cells, and that BC has a potent ability to quench these reactive molecular species.

In highlighting the need for further experimental investigation, Peto et al. (1981) suggested that it would be useful to discover whether dietary BC started some time after moderate (not massive) initiation of animals with DNA-binding carcinogens, has any material inhibitory effects, analogous to the inhibitory effects induced by natural and synthetic retinoids, on the later stage(s) of carcinogenesis.

As a direct result of the report of Peto et al. (1981), a relatively large numbers of human intervention studies (reviewed by Mathews-Roth 1985; Malone 1991; Szarka et al. 1994) have been established to investigate the activity of BC against human cancer. In contrast, there have been few reports of experimental investigations into the potential mechanisms of BC anti-cancer activity (Moon 1989; Rogers et al. 1993; Kelloff et al. 1994). The reason for this shortage of laboratory investigations has been the belief that the efficient conversion of dietary BC to retinol by experimental animals renders it very difficult to produce animals with appreciable tissues levels of BC (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994). However, there are several reports in the literature which indicate that feeding high levels of dietary BC resulted in the absorption of appreciable amounts of unconverted carotenoid in the blood and tissues of laboratory rats (Kornhauser et al. 1986), as well as mice and guinea pigs (Mathews-Roth et al. 1977). Hicks et al. (1984) fed a high level (5 mM) of BC to BL6D2F1 mice pre-treated with the carcinogen BBN, to examine the effect of dietary BC upon the incidence of experimentally-induced urinary bladder carcinomas. These workers found high plasma levels of unconverted BC in mice given the carotenoid in the diet. Furthermore, at post-mortem the body fat and certain internal organs of these mice, but not of the controls, appeared yellow/orange in colour, indicating that BC had been absorbed into the tissues. Finally, in this thesis (Trial VI), feeding high dietary BC to vitamin A-deficient rats for about 5 weeks (day 54 - day 90) was shown to produce appreciable levels of unconverted BC in the plasma and also to colour the tissues. Thus, it has been shown that appreciable amounts of unconverted BC can be absorbed by both rats and mice provided they are given a sufficiently high level of BC in the diet.

At the time that the present work was begun (1984), there were only 8 reports examining BC for anti-cancer activity in experimental animals (Dorogokulpa et al. 1973; Epstein 1977; Mathews-Roth 1982; Reider et al. 1983; Santamaria et al. 1983; Mathews-Roth 1983; Rettura et
al. 1984; Hicks et al. 1984). BC was administered in beadlet form in the diet in all of these studies, apart from that of Epstein (1977), who gave intraperitoneal injections. Dorogokulpa et al. (1973) and Reider et al. (1983) gave BC in the form of carrots. All these workers studied the activity of BC when given after treatment with the carcinogen. In this way, the activity of BC on the promotion-progression phases of carcinogenesis could be evaluated. A key feature of Trial VIII was the addition of BC to the experimental diets before the administration of carcinogen. Thus, the effect, if any, of BC upon the initiation phase as well as the later promotion-progression phases of carcinogenesis could be examined.

Of all the above studies, reported before the present work was begun, only that of Hicks et al. (1984) examined the effects of BC against experimental urinary bladder cancer. These workers found no effect when BC was administered to mice after treatment with moderate doses of BBN. Thus, the present investigation (Trial VIII) was designed to evaluate the activity of a high level of dietary BC given to rats prior to administration of the carcinogen. It was hoped that Trial VIII would demonstrate whether, or not, the administration of dietary BC could reduce the incidence of experimentally-induced bladder cancer in rats. Furthermore, if BC was found to possess anti-cancer activity, the design was intended to also show whether such activity was mediated through conversion to vitamin A, or whether some other mechanism was involved. To this end, BC was to be feed to rats which were receiving an adequate amount of pre-formed vitamin A in their diet and, to examine the possibility that BC might show anti-cancer activity as a result of its provitamin A potential, the carotenoid was to be feed as the only vitamin A source to vitamin A-deficient animals also.

B Experimental Design

(i) Animals

A total of 335 weanling female F344 rats (Category 3) were obtained from Harlan Olac Ltd, Bicester, Oxon. To reduce the chances of inducing asynchronous vitamin A deficiency in animals of widely differing body weights, the supplier had been requested to provide rats which were, on arrival, within the narrow range of 18-21 days of age. However, the large number of animals required could not be supplied as a single batch. Instead, the animals were delivered in 2 batches, the second arriving 2 days after the first. When delivered, the rats in each batch were 18-21 days of age. On arrival, all the animals were acclimatised for a period of 6 days. Each batch were weighed and fed a pelleted maintenance diet (SDS Ltd, Witham, Essex). After 48 hours, this diet was removed and the animals were starved for 18 hours (to prevent accumulation of substantial vitamin A reserves). Following this starvation period, the maintenance diet was administered.
again. The rats were randomised and assigned to the appropriate experimental groups. Animals from the first batch were allocated to Groups 1-4 (to be treated with carcinogen), while the second batch were placed in Groups 5-8 (to be given carcinogen-vehicle). After randomisation, the rats were held in cages, 5 or 6 animals to each cage. Six days after arrival (week 0, day 0), the maintenance diet was removed and each group of rats was given the appropriate experimental diet.

Despite careful monitoring, a number of weanling animals died after, and as a result of, the 18 hour starvation period. To replace some of these animals, a further batch of 12 female F344 rats aged 18-21 days were obtained from Harlan Olac Ltd. These rats were acclimatised for 6 days in a similar manner to the original animals. Although they arrived long after the trial had begun, these 12 animals were killed, after acclimatisation, at a time point equivalent to day 0, week 0 of the main trial. The plasma and tissue samples which were taken from these rats at post-mortem were considered to be representative of samples which would have been taken at this time point in the main trial had the original animals not died.

(ii) Trial design

The design of Trial VIII is shown in Table 7.3. There were 8 experimental groups, 4 to be treated with carcinogen (Groups 1-4) and 4 to receive carcinogen-vehicle (Groups 5-8). At the start of the trial (week 0, day 0) the total number of rats in all 8 groups was 305. The numbers of animals in each group were as follows: Group 1, 32; Group 2, 32; Group 3, 48; Group 4, 65; Group 5, 31; Group 6, 31; Group 7, 33; Group 8, 33.

As illustrated in Table 7.3, there were several distinct phases to Trial 8. In the initial phase, from the start of the trial at week 0 (day 0) until week 7 (day 45), Groups 3, 4, 7 and 8 were fed the unsupplemented basal vitamin A-deficient diet (SSD(ii)) to induce vitamin A deficiency. Concurrently, the other 4 experimental groups, Groups 1, 2, 5 and 6 were fed the basal diet plus a normal level of vitamin A in the form of VAA (2064 µgRE/kg).

The second phase of the trial was from week 7 to week 15. At week 7 (day 45), animals in Groups 3, 4, 7 and 8 demonstrated clinical evidence of vitamin A deficiency (body weight plateaux and eye lesions). From week 7, the basal vitamin A-deficient diet fed to rats in Groups 4 and 8 was supplemented twice weekly with freshly prepared VAP (0.1875 µgRE/ml) in the drinking water, to maintain the animals in a vitamin A-deficient but otherwise healthy condition. Simultaneously, animals in Groups 3 and 7 were fed the basal diet supplemented with BC, at a concentration of 3 mM/kg. BC was the only source of vitamin A fed to these rats. At the same time...
time, BC (3mM/kg) was given also to the vitamin A-normal rats in Groups 1 and 5. During this second phase, the rats in Groups 3 and 7 would be expected to return to normal vitamin A status as a consequence of vitamin A derived exclusively from BC. Meanwhile, the rats in Groups 1 and 5, which had never been vitamin A-deficient, would be receiving in their diet adequate amounts of pre-formed vitamin A and, in addition, BC. From week 7 until the end of the trial, these diets were fed to the respective groups.

The third phase of the trial (week 15 to week 19) involved dosing the animals with carcinogen (Groups 1-4) or carcinogen-vehicle (Groups 5-8). Five weekly doses of BBN (carcinogen) or 30% absolute ethanol (carcinogen-vehicle) were given by gavage (doses 1-3) or by stomach tube (doses 4-5). The amounts of carcinogen and the corresponding dosage volumes in which these were given to rats in Groups 1-4 were as follows: 1st dose, 100 mg in 0.5 ml; 2nd dose, 100 mg in 0.5 ml; 3rd dose, 110 mg in 0.5 ml; 4th dose, 150 mg in 0.75 ml; 5th dose, 175 mg in 0.875 ml. Identical dosage volumes of the vehicle were given to the rats in Groups 5-8. Thus, over 5 weeks, a total of 635 mg of BBN was given to Groups 1-4. The general dosing procedures were similar to those described for Trial VII. However, an unacceptable number of rats in Trial VIII died shortly after the first 3 doses, which were administered by gavage. The majority of these mortalities occurred following accidental dosing into the lungs. In an attempt to stop further deaths and maintain sufficient numbers of rats in each group, the fourth and fifth doses were given by stomach tube. For this purpose, a rubber dosing catheter (International Market Supply, Dane Mill, Broadhurst Lane, Congleton, Cheshire, CW12 1LA, UK) was attached to the tuberculin syringe containing the carcinogen or vehicle to be dosed. A further 12 rats died as a result of dosing accidents during the fourth dose and 2 more during the final dose. After dosing (week 19), the number of rats remaining in each group, with the total number of deaths from dosing accidents in parentheses, were as follows: Group 1, 27 (5); Group 2, 27 (5); Group 3, 28 (11); Group 4, 43, (13); Group 5, 28 (3); Group 6, 28 (3); Group 7, 30, (3) and Group 8, 32, (1).

The fourth phase of Trial VIII consisted of the post-dosing period from week 19 to week 54. During this phase, the animals were left to develop urinary bladder tumours. Tumour development was monitored by the periodic analysis of urine samples for haematuria and scheduled interim necropsies (see below). During the entire trial no significant haematuria was detected in any group. Therefore, the data have not been included in the thesis.

The final phase of Trial VIII was the terminal kill. Initially, it had been intended to kill the rats at about week 70-80, when approximately 100% of the most susceptible animals (Group 4) should
have demonstrated urinary bladder carcinomas. Owing to administrative constraints, however, the terminal kill had to be brought forward, the first rats being killed in week 54, or 35 weeks after the end of dosing. The terminal kill lasted from week 54 to week 57. However, to avoid artefactual time-related differences in tumour incidence between groups, rats from each group were killed throughout the period of the terminal kill.

The times of all the scheduled post-mortems and the numbers of animals involved are shown in Table 7.4. In addition, the numbers of unscheduled deaths, including deaths from dosing accidents, are shown in Table 7.4. To determine representative baseline data (bladder, trachea and kidney histology; plasma levels for retinol, α-tocopherol and BC), 12 rats (not shown in the main body of Table 7.4, but referred to in the footnotes) were necropsied at week 0. Although these animals were assigned to Groups 3 and 4 (6 rats per group), at the time of necropsy they were being fed maintenance diet and had not yet received their experimental diets. At week 7 (day 45), 5 rats from each of Groups 3 and 4 were necropsied, to determine whether rats in Groups 3 and 4 (and, by association, Groups 7 and 8) were vitamin A-deficient. To ascertain whether rats in Groups 3 and 7 had returned to a similar vitamin A status as the controls (Groups 2 and 6) prior to dosing, and, at the same time, to confirm that animals in Groups 4 and 8 were still vitamin A-deficient, 4 rats from each of Groups 3 and 4 were killed at week 15. To determine the rate of development of urinary bladder tumours in the most susceptible rats (Group 4), 2 sets of interim kills, each involving 5 animals from this group, were performed at weeks 44 and 49 (25 and 30 weeks after the end of dosing, respectively). All rats in scheduled kills and any rats killed in extremis (presented in Table 7.4 under 'other deaths') were necropsied and samples were taken for subsequent histology and plasma analysis. Animals which died as a result of dosing accidents were discarded without being necropsied, while rats which were found dead prior to a scheduled necropsy were subject to a gross post-mortem examination only.

At post-mortem, the urinary bladder, kidneys, a sample of trachea and any abnormal tissues were excised and fixed in phosphate-buffered neutral 10% formalin for histology. After fixation, 1 kidney from each rat was treated with decalcifying fluid for 48 hours prior to processing for paraffin histology. In rats showing substantial degrees of nephrocalcinosis this treatment facilitated the preparation of good sections from at least 1 kidney from every rat. Comparison of the mineralization found in decalcified and undecalcified kidney sections helped to determine the chemical nature of the mineralization. The liver also was removed and weighed at post-mortem (data not shown). Two standard sections of the liver (1 from the left lobe and 1 from the median lobe) were taken and fixed. The remainder of each liver was weighed again before being frozen.
and stored at -20°C for analysis of hepatic vitamin A, vitamin E and BC. Subsequently, however, none of the liver sections were assessed for histopathological changes, nor were the frozen liver samples analysed. Finally, plasma samples were obtained from freshly killed rats at post-mortem. These samples were frozen and stored at -20°C for retrospective analysis for retinol, α-tocopherol and BC.

Throughout Trial VIII, the health of all rats was assessed by monitoring their body weights and additionally, animals were regularly observed for any signs of vitamin A deficiency or other evidence of ill health. Any sick animals were killed and necropsied. Occasionally, animals were found dead, particularly over weekends. Such animals were usually too severely autolysed or cannibalised to be sampled for histology and, therefore, they were subject only to a gross examination.

(iii)  *Retrospective plasma analysis*

Retrospective analyses for retinol, α-tocopherol and BC were carried out on plasma samples stored under oxygen-free nitrogen at -20°C for up to 6 months.

(iv)  *Macroscopic assessment of fixed urinary bladders and determination of relative bladder weights and estimated tumour volumes*

After fixation, the urinary bladders were bisected longitudinally into left and right halves, using a safety razor blade. Excess formalin was removed by gentle dabbing with a paper tissue. Then, to obtain the weight of the bladder, both pieces were weighed together. The bladder weights were expressed relative to body weight (g of tissue/kg rat body weight). After weighing, both bladder halves were examined under a dissecting microscope (at x10 magnification) for the presence of macroscopic bladder tumours. During this examination, the bladder tissue was kept moist with formalin to prevent desiccation. Bladder maps were drawn for each bladder, to record the approximate position, size and shape of any macroscopic lesions. Using an eyepiece graticule, the length, width and depth/height of each bladder lesion was measured. After multiplication by a conversion factor, these data gave a rough estimate of the volume of each lesion in cubic millimetres (assuming each lesion was cubic in shape). Where multiple lesions were present, the total estimated tumour volume per bladder was derived by addition of the values for each individual lesion. After these procedures, the bladders were processed for paraffin histology as in Trial VII. The histological diagnosis recorded for each bladder was the most severe lesion observed.
(v) **Classification of urothelial lesions**

The histopathological classification system used for the diagnosis of all the urothelial lesions was the same as described for Trial VII. (For details of this system, refer to Table 7.1). Briefly, all proliferative lesions were classified according to whether they were hyperplasias or carcinomas. Only the worst lesion was recorded for each animal.

(vi) **Statistical analyses**

The results obtained for relative bladder weights and estimations of bladder tumour volumes were analysed for statistical significance (p < 0.05) by analysis of variance (ANOVA). The incidences of urinary bladder tumours in the different experimental groups (Groups 1-4) were analysed for statistical significance (p < 0.05) by the Fishers Exact test.

C **Results**

(i) **Body weights**

Mean body weights of the BBN-dosed rats (Groups 1-4) and the vehicle-dosed animals (Groups 5-8) are shown in Figures 7.2 and 7.3, respectively (for the means ± sd, please refer to Tables 7.5 and 7.6). Between weeks 14-34, no determinations of body weights were made. However, it was considered that this did not adversely affect the growth curves over the trial as a whole.

Over the first 7 weeks of the trial, rats fed the vitamin A-deficient diet alone (Groups 3, 4, 7 and 8) gained weight at a lower rate than animals fed control diets containing an adequate level of vitamin A (Groups 1, 2, 5 and 6). By week 12, supplementing Groups 3 and 7 with BC from week 7 had restored the mean body weights of these animals to the level of those fed diets containing adequate amounts of vitamin A. Over the same period, supplementing Groups 4 and 8 with low-level vitamin A in the drinking water (VAP) had maintained these animals in a healthy state such that they gained weight at a similar rate to rats in Groups 1, 2, 5 and 6. From week 12 until the end of the trial, all groups continued to gain weight slowly. By week 42, rats fed the vitamin A-deficient diet and supplemented with low-level VAP (Groups 4 and 8) were fractionally heavier than those fed the same diet and supplemented with BC (Groups 3 and 7).

(ii) **Clinical observations**

At week 6, individual rats in Groups 3, 4, 7 and 8, fed the unsupplemented vitamin A-deficient diet, demonstrated early eye lesions indicative of developing vitamin A deficiency. At this time, some animals in each of these groups showed grade 1 or grade 2 lesions (for details of the system used to grade the clinical signs of vitamin A deficiency, refer to Table 6.4). At week 7, several
rats in Groups 7 and 8 demonstrated grade 3 clinical signs; 1 of these animals (from Group 7) was subsequently found dead in week 8, by which time the clinical evidence of vitamin A deficiency shown by this rat had progressed to grade 5. A gross necropsy carried out on this rat revealed a complete lack of abdominal fat depots (emaciation) and swollen intestines (bloat by intestinal gas). Following the administration in week 7 of BC to Groups 3 and 7, and VAP to Groups 4 and 8, no further clinical evidence of vitamin A deficiency was observed for the remainder of the experiment. However, it was evident at each of the interim kills at week 44 and week 49 (25 weeks and 30 weeks post carcinogen dosing, respectively) that rats in Group 4 had plentiful body fat. This clinical observation indicated that these animals were probably not as vitamin A-deficient as it had been hoped they would be at these time points, a view which was supported by the body weight data. As a result, therefore, it was decided to reduce the amount of VAP supplementation to rats in Groups 4 and 8 by 50% at week 44 and, when similar findings were observed at week 49, by a further 25% after the second interim kill.

(iii) Analyses for plasma retinol, α-tocopherol and BC

The results of the retrospective analyses for plasma retinol, α-tocopherol and BC in Groups 3 and 4 throughout Trial VIII are shown in Tables 7.7, 7.8 and 7.9, respectively. At the start of the experiment, the mean plasma retinol values (µg/dl ± sd) in Groups 3 and 4 were 46.71 ± 6.21 and 50.34 ± 6.96, respectively. The mean α-tocopherol levels (mg/dl ± sd) in these two groups were 0.85 ± 0.09 and 0.95 ± 0.07. By week 7, plasma retinol values in both groups had fallen by more than 90%, to 3.81 ± 3.90 and 3.63 ± 3.39, respectively. Thus, by week 7, plasma retinol had fallen to about 10% of the normal values (22.0 - 25.0 µg/dl). This fall in plasma retinol confirmed the development of vitamin A deficiency by week 7 in Groups 3 and 4 (and, by analogy, Groups 7 and 8). Interestingly, the mean α-tocopherol levels (mg/dl ± sd) in Groups 3 and 4 had also fallen by week 7, to 0.38 ± 0.04 and 0.34 ± 0.11, respectively. At week 15, 8 weeks after the start of dietary BC administration to Group 3 (and immediately prior to the commencement of carcinogen dosing), the mean plasma BC level mg/100 ml ± sd) of this group was 101.7 ± 11.50, while plasma retinol (µg/dl ± sd) was 24.96 ± 0.42. This demonstrated that BC given to Group 3 had been absorbed by these animals and, furthermore, that some BC had been converted to vitamin A, while the remainder had been absorbed in the unconverted state. Supplementation of these animals with BC had reversed vitamin A deficiency by week 15, and restored the plasma retinol level to the normal value for adult female F334 rats. In contrast, the plasma retinol level of rats in Group 4 at 15 weeks was only 12.42 ± 1.74, or 50% of that in Group 3. It is noteworthy that the
administration of BC to Group 3 from week 7 had also increased plasma \( \alpha \)-tocopherol levels, such that by week 15 the mean value had almost returned to the level recorded at week 0.

At the interim kills, 25 weeks and 30 weeks after the end of carcinogen dosing, the plasma retinol level of rats in Group 4 was 21.24 ± 1.17 and 25.36 ± 4.27, respectively. These retrospective analyses confirmed that rats in Group 4 were not as vitamin A-deficient as would have been hoped at these time points, as suggested by the clinical observations and the body weight data at these time points.

The mean plasma retinol, \( \alpha \)-tocopherol and BC levels in all groups at the terminal kill, 38 weeks after the end of carcinogen dosing, are presented in Table 7.10. There was little difference between the mean plasma retinol values in all 8 groups. These ranged from 22.83 ± 2.64 mg/dl in Group 7 to 27.03 ± 3.12 mg/dl in Group 5. Most significantly, perhaps, neither of the groups expected to have greatly reduced retinol levels (Groups 4 and 8, fed the vitamin A-deficient diet and supplemented with low-level VAP) were markedly different from the other groups which had received adequate vitamin A in the diet, either pre-formed or as BC.

Animals in Groups 1 and 5 which had received BC from week 7, in addition to a diet containing an adequate amount pre-formed of vitamin A, demonstrated mean plasma BC levels at the terminal kills of 279.5 ± 146.7 \( \mu \)g/dl and 316.40 ± 155.64 \( \mu \)g/dl, respectively. Thus, these animals had absorbed high amounts of BC into their blood, although with a large degree of variation between individual rats. Animals in Groups 3 and 7 which had received BC from week 7 as the only source of dietary vitamin A, also demonstrated high levels of unconverted BC in the plasma (Group 3, 169.3 ± 164.8 \( \mu \)g/dl; Group 4, 181.61 ± 145.27 \( \mu \)g/dl, although the amounts were not as high as in the groups which had received an adequate level pre-formed vitamin A as well.

Again it was noteworthy that the mean \( \alpha \)-tocopherol levels in all groups given BC were higher than those in groups not given BC, regardless of the presence in the diet of adequate levels of pre-formed vitamin A. The lowest \( \alpha \)-tocopherol levels were 0.98 ± 0.49 mg/dl (Group 4) and 1.33 ± 0.64 mg/dl (Group 8). These two groups were presumed to have received the lowest amounts of vitamin A, pre-formed or otherwise.
(iv) **Examination of urinary bladders at the terminal kill**

a **Relative bladder weights**

The mean (± sd) relative bladder weights for all rats killed at the end of Trial VIII (terminal kill) are shown in Figure 7.4. The mean (± sd) relative bladder weights (g/kg rat body weight) in each group were as follows: Group 1, 0.467 ± 0.185; Group 2, 0.427 ± 0.181; Group 3, 0.504 ± 0.240; Group 4, 0.467 ± 0.177; Group 5, 0.406 ± 0.155; Group 6, 0.444 ± 0.179; Group 7, 0.402 ± 0.168; Group 8, 0.447 ± 0.459. Statistical analysis demonstrated no evidence of any significant differences in group mean relative bladder weight (p > 0.05, ANOVA).

b **Macroscopic assessment of gross bladder lesions**

All bladders from the BBN-treated groups were observed to contain macroscopic lesions under the low-power objective (x 10) of the dissecting microscope. These lesions ranged in gross appearance from tiny bleb-like lesions to large exophytic growths filling much of the bladder lumen. The total number of lesions of all types/number of bladders in each BBN-treated group were as follows: Group 1, 79/26; Group 2, 102/26, Group 3, 135/28; Group 4, 132/33. The mean number of lesions in each BBN-treated group was: Group 1, 3.039; Group 2, 3.923; Group 3, 4.821; Group 4, 4.000. These data show a slight reduction in mean lesion number in animals from Group 1 given BC in addition to a diet containing adequate vitamin A. Statistical analysis demonstrated that the observed differences in the numbers of macroscopic lesions in each group were not significant (p > 0.05, ANOVA).

c **Estimated bladder tumour volumes**

The mean (+ sd) values for the estimated bladder tumour volume (mm³/bladder) are shown in Figure 7.5. The mean (sd) values for each BBN-treated group were as follows: Group 1, 12.198 (26.506); Group 2, 20.518 (82.9350; Group 3, 5.576 (12.261); Group 4, 15.423 (31.185). In Group 2, one animal was found to have a much greater bladder tumour volume than any of the animals in any group. The volume of tumour in this bladder alone was 1746.69 mm³. Without this large outlier, the mean (+ sd) tumour volume for Group 2 was 4.281 (4.972). This data is also shown in Figure 7.5. Although the mean values vary from each other, there was no statistical significance in these differences (p > 0.05, ANOVA).

An attempt was made to examine whether there were any apparent treatment related differences in total tumour volume/bladder between the four BBN-dosed groups. The total tumour volume of each bladder in each group was plotted on log₁₀ graph paper. In each group, the range of total tumour volume/bladder was subdivided into the following log₁₀ subdivisions (mm³): 0-0.1,
0.1-1.0, 1.0-10, 10-100, 100-1000. For each group, the percentage distribution of total tumour volume/bladder, based on these $\log_{10}$ subdivisions of the range of tumour volumes, are shown in Figure 7.6. There were no obvious differences in the percentage distribution of tumour volumes within each of the BBN-treated groups.

With one exception, in which an urinary calculus had induced a diffuse hyperplastic response, all bladders from the vehicle-treated rats were free of macroscopic lesions (Figure 7.7). In contrast, all the bladders from BBN-treated animals were found to have macroscopic lesions including large exophyte lesions (Figure 7.8).

d **Histological examination of the urothelium**

The histological findings for all bladders in the terminal kill are summarised in Table 7.11. The histological results for all the urothelia in Groups 1-8 were classified, according to the worst lesion present, into 3 categories: 1) normal, 2) hyperplasia (ranging from small, simple, flat hyperplasias to complex papillary and/or nodular lesions), 3) carcinoma.

There was no statistical evidence to suggest that BC had reduced the incidence of carcinomas, as measured by the Fisher Exact Test ($p > 0.05$). The data may show a slight beneficial effect of BC, but the numbers of animals surviving to the terminal kill were too small to enable such an effect to be detected statistically. There was some evidence that the incidence of carcinomas in Group 4 was higher than that in the other 3 carcinogen-dosed groups, when the latter groups were pooled together. Although this difference was not statistically significant ($p > 0.05$, Fishers Exact Test)), the probability value was borderline ($p = 0.078$).

All carcinomas were TCC, with the greatest depth of invasion being into the lamina propria of the bladder wall (P1b) (Figures 7.9 - 7.12). However, the majority of carcinomas were papillary or papillary/nodular lesions (Figures 7.13 - 7.17), with invasion confined to the stromal core of the stalk (P1a). No cancers were found to have invaded into the superficial muscle of the bladder wall (P2), or beyond.

A small number of carcinomas with a solid growth pattern were present (Figures 7.18 - 7.22). These solid lesions were more often dysplastic than tumours of other growth patterns. Foci of squamous metaplasia, sometimes associated with keratinisation, were present in some lesions (Figures 7.23 and 7.24), but no unequivocal SCC was found. Occasionally, foci of a glandular/cystic/adenomatous growth pattern were present within the larger papillary or
papillary/nodular lesions (Figure 7.25). Cytologically, most carcinomas were well differentiated or moderately-well differentiated. Very few were dysplastic, although dysplastic foci within more differentiated tumours were not uncommon.

Most hyperplasias were of the papillary type. The majority of the non-malignant papillary lesions were of the larger, more complex papillary form (Pap Hyp. II) (Figures 7.23 - 7.24). These lesions would be categorised under some classification systems as 'papillomas', rather than as 'papillary hyperplasias' (Squire 1986). A few simple, leaf-like papillary hyperplasias (Pap Hyp I) were also observed (Figures 7.26 - 7.27).

Although not specifically recorded, there was no evidence of a particular preponderance towards one or more types of tumour morphology within a particular treatment group. Multiple proliferative lesions were present in the majority of bladders, as indicated by the macroscopic findings. Often, the full range of morphological forms a nodular hyperplasia (Figure 7.28) and simple flat hyperplasia (Figure 7.29), to frank carcinoma were present in the same bladder. As the main thrust of the present investigation was to determine the worst lesion in each bladder, the total numbers of all types of lesions other than carcinomas are not presented.

Whereas none of the bladders in the 4 BBN-treated groups (Groups 1-4) were normal at the terminal kill, all except one bladder from the vehicle-treated groups (Groups 5-8) were normal (Figures 7.30 - 7.33). The one abnormal vehicle-treated rat was from Group 8. A large urinary calculus was present in the bladder lumen and the entire urothelium was extensively hyperplastic (papillomatosis) (Figure 7.34). A large calculus was present in the bladder lumen. This is the probable cause of the extensive hyperplastic response (Capurro et al. 1960). No carcinomas were found in the vehicle-treated animals.

(v) Other histological results

The urinary bladder (Figure 7.35), trachea (Figure 7.36), and kidney (Figure 7.37) from rats killed at the start of the trial all appeared normal. From week 7 to the end of the trial, the kidneys demonstrated evidence of nephrocalcinosis (Figure 7.38). Many kidneys from the terminal kill demonstrated histological evidence of early nephropathy (Figure 7.39), but no other changes were evident in the kidney. Similarly, most tracheas were normal throughout the trial. However, at week 7, several small foci of squamous metaplasia (Figure 7.41) were evident in the tracheas of animals from group 4. These findings represented histological indications of vitamin A deficiency.
in these animals. However, there was no evidence of squamous metaplasia in any of the bladders from animals in Group 4 killed at week 7 (figure 7.40) or at week 25 (Figure 7.42).

The interim kills at week 44 and week 49 demonstrated that bladder tumours were developing in Group 4, the most susceptible BBN-dosed group. However, although large papillary tumours (Figure 7.43) were present, there were no carcinomas in these animals.

There were several incidental neoplastic lesions found in Trial VIII (Table 7.4). Two animals were found dead at week 43 and 53 with monocytic leukaemia (Figures 7.44 and 7.45). Another animal was killed in extremis in week 34 and found to have a large schwanoma (Figure 7.46) close to the spine. Another rat was killed in extremis in week 50 and found to have malignant phaeochromocytoma. None of these 4 animals were found to have bladder tumours. Two other animals demonstrated incidental lesions as well as bladder tumours. Both of these animals were killed in the terminal kill. Of these 2 animals, 1 was found to have adenocarcinoma of the mammary gland (Figure 7.47), while the other had monocytic leukaemia.

D Conclusions

Over the first 7 seven weeks of Trial VIII, rats fed the vitamin A-deficient diet alone (Groups 3, 4, 7 and 8) gained weight at a lower rate than animals fed control diets containing an adequate level of vitamin A (Groups 1, 2, 5 and 6). These findings (Figures 7.2 and 7.3) were indicative of developing vitamin A deficiency, a fact supported by the observation of clinical signs of vitamin A deficiency (eye lesions, emaciation and gastro-intestinal effects) in some of these animals during weeks 6-8. Further evidence of vitamin A deficiency in these animals was the observation of focal squamous metaplasia in the trachea of rats killed from Group 4 at week 7 (Figure 7.41). Confirmation of vitamin A deficiency in these rats was provided by the plasma retinol data for week 7 (Table 7.7). Following the administration in week 7 of BC to Groups 3 and 7, and VAP to Groups 4 and 8, no further clinical evidence of vitamin A deficiency was observed for the remainder of the experiment.

By week 12, supplementing Groups 3 and 7 with BC from week 7 had restored the mean body weights of these animals to the level of those fed diets containing adequate amounts of vitamin A (Figures 7.2 and 7.3). Over the same period, supplementing Groups 4 and 8 with low-level vitamin A in the drinking water (VAP) had maintained these animals in a healthy state such that they gained weight at a similar rate to rats in Groups 1, 2, 5 and 6. From week 12 until the end of the trial, all groups continued to gain weight slowly. By week 42, rats fed the vitamin A-deficient
diet and supplemented with low-level VAP (Groups 4 and 8) were fractionally heavier than those fed the same diet and supplemented with BC (Groups 3 and 7). This was an unexpected finding.

At each of the interim kills at week 44 and week 49 (25 weeks and 30 weeks after carcinogen dosing, respectively) it was evident that Group 4 had plentiful body fat. This clinical observation indicated that these animals were probably not as vitamin A-deficient as it had been hoped they would be at these time points, a view which was supported by the body weight data.

The plasma retinol values for these animals confirmed that between week 15 and the end of the trial, animals in Groups 4 and 8 became progressively less vitamin A-deficient (Tables 7.7 and 7.10). Although action was taken at both week 44 and week 49 to reduce the VAP intake of animals in Groups 4 and 8, this action was too late to prevent plasma retinol levels in these animals from rising to normal values by the terminal kill (Table 7.10).

The reason why animals in Groups 4 and 8 were no longer vitamin A-deficient (as defined by plasma retinol) is unclear. It would appear that these animals received more vitamin A than was necessary to merely maintain them in a vitamin A-deficient state. In other words, these animals were receiving more than enough VAP. It is possible, although unlikely, that this finding may be related to a change in the method of preparing the VAP used to supplement these animals with vitamin A. From week 7 until week 10, VAP had been prepared using deionised water. This was done to avoid using water from the mains supply (with a high nitrate content), thereby reducing VAP breakdown in the water bottles to a minimum. However, when the deioniser broke down in week 10, it was decided to revert to using the mains supply for VAP preparation, as had been the practice in Trials IV and Vb. Thus, from week 10 until the end of Trial VIII, VAP was prepared using tap water. It is extremely unlikely, however, that this change in protocol early in the experiment resulted in increased vitamin A intake. If anything, due to high nitrate levels, water from the mains supply would be expected to reduce the level of vitamin A in the water bottles in comparison with deionised water, not to increase it. However, lower than expected VAP breakdown could, perhaps, be explained if there was some seasonal variation in the level of nitrate, or other oxidant(s) in the mains supply water.

The decision to return to using water from the mains supply in Trial VIII was based on evidence from previous experiments that had shown that VAP in mains water was an effective means of maintaining vitamin A-deficient rats for long periods. For example, in Trial IV, VAP in mains water, at the same concentration that was used in the present experiment, had maintained vitamin A-deficient rats for 16 weeks without causing plasma retinol to rise. Furthermore, in the pilot
dosing study (Trial VII), the same protocol was used to maintain a single surviving vitamin A-deficient rat such that at the terminal kill, 50 weeks after BBN treatment, it was found to have a low plasma retinol value of 3.90 μg/dl.

Another possible explanation for a higher vitamin A intake could have been a rise in the absolute amount of water consumed by rats in Group 4 as they increased in age. The VAP concentration (0.1875 μgRE/ml) used for the bulk of the trial was based on a daily water consumption of 16 ml/rat.

The results of the plasma analyses confirmed that 3 mM/kg BC fed to animals in Group 3 and, by analogy, Group 7, effectively restored plasma retinol in these animals between week 7 and week 15 (Table 7.7). Furthermore, at week 15 these animals had also absorbed substantial amounts of unconverted BC into their plasma (Table 7.9). At the terminal kill, plasma samples obtained from all rats fed dietary BC, but not from any other groups, were coloured orange/yellow. Furthermore, the body fat and various organs (adrenals, liver and kidney) were also coloured, indicating that unconverted BC had been absorbed by these tissues. The urinary bladders of BC-fed animals were very slightly more yellowish in colour than those from animals not given BC. In part, this colouration may have been due to the presence of BC in fat associated with the bladder. However, it may also have indicated the accumulation of BC within the bladder tissue. Mathews-Roth et al. (1991) reported that the mean concentration of unconverted BC in mice fed a diet containing about 2 mM/kg BC was 396 μg/100g bladder wet weight, with individual concentrations ranging from 160-800 μg/100g bladder wet weight.

At the terminal kill, mean α-tocopherol levels in all groups given BC were higher than those in groups not given BC, regardless of the presence in the diet of adequate levels of pre-formed vitamin A. This interesting finding may be due to a protective antioxidant effect of the high level of BC in the diet and in the plasma. High levels of BC may preferentially remove reactive radicals which, in the absence of BC, are removed by α-tocopherol.

There was no statistical evidence of any significant differences in relative bladder weights. This was true when each BBN group was compared with the corresponding vehicle-dosed group, and also when the BBN groups were compared with each other. Similarly no significant differences were detected in the estimated tumour volumes in bladder from the 4 BBN-treated groups.
At the terminal kill, all BBN-treated bladders contained macroscopic lesions. Although no significant differences were found between the 4 BBN-treated groups, the mean number of macroscopic lesions per bladder was slightly reduced in Group 1, compared with the other 3 BBN groups.

No significant evidence was found that BC reduced the incidence of carcinomas as measured by the Fisher Exact Test. In rats treated with carcinogen and fed BC (Group 3), slightly fewer carcinomas (29%) and slightly more hyperplasias (71%) were found, compared with the rats fed the control diet in Group 2 (39% carcinomas, 61% hyperplasias). These results (Table 7.11) could indicate a slight beneficial effect of BC, but the numbers of animals surviving until the terminal kill were too low for such an effect to be detected statistically.

The histological findings summarised in Table 7.11 indicate a small enhancing effect of vitamin A deficiency upon the incidence of carcinomas in Group 4. This effect was not significant when compared statistically on a group by group basis ($p > 0.05$, Fisher Exact Test). However, when all the bladders from Groups 1, 2 and 3 were pooled together, the increase in the number of carcinomas in Group 4 over those in the pooled groups approached borderline significance.

The range of proliferative bladder lesions found after treatment with BBN in the present study is consistent with those reported for other similar investigations in the rat (Ito et al. 1969; Hicks et al. 1982b). The percentage incidence of carcinomas in the most susceptible group (Group 4) at the end of the trial was not high (52%). This would have been higher if a higher dose of carcinogen had been used, or if circumstances had allowed the trial to be maintained for a further 20 weeks or more weeks (Hicks et al. 1982b). In the present study, a total dose of 635 mg BBN/rat was administered. This was a moderate dose of carcinogen. However, if additional aliquots of BBN had been given, this would have risked further losses of animals from these groups, due to dosing accidents, reducing the statistical power of the experiment. Furthermore, the use of moderate, rather than massive, doses of powerful carcinogens were suggested by Peto et al. (1981) for experimental investigations into the chemopreventative activity of BC.

E Discussion of Trial VIII
The activity of dietary BC against BBN-induced urinary bladder cancer has been investigated in the rat. The experiment was terminated 35 weeks after moderate carcinogen treatment. No statistically significant evidence was found to indicate that BC had had any effect upon the incidence of TCC in BBN-treated rats fed a diet containing an adequate level of vitamin A (Group
1), or in BBN-treated rats fed a vitamin A-deficient diet supplemented with BC (Group 3). There was some evidence of a very small reduction in the numbers of TCC in BBN-treated rats fed the vitamin A-deficient diet and supplemented with BC (Group 3). However, the numbers of rats surviving to the terminal kill were too low to allow any small effects to be detected statistically. Also, there was some evidence, although not significant, in the rats given BC in addition to an adequate level of vitamin A (Group 1) that the mean number of macroscopic lesions per bladder was reduced slightly compared with the other groups. In addition, there was some evidence that vitamin A-deficiency had enhanced the incidence of TCC in BBN-treated animals fed a vitamin A-deficient diet supplemented only with a low-level of vitamin A in the drinking water (Group 4). However, this difference was not statistically significant, and was only of borderline significance when the incidence of TCC in Group 4 was compared with the pooled incidences of TCC in Groups 1, 2 and 3. There was no statistical evidence to suggest that BC had affected relative bladder weight or the estimated tumour volumes in the 4 BBN-treated groups. Relative bladder weights in these 4 groups were not significantly different, either from each other, or from the weights of bladders in the corresponding vehicle-treated groups.

At the time that the work described here was begun (1984), there were only 8 reports examining BC for anti-cancer activity in experimental animals (Dorogokulpa et al. 1973; Epstein 1977; Mathews-Roth 1982; Reider et al. 1983; Santamaria et al. 1983; Mathews-Roth 1983; Rettura et al. 1984; Hicks et al. 1984). Of these, only the study of Hicks et al. (1984) had examined the activity of BC against experimental bladder cancer. Hicks et al. (1984) found that feeding 5 mM/kg BC to mice after treatment with BBN had no effect on the final incidence of urinary bladder carcinomas. Since 1984, there have been two other reports of studies involving BC and murine urinary bladder cancer (Moon 1989; Mathews-Roth et al. 1991). In a review of the anti-cancer activity of carotenoids, Moon (1989) reported previously unpublished results showing no effect in BBN-treated mice of BC when given intraperitoneally (3-24 mg/mouse/week). This study formed part of the pre-clinical assessment of BC within the wider programme of research, funded by the NCI in the USA, to find effective chemopreventative agents for human cancer (Bertram et al. 1987; Boone et al. 1990; Kelloff et al. 1994). In a comparative study of the chemopreventative activity of BC and canthaxanthin, a carotenoid lacking vitamin A activity, Mathews-Roth et al. (1991) found that BC significantly protected mice against BBN-induced cancer, but that canthaxanthin did not. Thus, of the 3 experiments in the literature involving BBN-induced bladder cancer in mice, two have found negative results with BC (Hicks et al. 1984; Moon 1989). Only Mathews-Roth et al. (1991) have reported a significant protective effect for BC.
Very little information was given by Moon (1989) about the pre-clinical assessment of BC in mice treated with BBN. Therefore, it is difficult to discuss this study in relation to the other two investigations involving BC and experimental bladder cancer in mice reported by Hicks et al. (1984) and by Mathews-Roth et al. (1991). However, the method of BC administration reported by Moon (1989) is clearly different from the studies of Hicks et al. (1984) and Mathews-Roth et al. (1991). In the study mentioned by Moon (1989), mice were injected with a high level of BC (3-24 mg/week), in an attempt to ensure high tissue levels of the carotenoid without conversion to vitamin A (Kelloff et al. 1994). In contrast, Hicks et al. (1984) and Mathews-Roth et al. (1991) administered BC in the diet. Hicks et al. (1984) fed BC at 5 mM/kg, whereas Mathews-Roth et al. (1991) gave a diet containing about 2 mM/kg BC.

The use of the mouse/BBN model as a pre-clinical screen of potential chemopreventative agents as reported by Boone et al. (1990) is based upon the early developmental work of Becci et al. (1981) and McCormick et al. (1981). It is likely, therefore, that the unpublished investigation reported by Moon (1989) involved the administration of BC after BBN treatment, and the use of BL6D2F1 hybrid mice. The animals were probably killed 6 months after carcinogen treatment (Becci et al. 1981; Moon et al. 1982). Furthermore, BBN is likely to have been given in 9 weekly aliquots of 10 mg (90 mg total dose). This dose level of BBN has been used successfully by Moon et al. (1982) to investigate the anti-carcinogenic activity of a range of synthetic retinoids. The same mouse strain was used by Hicks et al. (1984) and by Mathews-Roth et al. (1991). Mathews-Roth et al. (1991) administered BBN in 9 weekly aliquots of 10 mg (total dose of 90 mg) and killed the animals 6 months later, as described by Moon et al. (1982). In contrast, Hicks et al. (1984) found no effect of dietary BC when the carotenoid was given for 6 months after a much lower dosage of carcinogen (10 weekly aliquots of 1.5 mg or 3.0 mg BBN, giving total dosages of 15 mg or 30 mg, respectively). Thus, no effect of BC has been observed in mice given two different doses of BBN (Hicks et al. 1984; Moon 1989). As Mathews-Roth and associates (1991) probably used the same dose level of carcinogen as that used in the unpublished study reported by Moon (1989), the positive effect of BC observed by Mathews-Roth et al. (1991) was not likely to have been due to differences in carcinogen treatment.

The only methodological difference in the treatment of the animals between these three studies was that Mathews-Roth et al. (1991) administered BC for 5 weeks prior to carcinogen dosing. At first sight, this difference would appear to account for the difference in results reported by the three groups of investigators. However, in the experiment performed by Mathews-Roth and associates, involving 24 animals in each group, the numbers of carcinomas found in mice fed BC or placebo
diet were 5/24 and 8/24, respectively (Mathews-Roth et al. 1991). This result was not statistically significant. The authors themselves stated that the numbers of animals in each group were too small to indicate that any particular type of lesion had been inhibited by BC. Therefore, in terms of the numbers of TCC in mice fed BC and mice fed a placebo diet, there is no difference between the results reported by Mathews-Roth et al. (1991) and those of Hicks et al. (1984). Moon (1989), when commenting on the lack of effect of BC in the unpublished mouse/BBN experiment, gave no details of the nature or numbers of bladder tumours involved. Therefore, dietary pre-treatment of mice with BC for 5 weeks prior to BBN dosing, did not significantly reduce the incidence of carcinomas in animals fed BC.

Mathews-Roth et al. (1991) reported their results in terms of TCC, carcinoma-in-situ (cis) and a third category labelled 'tumours'. The statistically significant effect of BC reported by Mathews-Roth et al. (1991) related to the numbers of lesions in this third category, and not to either category of malignant lesion. However, it is not clear what type of lesions were categorised into this third group. There is no mention of a separate group to include the numbers of non-neoplastic proliferative lesions (hyperplasias). It is probable, therefore, that hyperplasias have been included with any benign neoplastic lesions in the authors category of 'tumours'. It is also possible, although not stated, that the authors have included all types of lesions together (malignant and non-malignant) in their category of 'tumours', in a similar manner to the total numbers of macroscopic lesions presented in the Trial VIII.

In describing the method used to prepare bladder tumours for histology, Mathews-Roth et al. (1991) stated that all the tumours were 'removed' from the bladder and fixed prior to histological processing. If by this, the authors mean that tumours were excised from the bladder before fixation, it is probable that difficulties would have been encountered in diagnosing invasive carcinomas. This is because BBN-induced tumours in the mouse develop predominantly as flat endophytic lesions that invade down into the bladder wall, rather than as papillary exophytic lesions (Hicks et al. 1985).

Trial VIII is the first study to have examined the activity of BC against urinary bladder in the rat. No similar studies have been attempted in rats because of difficulties in accumulating appreciable levels of unconverted BC in the tissues (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kelloff et al. 1994). However, the work described here has shown that appreciable levels of unconverted BC do accumulate in the plasma of rats fed high levels of dietary BC (3 mM/kg or higher). At the terminal kill of Trial VIII, the mean BC levels in the plasma of the four
groups given BC (3 mM./kg) ranged from (169-316 µg/dl). Furthermore, various tissues and organs of these animal and those in Trial VI (fed 6 mM/kg BC), were coloured orange/yellow, indicating that BC had accumulated in these sites (please refer to Figures 6.22 and 6.23). In Trials VI and VIII, no measurements were made of the levels of unconverted BC within the tissues which had been coloured in BC-fed rats. However, Mathews-Roth (1977) measured the levels of BC within some of the tissues from mice fed high levels of dietary BC. Although BC was undetectable in tissues from control animals (fed a normal maintenance diet), BC was detected in many tissues including the liver (970 µg/100g wet weight of tissue), kidney (190 µg/100g wet weight of tissue), adrenals (170 µg/100g wet weight of tissue) and body fat (44 µg/100g wet weight of tissue). The mean amount of unconverted BC in the plasma of these mice was reported to be only 23 µg/dl (Mathews-Roth 1977). As the plasma BC levels were much higher, it is likely that the amounts of unconverted BC in the tissues of BC-fed rats in the present investigation (Trial VIII) would have been much higher than those reported for mice by Mathews-Roth (1977). In a separate study, Mathews-Roth et al. (1991) reported that in mice fed dietary BC at a level of about 2 mM/kg, the mean concentration of BC in the urinary bladder was 396 µg/100 g of bladder wet weight.

In addition to being the first investigation to examine the activity of BC against urinary bladder cancer in the rat, the work reported here (Trial VIII) is the first experimental study of the activity of BC against bladder cancer in vitamin A-deficient rats. Cohen et al. (1976) are the only other workers who have examined the effects of vitamin A deficiency on the incidence of experimental urinary bladder cancer. Vitamin A deficiency was reported by these workers to enhance the number of bladder tumours induced by the carcinogen FANFT (Cohen et al. 1976), a finding that supports the observation in the present study (Trial VIII) of a small though not significant enhancement of tumour incidence by vitamin A deficiency. As discussed previously, a problem arose in Trial VIII whereby animals in Group 4, which were vitamin A-deficient by week 7, were found to have normal plasma retinol values by the end of the trial. It appears likely from the plasma samples taken at scheduled kills throughout the trial, that the plasma retinol levels of rats in Group 4 did not suddenly return to normal, but rather there was a gradual rise to normal retinol levels. The data shown in Table 7.7 suggest that normal retinol levels were reached somewhere between week 15 and week 44. Thus, the animals in Group 4 were vitamin A-deficient for a reasonable proportion of the study. It is possible that a significant enhancement of tumour incidence by vitamin A deficiency would have been detected in Trial VIII, had the animals in Group 4 remained fully vitamin A-deficient for the entire experiment.
As well as reporting on the activity of BC against BBN-induced bladder cancer in mice, Mathews-Roth and associates (1991) also found that canthaxanthin, a carotenoid without provitamin A activity, had no effect on the final incidence of BBN-induced bladder tumours. This carotenoid has been shown to be more active than BC in some of the animal models in which BC has been tested (Mathews-Roth 1982; Santamaria et al. 1983; Moon 1989). Furthermore, Grubbs et al. (1991) recently reported that canthaxanthin led to a 65% reduction in the incidence of mammary tumours in rats treated with DMBA, when fed for 3 weeks prior to administration of the carcinogen. Results such as these showing protective effects of carotenoids lacking vitamin A activity, have increasingly led investigators to question whether provitamin A activity is required for the inhibition of cancer by carotenoids (Krinsky 1992). The study of Mathews-Roth et al. (1991) is the first to investigate the activity of canthaxanthin against experimental bladder cancer. The result suggests that the mechanism of action of canthaxanthin in other models of cancer, whatever that may involve, may not operate against BBN-induced bladder cancer.

There is much evidence to support the idea that vitamin A activity is important in urinary bladder carcinogenesis. The urinary bladder is sensitive to changes in the level of vitamin A. The urothelium is one of the epithelia of the body that exhibits squamous metaplasia in conditions of vitamin A deficiency (Wolbach and Howe 1925; Hicks 1968 and 1969). Experimental urinary bladder carcinogenesis is enhanced by vitamin A deficiency (Cohen et al. 1976). Supplemental vitamin A inhibits urinary bladder carcinogenesis in rats dosed with BBN (Miyata et al. 1978), and in mice given FANFT (Dawson et al. 1979). Furthermore, cancer of the bladder is one of relatively few neoplasms that is sensitive to synthetic retinoids, both in experimental animals (Sporn et al. 1977; Moon et al. 1982; Hicks et al. 1985; Hill and Grubbs 1992; Moon et al. 1992), and in man (Alfthan et al. 1983; Studer et al. 1984). Recent improvements in epidemiological techniques have resulted in fewer human cancers being linked with vitamin A than was the case in the early 1980s (Michels and Willett 1994). The epidemiological evidence linking lower than average vitamin A intake with a higher risk of human bladder cancer, however, is still relatively strong (Mettlin and Graham 1979; Kolonel et al. 1985; La Vecchia et al. 1989; Steineck et al. 1990; Shibata et al. 1992).

The epidemiological evidence linking human urinary bladder cancer with BC is not as strong as that for pre-formed vitamin A. The relationship between BC and bladder cancer has been examined in only one prospective study and found not to be significant (Shibata et al. 1992). Of all the retrospective dietary studies which have investigated BC intake and urinary bladder cancer (Kolonel et al. 1985; Claude et al. 1986; Risch et al. 1988; La Vecchia et al. 1989; Nomura et al.
1991; Riboli et al. 1991), only that of La Vecchia (1989) reported a significant inverse relationship between risk of bladder cancer and estimated carotenoid intake. In this analysis, however, total energy intake or other micronutrients were not taken into account. Claude et al. (1986) reported that frequent consumption of fruits and vegetables was associated with a reduced risk of bladder cancer, but these workers did not relate these findings to BC, or any other nutrients. Retrospective studies have shown no significant difference in pre-diagnostic blood levels of BC between cases and controls (Nomura et al. 1985; Wald et al. 1988; Helzlsouer et al. 1989; Comstock et al. 1991; Knekt et al. 1991a). Thus, the epidemiological data may suggest that pre-formed vitamin A is more important than BC in relation to protection from bladder cancer in man.

In the present study (Trial VIII), there was no statistically significant evidence that a high dietary level of BC, in addition to an adequate dietary level of vitamin A, reduced the incidence of urinary bladder cancer in carcinogen-treated rats (Group 1). However, there was a slight trend towards fewer macroscopic lesions in this group, compared with the other BBN-treated groups. In rats given BC as the sole source of vitamin A (Group 3), there was also no statistically significant evidence of any reduction in the numbers of bladder cancers when compared with the control group (Group 2). However, the number of carcinomas in Group 3 was slightly lower than in Group 2, possibly indicating a small reducing effect of BC (although the numbers of surviving animals available for analysis prevented such a small effect from being statistically detected).

Mechanistically, it is not possible to draw any firm conclusions from Trial VIII, as BC had no statistically significant effect upon carcinoma incidence. Conversion to vitamin A of BC fed to rats in Group 3 may have been important in reducing the numbers of carcinomas in this group, in relation to the numbers of malignant tumours in the vitamin A-deficient rats in Group 4. Although not statistically significant, there were far fewer carcinomas in animals from Group 3 than in Group 4. This finding has implications for human cancer. Mild vitamin A deficiency, although rare, is present in some members of the population in western countries, for example many elderly people, and is widespread in many other parts of the world (Hicks 1983; Pitt 1985). These individuals are at an increased risk of developing cancer as a result of having sub-optimal levels of dietary vitamin A. A simple increase in the consumption of vegetables and fruit rich in BC, or other provitamin A carotenoids, would eradicate the element of risk associated with vitamin A deficiency in these individuals.
In summary, the present study found no statistically significant evidence that BC reduced the incidence of urinary bladder carcinomas induced in rats by BBN. This negative result is supported by the results of all three other studies which found no effect of BC on urinary bladder TCC induced in mice by BBN. As in the mouse, the results reported here demonstrated no protective effect for pre-treatment of rats with BC prior to BBN administration. If further investigations are reported that also fail to demonstrate unequivocal reductions by BC of the numbers of carcinogen-induced bladder carcinomas, this may indicate that BC is not effective in preventing experimental bladder cancer. Even if further experimental investigations do show clear reductions in bladder cancer incidence by BC, this will not necessarily indicate that BC protects against human bladder cancer. The results of the on-going randomised, placebo-controlled, clinical intervention studies in humans, such as that involving 22,000 US physicians (Hennekens and Eberlain 1985), may demonstrate definitively whether dietary BC protects humans against the development of cancer in general (Hennekens et al. 1984; Greenwald et al. 1990; Szarka et al. 1994). Providing sufficient numbers of placebo-fed participants subsequently develop bladder cancer, these intervention studies may also demonstrate whether BC protects humans against urothelial cancer.
CHAPTER EIGHT

FINAL DISCUSSION

1  BC AND THE PREVENTION OF HUMAN CANCER

Prevention is undoubtedly the ideal method of cancer control (Greenwald et al. 1990).

Accumulated evidence indicates that dietary and smoking habits and other environmental or lifestyle factors contribute to at least 70% of cancer deaths (Doll and Peto 1981). These deaths are, therefore, theoretically amenable to preventative intervention (Doll and Peto 1981). The environmental determinants of cancer have been quantitatively estimated by Doll and Peto (1981). In their extensive review, these authors estimated that approximately 35% of cancers in the United States might be caused by dietary factors. If this is true, it follows that 35% of cancers in the USA, and probably in the rest of the western world, could be avoidable with appropriate dietary manipulation. These findings are extremely exciting, because they indicate the possibility, at least theoretically, of preventing a large proportion of cancer deaths.

Progress has been made already in reducing occupational risk factors for cancer. The elimination of manufacturing processes involving carcinogenic aromatic amines, for example, has led to a reduction in the numbers of associated urinary bladder cancers (Ferber et al. 1976; Doll 1992). Also, some progress has already been made in elucidating the dietary factors that may be responsible for increasing the occurrence of cancer, as well as agents that might have potential in preventing neoplastic disease.

Fruit and vegetables have been found to be associated with protection from a number of different forms of cancer, particularly those of the respiratory and alimentary tracts (Steinmetz and Potter 1991a; Block et al. 1992). Indeed, the evidence linking increased intake of fruit and vegetables with lower than average cancer risks has reached overwhelming proportions (Block et al. 1992; Michels and Willett 1994). However, it is unclear how this protective association is mediated (Michels and Willett 1994). Protection by a diet high in fruit and vegetables may be explained by a low intake of other risk enhancing foods, such as fat and protein. If, on the other hand, fruit and vegetables are essential elements of a low-risk diet, what individual component(s) within these foodstuffs is/are the important factor(s)? If specific protective components could be identified, appropriate advice could be given about which foods to eat in order to ensure a low-risk diet.
Fruit and vegetables contain a large number of potentially protective chemical components, both nutrients and non-nutrients (Steinnetz and Potter 1991b). Many of these substances have been shown experimentally to protect against carcinogenesis (Wattenberg 1978; Bertram et al. 1987). Among the many potential protective substances in fruit and vegetables, vitamin C and provitamin A carotenoids, particularly BC, have been the focus of much attention (Peto et al. 1981; Ziegler 1989; Block et al. 1992).

In order to be an effective chemopreventative agent in man, applicable to the general population, any potential agent must be extremely non-toxic (Greenberg 1993). Carotenoids, such as BC, easily fulfil this condition (Bagdon et al. 1960, Heywood et al. 1985; Bendich 1988). Indeed, with regard to toxicity, the chemoprevention of cancer by treatment with BC is a far more attractive option than the use of synthetic retinoids for the same purpose (Peto 1983; Greenberg et al. 1985). Although carotenoids such as BC have been found to be active in certain carcinogenic processes (Krinsky 1992), only limited evidence has been obtained from randomised human intervention studies that BC is effective against human cancer. In one intervention trial, BC was found to significantly reduce the incidence of stomach cancers in participants of a study in Linxian, China (Blot et al. 1993). Conversely, in another trial, carried out in Finland, an increased number of lung cancers were found in male smokers who were given BC (Heinonen and Albanes 1994). This last result was unexpected and runs contrary to the large body of epidemiological evidence that indicates a strong protective effect of BC in human lung cancer (Michels and Willett 1994). In view of this contradiction, the authors of the Finnish study themselves suggested that the result was probably due to chance. Conflicting data have also been obtained from clinical trials investigating the use of BC to prevent oral leukoplakia, a pre-malignant condition (Szarka et al. 1994). Stich et al. (1988a), using BC alone, found a 15% remission rate which increased to 27% when BC was given in addition to retinol. However, when double the dose of retinol was used alone, a 57% remission rate was obtained (Stich et al. 1988b). Toma et al. (1992) found that treating patients with high doses of BC for 6 months only produced a 27% remission rate. Lippmann et al. (1993) reported that low-dose 13-cis-RA was more effective in maintaining clinical and histological remission than BC.

In view of the strong belief within the scientific community that BC was protective against lung cancer, the findings reported by Heinonen and Albanes (1994) of a higher lung cancer incidence in male smokers given BC compared to matched placebo-treated controls were greeted with great surprise (Nowak 1994). This surprise was also tinged with uncertainty, as the Finnish study was a randomised, placebo-controlled investigation. Clinical trials of this design are regarded as the
gold standard to evaluate the efficacy of any dietary intervention (Hennekens et al. 1984). Yet Michels and Willett (1994) recently pointed out that even this experimental design can run into problems, leading to false positive and false negative results. Nevertheless, the results of further randomised intervention trials, such as the BC-arm of the Physicians Health Study (Hennekens and Eberlain 1985), will be known in the next few years, and it is hoped that the data from these studies will clarify whether supplemental BC protects against the development of neoplastic disease in man (Steinmetz and Potter 1991b; Rousseau et al. 1992).

2 RATIONALE FOR BC AS A CHEMOPREVENTATIVE AGENT FOR CANCER
The rationale behind the idea that BC is a potentially effective anti-cancer agent is based on 3 criteria: 1) epidemiological evidence that BC is protective against the development of human lung cancer, 2) the ability of BC to trap free radicals and quench singlet oxygen and, 3) the provitamin A activity of BC allowing conversion to retinol (Kelloff et al. 1994).

A Epidemiological Association between BC and Human Lung Cancer
The relationship between intake of fruit and vegetables, or their associated serum nutrients, and lung cancer has been investigated more often than the relationship of these dietary constituents to any other form of cancer (Block et al. 1992). Positive evidence of a protective effect of fruit and vegetable intake has been observed in almost all of the more than 30 studies involving lung cancer (Block et al. 1992). BC has been implicated as the protective factor present in these foods (Fontham 1990), though most studies have not been sufficiently comprehensive to distinguish with confidence an effect of BC from that of other factors present in these foods (Ziegler 1989; Willett 1994). Plasma levels of BC in blood collected prior to the diagnosis of malignant disease have largely been found to be inversely associated with the subsequent risk of lung cancer (Stahelin et al. 1984; Nomura et al. 1985; Menkes et al. 1986; Knekt et al. 1990; Stahelin et al. 1991). No association between serum BC and the risk of subsequent lung cancer was reported by Willett et al. (1984), but the number (17) of lung cancer cases in this study was relatively small.

At this point it is important to note that some studies, particularly the early ones (Hiyarama 1979; Shekelle et al. 1981; Colditz et al. 1985), did not adequately control for the effects of cigarette smoking (Ziegler 1989). Intake of fruit and vegetables, and therefore carotenoids, is decreased among smokers (Stryker et al. 1988). Thus, uncontrolled confounding due to smoking might result in an apparent protective effect for diet in studies of lung cancer, as well as other smoking related neoplasms such as cancer of the head and neck, cervix and urinary bladder (Ziegler 1989). Nevertheless, there is overwhelming epidemiological support to suggest that a diet rich in
BC-containing fruit and vegetables is protective against the development of human lung cancer (Steinmetz and Potter 1991; Block et al. 1992; Willett 1994). Although BC has not been confirmed as the protective factor in these foods, the epidemiological evidence remains supportive that BC is protective against lung cancer (Michels and Willett 1994). Although positive associations between BC and other forms of cancer have been detected (Mettlin and Graham 1977; La Vecchia et al. 1989; Graham et al. 1990; Hunter et al. 1993), too few studies have been carried out to be certain of the apparent link between BC intake and cancer at sites other than the lung.

The strong association between lung cancer and BC in epidemiological studies is not mirrored by a similar relationship for pre-formed vitamin A (Ziegler 1989; Michels and Willett 1994), suggesting that in lung cancer BC may not be protective through conversion to retinol (Ziegler 1989; Rousseau et al. 1992).

One of the most extensive early epidemiological studies was that of Shekelle et al. (1981) who examined the intake of all the major nutrients to the risk of cancer at all sites. Carotenoid intake alone was significantly associated with lung cancer risk; there was no increased lung cancer risk with low intake of pre-formed vitamin A. In the USA, where Shekelle and associates (1981) carried out this study, the major dietary source of vitamin A is pre-formed vitamin A, rather than carotenoids (Ziegler 1989). It was therefore very significant that a reduced lung cancer risk was found with consumption of carotenoids but not pre-formed vitamin A. This finding strongly suggests that the active carotenoids do not have to be converted to vitamin A to be protective against human lung cancer (Ziegler 1989).

**B  BC, Singlet Oxygen and Free Radicals**

The ability of BC to trap free radicals and quench singlet oxygen has been put forward as a possible mechanism whereby BC could prevent cancer, without conversion to vitamin A (Peto et al. 1981). BC is an efficient quencher of singlet oxygen, a function that it performs in plants to protect the photosynthetic apparatus from oxidative damage (Rousseau et al. 1992). Thus, BC could quench the singlet oxygen that is generated as a by-product of normal cellular metabolism, thereby preventing oxidative damage to DNA and any subsequent genotoxic effects. The ability to trap free radicals might allow BC to trap the reactive intermediates formed by the biotransformation of procarcinogens to ultimate carcinogens. Experimental evidence exists to support the idea that BC protects against genotoxicity (Rousseau et al. 1992). *In vitro*, BC 1) prevents cyclophosphamide-induced mutagenesis in *Salmonella typhimurium* (Belisario et al. 2000).
1985), 2) inhibits MCA-induced neoplastic transformation in C3H/10T1/2 cells (Bertram et al. 1991), and 3) protects Chinese Hamster Ovary cells against phagocyte-generated oxygen metabolites as demonstrated by a decrease in sister chromatid exchanges (Weitberg et al. 1985). Furthermore, in man, BC reduces the number of micronuclei (markers of aberrant mitotic division) in exfoliated cells from the buccal cavity in individuals who chew betel nut (Stich and Rosin 1984).

Although the mechanism by which free radicals are trapped by BC is unknown, (Rousseau et al. 1992), it appears to be related to the structure of the carotenoid molecule. Conversion to vitamin A is not required. Free radicals are implicated in UVB-induced carcinogenesis, although again the mechanism is unknown (Mathews-Roth 1982). UVB-induced skin tumours in mice are inhibited not only by BC, but also by carotenoids lacking vitamin A activity, such as phytoene and canthaxanthin (Mathews-Roth 1982). In contrast, vitamin A and synthetic retinoids have no effect on UVB-induced tumours (Hill and Grubbs 1992), further supporting the fact that carotenoids are anti-carcinogenic in this system through a mechanism that is distinct from vitamin A.

Free radicals are produced during cigarette smoking (Rousseau et al. 1992). The trapping and deactivation of these reactive moieties by BC may be involved in the apparent protection afforded by this carotenoid against lung cancer. This would explain the divergent epidemiological findings between BC and pre-formed vitamin A in lung cancer.

C Conversion of BC to vitamin A

Notwithstanding the evidence suggesting that carotenoids possess inherent anti-cancer activity independent of retinol, conversion of BC to vitamin A is, perhaps, the most obvious mechanism through which any anti-cancer activity of the carotenoid could be mediated. Historically, BC became implicated in human cancer through association with vitamin A. Early epidemiological studies investigating the link between vitamin A intake and cancer risk tended to estimate total vitamin A intake, including carotenoid-rich foods (Bjelke 1975). Later, the carotene-containing foods (essentially orange fruit, and yellow, orange and dark green leafy vegetables) were found to be associated with reduced cancer risk, independent of pre-formed vitamin A (Shekkele et al. 1981; Colditz et al. 1985). These foods had only been included in the dietary questionnaires because they were sources of provitamin A carotenoids. As BC is the most common provitamin A carotenoid, it was assumed that BC was the protective factor responsible for the lower cancer risk associated with higher consumption of fruit and vegetables. There was a limited amount of experimental evidence to support this view at the time. In 1973, Dorogokupla et al. had shown
that feeding diets supplemented with unlimited amounts of carrots, retarded the development of DMBA-induced subcutaneous tumours in rats and DMBA-induced skin tumours in mice. The authors suggested that BC from the carrots was responsible for these observations, through conversion into vitamin A. Thus, in the 1980s, the idea grew that BC possessed anti-cancer activity via conversion to vitamin A.

(i) The Anti-Cancer Activity of Vitamin A

Experimental systems, both in vivo and in vitro have clearly demonstrated the anti-carcinogenic activity of natural and synthetic retinoids (Chu and Malmgren 1965; Saffioti et al. 1967; Bollag 1971; Sporn et al. 1977; Becci et al. 1978, Harisiadis et al. 1978; Merriman and Bertram 1979, Lotan 1980; Moon et al. 1982; Hicks et al. 1982b and 1985). In the skin and mammary gland, retinoids are generally thought to the development of cancer via an anti-promoting effect, but the mechanism by which vitamin A and its synthetic derivatives are able to prevent cancer at other sites is not yet known (Hill and Grubbs 1992). It seems likely, however, that retinoids are also active during the promotional stages of carcinogenesis at other sites. In the urinary bladder, for example, the development of carcinogen-induced tumours is delayed, but not prevented, by synthetic retinoids such as 13-cis-RA or N-ethylretinamide (Hicks et al. 1982b) or 4-HPR (Hicks et al. 1985). The mechanism by which retinoids inhibit carcinogenesis is thought to involve alterations of gene expression, through the mediation of RARs and RXRs, leading to changes in the rate of cell proliferation and/or modulation of cell differentiation (Sporn and Roberts 1984; Bollag and Holdener 1992; Hill and Grubbs 1992; Holdener and Bollag 1993).

Cancer is regarded by some investigators as a disease of abnormal cell differentiation (Sporn and Roberts 1984). Others have regarded cancer as a disease, not necessarily of differentiation, but one in which some concomitant phenotypic change in differentiation is obligatory (Hicks 1978). Vitamin A has long been known to play a key role in the control of normal cell differentiation (Wolbach and Howe 1925; Hicks 1983). In many epithelial tissues, vitamin A deficiency induces a change in cell differentiation from a secretory phenotype to a squamous phenotype (Mori 1922; Wolbach and Howe 1925), indicating that deficiency of the vitamin results in failure of normal differentiation (Hicks 1983). In addition to the squamous metaplastic changes seen in vitamin A deficiency, there is often increased proliferative activity leading to hyperplasia of the affected epithelium (Wolbach and Howe 1925; Hicks 1983; Pitt 1985). These profound changes in differentiation and proliferation in vitamin A deficiency are completely reversed by the restoration of normal vitamin A levels (Hicks 1983), a finding which is true both in vivo and also in vitro in organ culture (Wolbach and Howe 1933; Lasnitzki 1955). Indeed, it was Lasnitzki (1955) who
demonstrated that vitamin A could suppress the abnormal cellular differentiation induced in mouse prostate organ cultures by treatment with carcinogens and restore a more normal pattern of differentiation. Other investigators demonstrated that retinoids could suppress malignant transformation of non-neoplastic cells grown in culture and treated with carcinogens (Harisiadis et al. 1978; Merriman and Bertram 1979), thereby showing that vitamin A possessed anti-carcinogenic potential.

These observations were drawn together by Sporn and Roberts (1984) who were not the first to put forward the hypothesis that vitamin A is able to control cell differentiation through effects upon gene expression. The discovery of the RARs and RXRs have added further support to this idea (Giguere et al. 1987; Petkovich et al. 1987). By binding with these receptors, which in turn bind to DNA, vitamin A is thought able to induce and/or repress the expression of target genes (Allenby 1995). In particular, vitamin A is thought to control the expression of genes involved with cell differentiation and cell proliferation (Bollag and Holdener 1992).

RA, the active metabolite of vitamin A, is known to exert a regulatory effect upon the expression of proto-oncogenes involved in the control of growth (Sporn and Roberts 1984; Allenby 1995). Recently, RA has been shown to inhibit the expression in culture of the viral oncogenes E6 and E7 from HPV-18 (Holdener and Bollag 1993). HPV-18 is involved in the pathogenesis of human cervical cancer and these findings could have implications for the treatment of this form of neoplastic disease. Indeed, as evidence grows of the possible aetiological involvement of HPVs in other forms of human cancer including human bladder cancer (Chetsanga et al. 1992; Reznikoff et al. 1993), such findings could have widespread implications for the prevention of cancer.

Roberts and Sporn (1992) have recently highlighted another possible mechanism by which retinoids could exert a cancer chemopreventative action, namely through their ability to stimulate the local production of the cytokine, transforming growth factor-beta (TGF-β). This growth factor is a potent inhibitor of epithelial cell proliferation, and there is growing evidence that loss of responsiveness of cells to TGF-β is often associated with neoplastic progression (Roberts and Sporn 1992). However many tumours still remain responsive to inhibition by TGF-β, especially in the early stages of malignant progression. Retinoids have been demonstrated to induce expression of TGF-β as well as of TGF-β receptors (Glick et al. 1989, 1990), suggesting that local enhancement of responsiveness to TGF-β may have a role in the mechanism of cancer chemoprevention by retinoids (Roberts and Sporn 1992).
There is some evidence that immuno-potentiation by retinoids may be involved in their anti-cancer action (Hicks 1983; Goss and McBumey 1992). RA and other retinoids stimulate T-killer-cell induction in vitro and increase cytotoxicity in vivo. Clinically, retinoids have been claimed to have an immuno-potentiating effect in the treatment of lung cancer (Micksche et al. 1977). Other effects of retinoids on the immune system include the enhancement of skin graft rejection and the enhancement of antibody response to a variety of antigens (Goss and McBumey 1992). These effects upon the immune system might well account, in part, for the anti-tumour effects of retinoids (Goss and McBumey 1992). Carotenoids are also known to stimulate the immune system (Krinsky 1992). Thus, immuno-potentiation may also be a mechanism through which carotenoids can exert anti-carcinogenic activity.

3 EXPERIMENTAL EVIDENCE FOR THE ANTI-CANCER ACTIVITY OF BC

In reviewing the available data from epidemiological studies and from experimental investigations in animals, Peto et al. (1981) called for clinical trials to examine the activity of BC against human cancer, and also for further experimental work in animals in order to identify the mechanism by which any activity is mediated. These workers were the first to point out that the ability of BC to deactivate singlet oxygen and other free radicals could be a possible mechanism of action independent of conversion to vitamin A.

Experimental evidence to support the idea that BC possesses anti-cancer activity is relatively scarce. This has been due to difficulty in obtaining laboratory rodents with appreciable levels of unconverted BC within the body tissues (Rogers and Longnecker 1988; Moon et al. 1989; Rogers et al. 1993; Kellogh et al. 1994). Work described in this thesis (Trials VI and VIII) has shown that feeding a high dietary level of BC to rats for 5 or more weeks results in the accumulation of high levels of unconverted BC in the plasma. In addition, qualitative evidence was found of the accumulation of unconverted BC in the body fat and various organs including the urinary bladder. This work supports the view of Kornhauser et al. (1986) that rats fed a high level of BC form a useful model in which to study the protective effects of BC.

To date, there have been few animal studies of the action of BC against experimental tumours. BC has been reported to inhibit the development of skin tumours in mice exposed to UVB (Epstein 1977; Mathews-Roth 1983), or a combination of UVB and chemical carcinogens such as DMBA (Mathews-Roth 1983), or BP (Santamaria et al. 1981; Santamaria et al. 1983), or MNNG (Santamaria et al. 1985). Other systems in which BC has been shown to be protective against tumour formation include, 1) salivary gland tumours induced in mice by DMBA (Alam et al.)
1984; Alam and Alam 1987; Alam et al. 1988), or DMH (Basu et al. 1988), 2) mammary tumours induced by DMBA in rats (Rettura et al. 1984). Negative findings have been reported for DMBA-induced skin tumours in SENCAR mice (Lambert et al. 1990), and for BBN-induced urinary bladder tumours in B6D2F1 mice (Hicks et al. 1984; Moon 1989).

The prevention of DNA damage through the deactivation of free radicals would involve BC in the prevention of initiation. BC is able to prevent genotoxic damage in vitro (Belisario et al. 1985; Bertram et al. 1991), as are other carotenoids that lack provitamin A activity such as canthaxanthin (Krinsky 1992). Very few animal studies have examined the effects of pre-treatment with BC prior to administration of genotoxic carcinogens.

When administered 6 weeks prior to UVB irradiation, BC failed to prevent skin cancers in hairless mice (Mathews-Roth and Krinsky 1987). When administered after irradiation a 45% reduction in tumour incidence was found. This indicates that in this system BC protected during the promotion/progression phases rather than during initiation. In contrast, canthaxanthin (2-6 mM/kg of diet significantly inhibited DMBA-induced rat mammary carcinogenesis when administered 3 weeks prior to carcinogen treatment (Grubbs et al. 1991). This supports the idea that carotenoids lacking vitamin A activity can inhibit carcinogenesis at the early stages of the process, possibly through radical scavenging activity. Two experiments - the present investigation and that reported by Mathews-Roth et al. (1991) have attempted to examine the influence of carotenoid exposure prior to carcinogen treatment in urinary bladder carcinogenesis. No conclusions could be drawn from these studies regarding the stage of carcinogenesis during which BC was active, as both investigations found no effect of BC against malignant tumours.

Only the present investigation (Trial VIII) and the experiment reported by Mathews-Roth et al. (1991) have attempted to examine the importance of the conversion of BC to vitamin A for the expression of anti-cancer activity by BC. To do this, the work reported here involved a study of the activity of BC against carcinogen-induced bladder cancer in vitamin A-deficient and vitamin A-normal animals. The results support the idea that vitamin A deficiency enhances the development of cancer (Newberne and Rogers 1973; Rogers et al 1973; Nettesheim and Williams 1976; Cohen 1976; Newberne and Suphakarn 1977; Dogra et al. 1985; Bansal and Gupta 1987). The number of malignant tumours in vitamin A-deficient animals was higher than that present in normal controls, although the difference was not statistically significant. A very small non-significant reduction compared with control animals was found in the number of carcinomas in animals that had been vitamin A-deficient but were subsequently brought back to vitamin
A-sufficiency by a high level of dietary BC prior to administration of BBN. The total number of macroscopic lesions in animals fed BC in addition to a normal level of vitamin A was also slightly reduced in comparison with the controls, but the difference was not significant. There was no evidence of any reduction in carcinoma incidence in rats given BC in addition to an adequate dietary level of pre-formed vitamin A. Plasma levels of retinol in these animals were normal, while the levels of unconverted BC were high. As plasma retinol levels do not rise beyond a homeostatically set 'normal' level independent of dietary intake (Underwood *et al.* 1979; Wolf 1984; Olson 1984; Pitt 1985), the observed lack of effect upon carcinoma incidence in these animals demonstrates no effect of unconverted BC on these malignant lesions.

The use of vitamin A-deficient animals for carcinogenicity studies is acknowledged as technically difficult (Ong and Chytil 1983; Rogers and Longnecker 1988). To investigate the role of vitamin A in the anti-cancer activity of BC, Mathews-Roth and associates used a comparative approach involving feeding BC or canthaxanthin to BBN-treated mice. BC possesses vitamin A activity, but canthaxanthin, a carotenoid with proven anti-cancer activity in other models (Mathews-Roth 1982; Grubbs *et al.* 1991), does not (Mathews-Roth *et al.* 1991). These authors found no statistically significant reductions in the incidence of carcinomas in animals fed either carotenoid, but did report a significant reduction by BC, but not by canthaxanthin, in the number of lesions categorised as 'tumours'. The nature of these lesions is unclear, though it is likely that they consisted of hyperplasias and benign tumours, as they were separated from malignant tumours in the published results. Although the nature of these 'tumours' is unclear, this latter observation is reminiscent of the reduction in total macroscopic lesions found in rats given BC in addition to a normal level of vitamin A described in this thesis (Trial VIII).

Thus, although there are reports of inhibition by BC of experimentally-induced cancer, there are very few reports indicating the possible mechanism of that inhibition. Of those that have addressed this subject, none have reported conclusive evidence that any particular mechanism is involved.

### 4 BC, VITAMIN A AND URINARY BLADDER CANCER

In the western world, the most important aetiological components in human bladder cancer are cigarette smoking (Price 1971; Wynder and Goldsmith 1977; Morrison *et al.* 1984; Auerbach and Garfinkel 1989; Burch *et al.* 1989; Clavel *et al.* 1989; Harris *et al.* 1990; Hartge *et al.* 1990; Silverman *et al.* 1992) and occupational exposure to environmental carcinogens (Silverman *et al.* 1989), particularly aromatic amines (Rehn 1895; Scott 1952; Case *et al.* 1954; Case and Hosker 1954; Pitt 1985; Wolf 1984; Olson 1984).
1954; Melick et al. 1955; Veys 1969; Ward et al. 1991). In certain parts of Africa, schistosomiasis appears to be the most important risk factor (Ferguson 1911; Price 1971; Tawfik 1987), although convincing epidemiological evidence for this association are lacking (Silverman et al. 1992).

A dietary component may also be an important factor in the development of urinary bladder cancer (Wynder and Goldsmith 1977). In reviewing the epidemiology of human bladder cancer, Wynder and Goldsmith (1977) reported that the relationship of cigarette smoking to bladder cancer is not as strong as for lung cancer. They pointed out that, to the extent that cigarette smoking affects both the bladder and the lung, it can be understood that the effects of smoking in bladder carcinogenesis are both indirect and more diluted than for lung cancer. Bladder cancer is a much less common disease than lung cancer, and a greater proportion of bladder cancers occur among non-smokers and persons without occupational exposure than does cancer of the lung (Wynder and Goldsmith 1977). In evaluating the relatively large number of non-smokers and non-occupationally related bladder cancers, Wynder and Goldsmith (1977) were unable to find any other factors that could account for the pathogenesis of cancer in this population suggesting that other factors, perhaps those related to diet, could affect the development of bladder cancer.

Much evidence supports the idea that vitamin A activity is important in urinary bladder carcinogenesis. The urinary bladder is sensitive to changes in the level of vitamin A (Hicks 1968; Tallman and Wiemik 1992). The urothelium is one of the epithelia of the body that exhibits squamous metaplasia in conditions of vitamin A deficiency (Wacht and Howe 1925; Hicks 1968 and 1969). Experimental urinary bladder carcinogenesis is enhanced by vitamin A deficiency (Cohen et al. 1976). Supplemental vitamin A inhibits urinary bladder carcinogenesis in rats dosed with BBN (Miyata et al. 1978), and in mice given FANFT (Dawson et al. 1979). Furthermore, cancer of the bladder is one of relatively few neoplasms that is sensitive to synthetic retinoids (Hill and Grubbs 1992).

The responsiveness of urinary bladder carcinogenesis to synthetic retinoids has been demonstrated extensively in experimental animals (Grubbs et al. 1977; Squire et al. 1977; Sporn et al. 1977; Becci et al. 1979a and 1979b; Becci et al. 1981; McCormick et al. 1981; Moon et al. 1982; Moon and McCormick 1982; Hicks et al. 1982b; Hicks 1983; Hicks et al. 1985; Hicks and Turton 1986; Hill and Grubbs 1992; Moon et al. 1992). Human bladder cancer is also responsive to retinoids, as shown by the successful prevention of subsequent primary tumours in patients with an existing neoplasm (Alfthan et al. 1983; Studer et al. 1984). Synthetic derivatives behave like -235-
natural vitamin A, although there are some differences in pharmacokinetics and tissue storage between synthetic retinoids and natural retinoids (Kelloff et al. 1994). For example, synthetic retinoids are able to reverse squamous metaplasia in organ cultures of vitamin A-deficient hamster trachea, (Spor n et al. 1975 and 1976), and they are associated with all the classical clinical signs of hypervitaminosis A (Pitt 1985; Kistler 1986; Turton et al. 1991; Wan 1993). The mechanism through which synthetic retinoids inhibit carcinogenesis is not known. However, like the natural compounds from which they are derived, the anti-cancer activity of synthetic retinoids appears to be mediated through the inhibition of cell proliferation or the induction of cell differentiation (Holdener and Bollag 1993). This is thought to be achieved through interaction with RARs and RXRs leading to modulatory effects on gene expression (Sporn and Roberts 1984), including possibly alteration in the expression of cellular oncogenes (Steinmetz and Potter 1991).

There is relatively strong epidemiological evidence linking lower than average vitamin A intake with a higher risk of human bladder cancer (Mettlin and Graham 1979; Kolonel et al. 1985; La Vecchia et al. 1989; Steineck et al. 1990; Shibata et al. 1992). It must be noted, however, that the protective effect of vitamin A reported by Kolonel et al. (1985) was weak, and the decrease in the number of bladder cancer cases in people consuming vitamin supplements observed by Steineck et al. (1990) was of borderline statistical significance. Furthermore, a few retrospective dietary studies have not found any evidence of a significant reduction in bladder cancer incidence in individuals with a higher than average consumption of vitamin A (Risch et al. 1988; Nomura et al. 1991; Riboli et al. 1991). Mettlin and Graham (1979) reported a strong inverse relationship between bladder cancer incidence and intake of vitamin A, but these authors did not adjust for smoking in their analysis (Michels and Willett 1994). Finally, in all 3 of the blood-based case-control studies carried out to date, pre-diagnostic blood levels of retinol were not significantly different in bladder cancer cases and controls (Nomura et al. 1985; Helzlsouer et al. 1989; Knekt et al. 1991). Notwithstanding the lack of association between vitamin A and bladder cancer that has been found in some epidemiological investigations, overall the data are still relatively supportive of some degree of protection afforded by high vitamin A intake (Michels and Willett 1994).

In contrast to vitamin A, very little epidemiological evidence links human urinary bladder cancer with BC (Michels and Willett 1994). Only one prospective study has examined the relationship between BC and bladder cancer, but in this case the relationship was found not to be significant (Shibata et al. 1992). Of all the retrospective dietary studies which have investigated BC intake and urinary bladder cancer (Kolonel et al. 1985; Claude et al. 1986; Risch et al. 1988; La
Vecchia et al. 1989; Nomura et al. 1991; Riboli et al. 1991), only that of La Vecchia et al. (1989) reported a significant inverse relationship between risk of bladder cancer and estimated carotenoid intake. This analysis, however, did not take into account total energy intake or other micronutrients. Claude et al. (1986) reported that frequent consumption of fruit and vegetables was associated with a reduced risk of bladder cancer, but did not relate these findings to BC, or any other nutrients. Retrospective studies have shown no significant difference in pre-diagnostic blood levels of BC between cases and controls (Nomura et al. 1985; Wald et al. 1988; Helzlsouer et al. 1989; Comstock et al. 1991; Knekt et al. 1991a). The epidemiological data, therefore, suggest that pre-formed vitamin A may be more important in protecting against bladder cancer than BC.

An interesting observation was made in the investigation reported by Helzlsouer et al. (1989). In this retrospective study, bladder cancer was found to be significantly associated with pre-diagnostic blood levels of lycopene. Lycopene is an oxygenated carotenoid, lacking in vitamin A activity, found in tomatoes (Steinmetz and Potter 1991). The results of Helzlsouer et al. (1989) are worth further examination in experimental bladder cancer models. Lycopene and lutein, another oxygenated carotenoid, are the carotenoids that are found at the highest levels in human plasma (Steinmetz and Potter 1991). Furthermore, Lycopene has twice the activity of BC as a quencher of singlet oxygen (Rousseau et al. 1992). Lycopene is found to be protective in animal studies, or in further epidemiological investigations, this may indicate a useful potential chemopreventative agent for human bladder cancer. Furthermore, should lycopene be demonstrated to be an effective anti-cancer agent, the mechanism of such activity would not be via conversion to vitamin A, but through an inherent property of the carotenoid molecule.

If BC is active in protecting against urothelial neoplasia, it seems likely from the epidemiological evidence that such activity would be mediated through conversion to vitamin A, rather than through some inherent property of the BC molecule. To date, the prevention of bladder cancer by BC has been examined in only 4 animal studies, including that described in this thesis (Trial VIII). The 3 other studies (Hicks et al. 1984; Moon 1989; Mathews-Roth et al. 1991) were all performed in the mouse. The present study is the only one to have used the rat as a model system. All 4 investigations examined the effect of BC against urinary bladder cancer induced by the specific bladder carcinogen BBN (Druckrey et al. 1964). The study reported by Moon (1989) is unpublished and only a very brief outline was given in the author's 1989 review, which makes discussion of this study rather difficult. It is likely, however, that the study would have been conducted along the lines of the standard protocol described by Moon et al. (1982). If so, the
experiment would have involved the treatment of B6D2F1 mice with 10 weekly aliquots of
carcinogen to give a total dose of 90 mg BBN. The animals would have been killed 6 months later
and the numbers of carcinomas assessed histologically. These same experimental details were
used in the other mouse studies reported by Hicks et al. (1984) and by Mathews-Roth et al.
(1991). The only procedural differences between the 3 studies related to the administration of BC.
Moon (1989), gave BC intraperitoneally (3-24 mg/week), administering the carotenoid in this way
in an attempt to avoid conversion to retinol by the gut, thereby ensuring a high level of
unconverted BC in these animals (Kelloff et al. 1994). Hicks et al. (1984) and Mathews-Roth et
al. (1991) fed BC in the diet, at 5 mM/kg and about 2 mM/kg respectively. However,
Mathews-Roth (1991) fed the BC diet for 5 weeks prior to carcinogen treatment as well as 26
after dosing, whereas Hicks et al. (1984) only administered BC after BBN dosing.

Whether administered intraperitoneally, or in the diet, no evidence was found in these mouse
studies to suggest that BC had prevented the development of TCC (Hicks et al. 1984; Moon 1989;
Mathews-Roth et al. 1991). Furthermore, there was no anti-carcinogenic effect regardless of
whether BC was given before BBN dosing, or otherwise. The results presented here (Trial VIII),
of the first investigation of the anti-cancer activity of BC in the rat, also demonstrated no
significant effect of BC upon the incidence of TCC. In addition, the results from the rat confirm
the findings of Mathews-Roth and associates (1991) in the mouse that pre-treatment of animals
with BC before the administration of carcinogen has no effect on the incidence of TCC. Together,
the results of the studies in the mouse, and now the rat, appear to emphasise the lack of
epidemiological evidence supporting a role for BC in the prevention of urinary bladder cancer.

Mathews-Roth et al. (1991) reported that appreciable levels of unconverted BC were measured in
the bladder tissue of mice given the same dietary level of BC as used in their carcinogenicity study
(2 mM/kg). Thus, BC was present in the target tissue. Similar analyses were not performed in the
rat study in which BC was fed at a concentration of 3 mM/kg. However, it seems likely that
appreciable amounts of BC were also present in the target tissue, as the bladders of BC-fed rats at
post-mortem were more yellow in colour than those from control animals.

Both the present rat study (Trial VIII) and the mouse study of Mathews-Roth et al. (1991)
investigated the relevance of the provitamin A activity of BC for any anti-cancer activity that may
have been observed. Mathews-Roth et al. (1991) did not observe an effect on the incidence of
carcinomas, but did report a significant reduction by BC of a broad range of lesions that they
categorised as non-malignant 'tumours'. These lesions probably included reversible hyperplasias
as well as benign tumours. In the same experiment, canthaxanthin, a carotenoid lacking vitamin A
activity, had no effect, either on malignant lesions or on the broad range of proliferative lesions described by the authors as 'tumours'. These findings were interpreted by the authors as evidence that BC protects against BBN-induced bladder cancer, through its provitamin A activity, rather than any property inherent in the carotenoid molecule.

Similarly, the rat study reported here (Trial VIII) found no significant evidence of any effect of BC on the incidence of malignant bladder lesions. Some evidence of an effect by BC on the total number of macroscopic lesions was observed. Though not statistically significant, this finding is probably analogous to the reduction in the number of 'tumours' referred to by Mathews-Roth and associates (1991). Taken together, the results of Trial VIII and the study of Mathews-Roth et al. (1991) suggest that BC does not have any effect upon the incidence of malignant lesions, but may have some slight effect on the incidence of early proliferative lesions. This slight effect may have been mediated through conversion to vitamin A, although, in both Trial VIII and the study of Mathews-Roth et al. (1991), it was only evident in animals fed BC in addition to normal level of vitamin A.

The importance of conversion to vitamin A for the anti-cancer activity of BC was evident in Trial VIII, when the incidence of carcinomas in vitamin A-deficient rats was compared with that in a population of formerly vitamin A-deficient rats that subsequently had been restored to vitamin A-sufficiency by BC. The difference was not significant. However, the trend demonstrated that administration of BC to vitamin A-deficient animals may have reduced their risk of developing carcinomas to that of rats fed a control diet. This observation may have important implications for human cancer, for example, the higher than average risks of bladder cancer observed in individuals who consume a lower than average amount of vitamin A (Mettlin and Graham 1977; Kolonel et al. 1985; La Vecchia et al. 1989; Shibata et al. 1992) may be reduced to the level of average risk, simply by increasing the intake of BC containing foods.

The numbers of animals that survived to the terminal kills in each of these 2 studies were too small to allow small differences in lesion numbers between groups to be statistically detected. Had there been more animals, it is possible that the outcome of each of these experiments may have been different.

The experimental work described here (Trial VIII) reports a negative finding for the activity of BC against urinary bladder cancer in rats. Moon (1989) commented on the negative findings from an unpublished study of the activity of BC against BBN-induced bladder cancer in the mouse. This
experiment is only the third investigation of its type to be carried out in mice, and yet remains unpublished. Greenberg (1993) commented that there was a tendency amongst clinical researchers not to report negative results from trials of potential chemopreventative agents. This tendency may explain why Moon (1989) has not as yet reported fully on the pre-clinical investigation of the activity of BC against BBN-induced urinary bladder cancer in mice. Negative findings can be as significant for scientific progress as positive ones and ought to be published (Rousseau et al. 1992).

In summary, the work described in this thesis has investigated the anti-cancer activity of dietary BC against carcinogen-induced urinary bladder cancer in the rat. No statistically significant evidence was found that demonstrated any anti-carcinogenic effect of BC on the incidence of malignant bladder tumours. This finding is in agreement with the published literature. As there were no significant findings related to a protective effect of BC, it is not possible to draw any firm conclusions about the mechanism by which BC may protect against bladder cancer. However, the results of this experiment, and those reported by Mathews-Roth et al. (1991), show a trend indicating a slight protective effect against non-malignant proliferative lesions in the urinary bladder. Furthermore, if this trend is indicative of a true effect, these 2 studies also suggest that the effect may be mediated through conversion of BC to retinol, although it is possible that some other mechanism may also be responsible. If conversion of BC to vitamin A were an effective mechanism in protection from bladder cancer, this conclusion would be supported by the epidemiological evidence and, indirectly, by the wealth of experimental evidence demonstrating that the urinary bladder is sensitive to vitamin A.

No experimental studies to date have demonstrated a significant protective effect of BC against carcinogen-induced urinary bladder cancer. If these results are repeated in other experimental models of bladder cancer, this may indicate that BC is not active against this neoplasm.

Whether or not BC is truly protective against human bladder cancer, the overwhelming evidence from more than 200 epidemiological investigations supports the idea that a diet rich in BC-containing foods - yellow and orange fruit and vegetables and dark green leafy vegetables - is protective against the development of most forms of cancer (Steinmetz and Potter 1991a; Block et al. 1992, Greenberg 1993) including urinary bladder cancer (Metttlin and Graham 1977). It is hoped that the results of on-going clinical intervention studies in humans will clarify whether BC itself is protective (Hennekens and Eberlain 1985; Steinmetz and Potter 1991b).
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THE ACTIVITY OF DIETARY BETA-CAROTENE AGAINST CARCINOGEN-INDUCED URINARY BLADDER CANCER IN THE RAT

VOLUME 2

A thesis presented by

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Doctor of Philosophy

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June 1995
Table 1.1  Occupations associated with increased risk of bladder cancer

<table>
<thead>
<tr>
<th>Occupation</th>
<th>Occupation</th>
<th>Occupation</th>
<th>Occupation</th>
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<tbody>
<tr>
<td>Aluminium processors</td>
<td>Coke oven workers</td>
<td>Leather workers</td>
<td>Printers</td>
</tr>
<tr>
<td>Architects</td>
<td>Construction workers</td>
<td>Lorry drivers</td>
<td>Punch and stamping press operators</td>
</tr>
<tr>
<td>Artistic painters</td>
<td>Cooks and kitchen workers</td>
<td>Lumbermen</td>
<td>Railway switchmen</td>
</tr>
<tr>
<td>Asbestos workers</td>
<td>Cutting operatives</td>
<td>Machine operators</td>
<td>Roofers</td>
</tr>
<tr>
<td>Assessors</td>
<td>Drill press operators</td>
<td>Medical workers</td>
<td>Sailors</td>
</tr>
<tr>
<td>Blasters</td>
<td>Dry cleaners</td>
<td>Metal fabricators</td>
<td>Security guards</td>
</tr>
<tr>
<td>Bootblacks</td>
<td>Dye workers</td>
<td>Metal filing, polishers, sanders, buffers</td>
<td>Tailors</td>
</tr>
<tr>
<td>Butchers</td>
<td>Electrical workers</td>
<td>Miners</td>
<td>Taxi drivers</td>
</tr>
<tr>
<td>Car mechanics</td>
<td>Engineers</td>
<td>Nurserymen</td>
<td>Telephone operators</td>
</tr>
<tr>
<td>Cardboard container worker</td>
<td>Food processing workers</td>
<td>Painters</td>
<td>Textile workers</td>
</tr>
<tr>
<td>Cement and concrete finishers</td>
<td>Furnace operators</td>
<td>Paper and pulp workers</td>
<td>Tyre and rubber workers</td>
</tr>
<tr>
<td>Checkers and graders in manufacturing</td>
<td>Gardeners</td>
<td>Petroleum workers</td>
<td>Welders</td>
</tr>
<tr>
<td>Chemical workers</td>
<td>Gas workers</td>
<td>Pharmacists</td>
<td>Woodworkers, carpenters</td>
</tr>
<tr>
<td>Clerical workers</td>
<td>Glass processors</td>
<td>Photographic workers</td>
<td>Writers</td>
</tr>
<tr>
<td>Coarse fishermen</td>
<td>Hairdressers</td>
<td></td>
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</tr>
</tbody>
</table>

Table adapted from Cohen and Johansson (1992) and Silverman *et al.* (1992). Those occupations which are most strongly associated with bladder cancer are underlined. The association between the other occupations and bladder cancer are not as strong, and in many cases require further corroboration (Silverman *et al.* 1992).
Figure 1.1  Simplified summary of the process of multistage carcinogenesis induced by exposure to a typical genotoxic chemical requiring metabolic activation.
<table>
<thead>
<tr>
<th>Initiation</th>
<th>Promotion</th>
<th>Progression</th>
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<tbody>
<tr>
<td>Pro-carcinogen</td>
<td>DNA adducts</td>
<td>Fixation of genetic damage</td>
</tr>
<tr>
<td>Metabolic activation</td>
<td>Preneoplastic lesion</td>
<td>Preneoplastic cells</td>
</tr>
<tr>
<td>Ultimate carcinogen</td>
<td>Benign tumour</td>
<td>Neoplastic cells</td>
</tr>
<tr>
<td>Cell replication</td>
<td>Invasive cancer</td>
<td></td>
</tr>
<tr>
<td>Normal cell</td>
<td>1-2 days</td>
<td>1+ years</td>
</tr>
<tr>
<td></td>
<td>10+ years</td>
<td></td>
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</tbody>
</table>
Figure 2.1  Chemical structures of selected natural and synthetic retinoids.

Figure 2.2  Chemical structures of three provitamin A carotenoids (BC, α-carotene, and cryptoxanthin), and two carotenoids without vitamin A activity (lutein and canthaxanthin).
Basic Retinoid Structure

Natural Retinoids

R = CH$_2$OH = All-trans-Retinol
R = CH$_2$OCO(CH$_2$)$_{14}$CH$_3$ = All-trans-Retinyl Palmitate (VAP)
R = CHO = All-trans-Retinal
R = COOH = All-trans-Retinoic Acid (RA)

Synthetic Retinoids

R = CH$_3$OCOCH$_3$ = All-trans-Retinyl Acetate (VAA)
R = CONHC$_2$H$_5$ = N-Ethyl-Retinamide
R = CONH — OH = N-4-Hydroxyphenyl-Retinamide (4HPR)

13-cis-Retinoic Acid

Etretinate

Figure 2.1
Figure 2.2
### Table 5.1

**Trial I: Experimental design**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Number of rats</th>
<th>Scheduled post-mortems (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>SSD(i)</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>SSD(i) plus VAA*</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>LVAD</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>LVAD plus VAA*</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>LVAD plus BC*</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

**Notes:**

- **VAA**, 2064 μgRE/kg
- **BC**, 4μM/kg
Figure 5.1  Mean body weight increases of female F344 rats fed diets in Trial I. The growth curves for rats fed each diet are shown as follows:

Group 1, Maintenance (closed circles); Group 2, SSD (i) (open triangles);
Group 3, SSD (i) plus 2064 μgRE/kg VAA (closed triangles); Group 4, LVAD (open squares); Group 5, LVAD plus 2064 μgRE/kg VAA (closed squares);
Group 6, LVAD plus 4 μM/kg BC (open circles)
# Table 5.2

**Trial I:** Mean absolute food consumption and mean body weight

| Group | Diet        | Weeks on diet$^a$ |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|-------|-------------|-------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|       |             | 1                | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10| 11 | 12| 13| 14| 15| 16| 17|
| 1     | Maintenance | Diet consumed    | 10.1| 12.1| 13.6| 14.5| 15.0| 15.4| 16.2| 16.0| 16.7| 15.8| 15.5| 16.3| 15.7| 15.4| 14.9| 14.5| 13.4| 17.4|
|       |             | Body weight$^a$  | 55.8| 96.0| 95.5| 126.6| 139.3| 147.0| 166.0| 166.7| 174.8| 182.1| 187.9| 187.4| 203.4| 208.2| 203.9| 212.3| 212.3| 215.3|
| 2     | SSD(i)      | Diet consumed    | 10.4| 12.0| 13.2| 12.8| 13.9| 13.9| 14.7| 14.1| 13.2| 14.9| 15.3| 19.7| 10.5| 15.6| 15.7| 13.6| 2.8| 11.9|
|       |             | Body weight$^a$  | 60.3| 81.6| 99.9| 127.8| 140.9| 150.3| 166.5| 168.6| 175.8| 182.7| 188.7| 188.1| 206.2| 208.3| 205.6| 209.8| 209.8| 211.8|
| 3     | SSD(i) plus VAA | Diet consumed | 10.6| 11.9| 16.2| ND$^b$| 13.8| ND| 11.6| ND| 14.2| 12.5| 12.6| 13.9| 14.0| 14.2| 13.0| 12.5| 12.1| 12.1|
|       |             | Body weight$^a$  | 71.4| 91.7| 111.4| -| 149.8| -| 171.0| -| 179.1| 185.0| 185.0| 191.3| 190.1| 204.6| 210.0| 210.0| 217.3| 217.3| 219.0|
| 4     | LVAD        | Diet consumed    | 9.4 | 11.0| 12.0| 13.2| 13.4| 13.5| 13.5| 13.4| 13.5| 12.8| 12.4| 13.1| 12.1| 14.6| 11.7| 11.0| 9.9| 11.9|
|       |             | Body weight$^a$  | 53.9| 72.4| 90.5| 119.4| 133.5| 139.1| 157.0| 159.2| 167.6| 174.1| 180.5| 180.7| 192.5| 195.5| 193.5| 199.6| 199.6| 202.3|
| 5     | LVAD plus VAA | Diet consumed     | 9.4 | 11.5| 12.4| 13.5| 13.1| 13.5| 13.4| 12.6| 12.8| 12.2| 12.7| 13.7| 7.4| 13.9| 12.7| 11.9| 11.0| 13.2|
|       |             | Body weight$^a$  | 56.0| 77.1| 96.9| 127.8| 140.9| 146.0| 163.6| 165.5| 172.2| 177.5| 182.0| 182.0| 194.9| 198.6| 197.5| 203.5| 203.5| 205.3|
| 6     | LVAD plus BC | Diet consumed     | 9.3 | 11.2| 12.0| 13.4| 13.5| 12.9| 14.1| 14.1| 13.8| 13.1| 12.7| 13.4| 8.1| 13.7| 12.6| 11.8| 10.7| 12.1|
|       |             | Body weight$^a$  | 54.7| 74.7| 93.8| 122.4| 138.6| 145.2| 163.1| 166.8| 174.8| 180.1| 187.0| 187.2| 200.8| 204.8| 204.5| 210.0| 210.0| 218.3|

**Notes:**

- Group mean daily consumption (g eaten/rat/day)
- Continuous consumptions determined on all rats every 5-6 days
- No consumption determined in week 12 and 14
- Group mean value (g) nearest consumption
- ND: Not done
Table 5.3
Trial I: Mean* absolute water consumption and mean body weight

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Notes:

- a: Group mean daily consumption (ml drunk/rat/day)
- b: Consumptions determined discontinuously for 2-3 days each week on all rats
- c: No consumption determined in week 14, 16 and 17
- d: Group mean value (g) nearest consumption
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<th>α-tocopherol (mg/dl)</th>
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Notes:
- All values are group means ±SD
- All groups n=6
- Observed ranges in parentheses
- ND=Not done
Table 5.5
Trial II: Experimental Design

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<th>SSD(i) plus VAA&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>LVAD plus VAA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Brompton</th>
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Notes:
- Unscheduled post-mortems are in parentheses
- Vitamin A acetate (2064 µgRE/kg)
Figures 5.2 - 5.5

Mean body weight increases for rats fed diets in Trial II: Group 1 (female F344 rats); Group 2 (male F344 rats); Group 3 (female Wistar rats); Group 4 (female Sprague-Dawley rats).

Figure 5.2 Mean body weight increases of female F344 rats. The growth curves for rats fed each diet are shown as follows: Maintenance diet (closed circles); SSD (i) (open triangles); SSD (i) plus 2064 μgRE/kg VAA (closed triangles); LVAD (open squares); LVAD plus 2064 μgRE/kg VAA (closed squares); Brompton diet (open circles).

Figure 5.3 Mean body weight increases of male F344 rats. The growth curves for rats fed each diet are shown as follows: Maintenance diet (closed circles); SSD (i) (open triangles); SSD (i) plus 2064 μgRE/kg VAA (closed triangles); LVAD (open squares); LVAD plus 2064 μgRE/kg VAA (closed squares); Brompton diet (open circles).

Figure 5.4 Mean body weight increases of female Wistar rats. The growth curves for rats fed each diet are shown as follows: Maintenance diet (closed circles); SSD (i) (open triangles); SSD (i) plus 2064 μgRE/kg VAA (closed triangles); LVAD (open squares); LVAD plus 2064 μgRE/kg VAA (closed squares).

Figure 5.5 Mean body weight increases of female Sprague-Dawley rats. The growth curves for rats fed each diet are shown as follows: Maintenance diet (closed circles); SSD (i) (open triangles); SSD (i) plus 2064 μgRE /kgVAA (closed triangles); LVAD (open squares); LVAD plus 2064 μgRE/kg VAA (closed squares).
Figure 5.2
Figure 5.3
Table 5.6

Trial II: Body weights of female F344 rats (Group 1) fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Number of rats</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>6</td>
<td>61.8</td>
<td>74.8</td>
<td>101.5</td>
<td>118.0</td>
<td>136.5</td>
<td>147.9</td>
<td>156.5</td>
<td>162.8</td>
<td>173.0</td>
<td>178.0</td>
<td>185.5</td>
<td>190.5</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>6</td>
<td>65.8</td>
<td>72.8</td>
<td>107.8</td>
<td>123.8</td>
<td>134.9</td>
<td>143.7</td>
<td>148.3</td>
<td>159.2</td>
<td>163.5</td>
<td>165.5</td>
<td>170.5</td>
<td>175.5</td>
</tr>
<tr>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>60.5</td>
<td>71.5</td>
<td>95.0</td>
<td>113.1</td>
<td>130.6</td>
<td>144.9</td>
<td>153.3</td>
<td>161.5</td>
<td>173.8</td>
<td>182.7</td>
<td>185.2</td>
<td>187.4</td>
</tr>
<tr>
<td>LVAD</td>
<td>7</td>
<td>74.1</td>
<td>66.3</td>
<td>91.1</td>
<td>109.3</td>
<td>125.9</td>
<td>137.9</td>
<td>145.7</td>
<td>149.6</td>
<td>162.0</td>
<td>163.7</td>
<td>169.0</td>
<td>172.1</td>
</tr>
<tr>
<td>LVAD plus VAA</td>
<td>7</td>
<td>55.7</td>
<td>74.4</td>
<td>98.1</td>
<td>114.9</td>
<td>134.3</td>
<td>149.1</td>
<td>158.3</td>
<td>165.7</td>
<td>178.4</td>
<td>180.6</td>
<td>186.0</td>
<td>190.7</td>
</tr>
<tr>
<td>Brompton</td>
<td>4</td>
<td>47.3</td>
<td>60.8</td>
<td>79.0</td>
<td>90.3</td>
<td>98.1</td>
<td>100.8</td>
<td>114.5</td>
<td>114.5</td>
<td>114.5</td>
<td>114.5</td>
<td>114.5</td>
<td>114.5</td>
</tr>
</tbody>
</table>

Notes:

a. In g, mean (±sd); body weights were determined weekly

b. No body weights determined in week 9

c. Mean and sd calculated from 2 body weight determinations in week 5 and 13

d. Mean and sd calculated from 3 body weight determinations in week 6

e. At week 6, n=3 for 2 of the 3 body weight determinations

f. At week 7, n=2
Table 5.7

Trial II: Body weights* of male F344 rats (Group 2) fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Number of rats</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5*</th>
<th>6*</th>
<th>7</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>6</td>
<td>51.3 (12.8)</td>
<td>71.5 (18.3)</td>
<td>106.0 (24.1)</td>
<td>136.3 (24.1)</td>
<td>177.3 (28.7)</td>
<td>209.4 (27.2)</td>
<td>227.0 (25.4)</td>
<td>245.8 (25.5)</td>
<td>277.2 (25.2)</td>
<td>289.5 (27.3)</td>
<td>296.4 (25.6)</td>
<td>313.5 (25.6)</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>6*</td>
<td>51.0 (10.1)</td>
<td>75.7 (11.9)</td>
<td>106.0 (15.0)</td>
<td>129.0 (17.3)</td>
<td>154.2 (18.5)</td>
<td>172.9 (16.5)</td>
<td>186.7 (15.1)</td>
<td>195.0 (17.0)</td>
<td>205.5 (14.4)</td>
<td>203.3 (18.6)</td>
<td>198.3 (28.1)</td>
<td>189.3 (13.4)</td>
</tr>
<tr>
<td>SSD(i) plus SSD(i)</td>
<td>6</td>
<td>49.5 (10.7)</td>
<td>73.2 (11.4)</td>
<td>108.7 (14.3)</td>
<td>139.8 (14.0)</td>
<td>180.2 (11.9)</td>
<td>207.2 (12.3)</td>
<td>230.3 (9.8)</td>
<td>251.5 (10.8)</td>
<td>280.2 (12.3)</td>
<td>293.7 (11.0)</td>
<td>312.9 (11.7)</td>
<td></td>
</tr>
<tr>
<td>LVAD</td>
<td>7</td>
<td>53.6 (9.5)</td>
<td>77.3 (13.7)</td>
<td>113.4 (16.3)</td>
<td>140.7 (18.1)</td>
<td>167.1 (17.3)</td>
<td>194.9 (16.5)</td>
<td>216.0 (16.2)</td>
<td>229.6 (14.5)</td>
<td>254.3 (16.6)</td>
<td>261.3 (16.3)</td>
<td>272.9 (15.7)</td>
<td>278.1 (15.5)</td>
</tr>
<tr>
<td>LVAD plus VAA</td>
<td>7</td>
<td>49.9 (4.1)</td>
<td>75.1 (6.2)</td>
<td>103.9 (14.7)</td>
<td>128.0 (19.0)</td>
<td>172.4 (18.6)</td>
<td>203.0 (20.3)</td>
<td>223.4 (20.7)</td>
<td>240.3 (22.4)</td>
<td>269.0 (24.3)</td>
<td>280.0 (24.9)</td>
<td>293.1 (26.5)</td>
<td>305.6 (27.3)</td>
</tr>
<tr>
<td>Brompton</td>
<td>4*</td>
<td>47.8 (11.0)</td>
<td>67.8 (12.4)</td>
<td>88.5 (15.4)</td>
<td>96.8 (21.5)</td>
<td>101.1 (27.1)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
<td>111.8 (34.7)</td>
</tr>
</tbody>
</table>

Notes:

a In g, mean (±sd); body weights were determined weekly
b No body weights determined in week 9
c Mean and sd calculated from 2 body weight determinations in week 5 and 13
d Mean and sd calculated from 3 body weight determinations in week 6
e At week 13, n=4
f At week 6, n=2 for 2 of the 3 body weight determinations
Table 5.8

Trial II: Body weights* of female Wistar rats (Group 3) fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Number of rats</th>
<th>Weeks* fed diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Maintenance</td>
<td>6</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.1)</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>6</td>
<td>42.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.2)</td>
</tr>
<tr>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.0)</td>
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<tr>
<td>LVAD</td>
<td>6</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.8)</td>
</tr>
<tr>
<td>LVAD plus VAA</td>
<td>6</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.5)</td>
</tr>
</tbody>
</table>

Notes:

a In g, mean (±sd); body weights were determined weekly
b No body weights determined in week 9
c Mean and sd calculated from 2 body weight determinations in week 5 and 13
d Mean and sd calculated from 3 body weight determinations in week 6
Table 5.9

**Trial II:** Body weights* of female Sprague Dawley rats (Group 4) fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Number of rats</th>
<th>Week* fed diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Maintenance</td>
<td>6</td>
<td>55.8(9.6)</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>6</td>
<td>59.7(11.8)</td>
</tr>
<tr>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>68.8(9.5)</td>
</tr>
<tr>
<td>LVAD</td>
<td>7</td>
<td>57.9(13.5)</td>
</tr>
<tr>
<td>LVAD plus VAA</td>
<td>7</td>
<td>45.1(5.9)</td>
</tr>
</tbody>
</table>

Notes:

a  In g, mean (±sd); body weights were determined weekly
b  No body weights determined in week 9
c  Mean and sd calculated from 2 body weight determinations in week 5 and 13
d  Mean and sd calculated from 3 body weight determinations in week 6
Table 5.10
Clinical signs of vitamin A deficiency in F344 rats fed the Brompton diet

<table>
<thead>
<tr>
<th>Clinical sign</th>
<th>Appearance&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight plateau</td>
<td>24-33</td>
</tr>
<tr>
<td>Pylo-erection, untidy coat</td>
<td>36-39</td>
</tr>
<tr>
<td>Thin appearance</td>
<td></td>
</tr>
<tr>
<td>Eye lesions - crusty red exudate</td>
<td>33-38</td>
</tr>
<tr>
<td>- hair loss around eye socket</td>
<td>39-45</td>
</tr>
<tr>
<td>- eyeball slightly sunken into socket</td>
<td></td>
</tr>
<tr>
<td>Breathing difficulties</td>
<td></td>
</tr>
<tr>
<td>Paresis</td>
<td></td>
</tr>
<tr>
<td>Eye lesions - xeropthalmia</td>
<td>38-45</td>
</tr>
<tr>
<td>Brown/yellow staining of fur around anus</td>
<td>45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Swollen abdomen - gas in intestines</td>
<td></td>
</tr>
<tr>
<td>Hunched posture</td>
<td></td>
</tr>
<tr>
<td>Emaciation - no body fat</td>
<td></td>
</tr>
<tr>
<td>Moribund</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

a  Days fed the Brompton diet after which, depending on initial body weight, rats showed clinical signs of vitamin A deficiency

b  Three of 4 female F344 rats were killed before later clinical signs could develop
Table 5.11

Trial II: Group 1- Female F344 rats: Plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Post-mortem (week)</th>
<th>Diet</th>
<th>No of rats</th>
<th>Retinol*</th>
<th>α-tocopherol*</th>
<th>β-carotene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Maintenance</td>
<td>12*</td>
<td>40.48±0.01 (35.10-46.30)</td>
<td>0.76±0.14 (0.56-0.87)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>7</td>
<td>Brompton</td>
<td>4*</td>
<td>2.90±0.18 (2.70-3.10)</td>
<td>0.21±0.08 (0.12-0.31)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>13</td>
<td>Maintenance</td>
<td>6</td>
<td>26.62±1.70 (24.30-29.20)</td>
<td>0.46±0.30 (0.10-0.74)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i)</td>
<td>5</td>
<td>5.44±0.87 (4.30-6.30)</td>
<td>0.33±0.07 (0.24-0.41)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>28.23±2.21 (25.40-30.60)</td>
<td>0.72±0.31 (0.14-1.03)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD</td>
<td>7</td>
<td>12.50±3.21 (9.50-18.20)</td>
<td>0.40±0.04 (0.35-0.44)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD plus VAA</td>
<td>7</td>
<td>24.84±0.31 (24.40-25.40)</td>
<td>0.65±0.12 (0.50-0.79)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes:

a All values are group means ±SD, with observed ranges in parentheses
b Values expresses as µg/dl
c Values expressed as mg/dl
d Values are from 6 pairs of pooled samples
e Mean of 1 rat killed in week 6 and 3 rats killed in week 7
Table 5.12

Trial II: Group 2- Male F344 rats: Plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Post-mortem (week)</th>
<th>Diet</th>
<th>No of rats</th>
<th>Retinol[^a]</th>
<th>α-tocopherol[^b]</th>
<th>β-carotene[^b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Brompton</td>
<td>2[^d]</td>
<td>4.50±1.41 (3.50-5.50)</td>
<td>0.04±0.06 (0.00-0.08)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>13</td>
<td>Maintenance</td>
<td>6</td>
<td>53.63±7.73 (42.50-65.00)</td>
<td>0.30±0.09 (0.24-0.47)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i)</td>
<td>4</td>
<td>4.68±1.16 (3.20-6.00)</td>
<td>0.16±0.13 (0.03-0.33)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>56.2±5.39 (48.20-64.30)</td>
<td>0.56±0.08 (0.4-0.62)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD</td>
<td>7</td>
<td>9.90±4.56 (8.50-14.60)</td>
<td>0.32±0.07 (0.24-0.47)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD plus VAA</td>
<td>7</td>
<td>51.50±2.60 (48.50-55.2)</td>
<td>0.43±0.06 (0.36-0.52)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes:

a  All values are group means ±SD, with observed ranges in parentheses
b  Values expresses as μg/dl
c  Values expressed as mg/dl
d  Mean of rat killed in week 6 and 1 rat killed in week 7
Table 5.13

Trial II: Group 3-Female Wistar rats: Plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Post-mortem (week)</th>
<th>Diet</th>
<th>No of rats</th>
<th>Retinol a</th>
<th>α-tocopherol b</th>
<th>β-carotene c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maintenance</td>
<td>6</td>
<td>17.33±1.59 (15.10-19.70)</td>
<td>0.57±0.13 (0.45-0.74)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i)</td>
<td>6</td>
<td>5.27±1.30 (3.60-6.80)</td>
<td>0.48±0.12 (0.35-0.61)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>19.5±3.39 (15.80-25.90)</td>
<td>1.08±0.26 (0.70-1.46)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD</td>
<td>6</td>
<td>9.33±1.75 (6.20-11.10)</td>
<td>0.67±0.34 (0.12-0.97)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td>LVAD plus VAA</td>
<td>6</td>
<td>19.72±2.71 (17.10-23.90)</td>
<td>0.98±0.18 (0.75-1.22)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes

a All values are group means ±SD, with observed ranges in parentheses
b Values expresses as μg/dl
c Values expressed as mg/dl
Table 5.14

Trial II: Group 4-Female Sprague Dawley rats: Plasma analysis for retinol, \( \alpha \)-tocopherol and \( \beta \)-carotene

<table>
<thead>
<tr>
<th>Post-mortem (week)</th>
<th>Diet</th>
<th>No of rats</th>
<th>Retinol&lt;sup&gt;a&lt;/sup&gt;</th>
<th>( \alpha )-tocopherol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>( \beta )-carotene&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maintenance</td>
<td>5</td>
<td>22.74±3.09</td>
<td>0.86±0.20</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>(19.20-27.70)</td>
<td></td>
<td>(0.69-1.10)</td>
<td>(0.00-0.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SSD(i)</td>
<td>6</td>
<td>4.27±0.91</td>
<td>0.65±0.21</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>(3.00-5.00)</td>
<td></td>
<td>(0.41-1.03)</td>
<td>(0.00-0.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SSD(i) plus VAA</td>
<td>6</td>
<td>22.10±4.23</td>
<td>1.17±0.18</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>(16.70-26.00)</td>
<td></td>
<td>(1.00-1.45)</td>
<td>(0.00-0.00)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LVAD</td>
<td>7</td>
<td>5.96±0.70</td>
<td>0.78±0.19</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td>(3.00-6.70)</td>
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<td>(0.55-1.10)</td>
<td>(0.00-0.00)</td>
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<td></td>
<td>(19.60-23.70)</td>
<td></td>
<td>(0.57-1.36)</td>
<td>(0.00-0.00)</td>
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</tr>
</tbody>
</table>

Notes:

a All values are group means ±SD, with observed ranges in parentheses

b Values expressed as \( \mu \)g/dl

c Values expressed as mg/dl
Table 5.15
Trial III: Experimental Design

<table>
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<tr>
<th>Group</th>
<th>Rats</th>
<th>Strain</th>
<th>Sex</th>
<th>Number</th>
<th>Diet</th>
<th>Post-mortem (weeks)*</th>
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<td>SDS(ii)</td>
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<tr>
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<td>6</td>
<td>SSp(ii) plus VAA³</td>
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<td>SSp(ii) + 25% LVAD</td>
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<td>SSp(ii) + 50% LVAD</td>
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<td></td>
<td></td>
<td>6</td>
<td>SSD(ii)</td>
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</table>

Notes:

a Unscheduled post-mortems are in parentheses

b VAA 2064 µgRE/kg
Figures 5.6 - 5.9

Mean body weight increases for rats fed diets in Trial III: Group 1 (female F344 rats); Group 2 (male F344 rats); Group 3 (female Wistar rats); Group 4 (female Sprague-Dawley rats).

Figure 5.6
Mean body weight increases of female F344 rats. The growth curves for rats fed diets are shown as follows: Maintenance diet (closed triangles); SSD (i) (open squares); SSD (ii) (open circles); SSD (ii) plus 2064 µgRE/kg VAA (closed circles); LVAD (closed squares); Brompton diet (open triangles).

Figure 5.7
Mean body weight increases of male F344 rats. The growth curve of rats fed Maintenance diet is indicated by closed circles, while that of rats fed the SSD (ii) diet is shown by open circles.

Figure 5.8
Mean body weight increases of female Wistar rats. The growth curve of rats fed Maintenance diet is indicated by closed circles, while that of rats fed the SSD (ii) diet is shown by open circles.

Figure 5.9
Mean body weight increases of female Sprague-Dawley rats. The growth curve of rats fed Maintenance diet is indicated by closed circles, while that of rats fed the SSD (ii) diet is shown by open circles.
Figure 5.6
Figure 5.7
Figure 5.8
Figure 5.9
Table 5.16  
**Body weights** of Female F344 rats fed experimental diets

**Notes:**
- a In g, mean(±SD); body weights were determined weekly
- b No body weights were determined in week 1
- c From week 8, n=4
- d From week 9, n=3
- e All rats were necropsied in week 6
- f All rats were necropsied in week 8
- g From week 10, n=4

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<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
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<th>3</th>
<th>4</th>
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<th>7</th>
<th>8</th>
<th>9</th>
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<td>LVAD 5</td>
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<td></td>
</tr>
<tr>
<td>SSD(ii)</td>
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</tr>
<tr>
<td>SSD(ii) plus SSD(ii) plus VAA 6</td>
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</tr>
<tr>
<td>SSD(ii) plus 25% LVAD 5</td>
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<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>SSD(ii) plus 50% LVAD 5</td>
<td>31.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SSD(ii) plus 75% LVAD 5</td>
<td>31.8</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Notes:
- In g, mean(±SD); body weights were determined weekly
- No body weights were determined in week 1
- From week 8, n=4
- From week 9, n=3
Table 5.17

Trial III: Body weights* of Male F344 rats fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Time (weeks)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  12  13</td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>5</td>
<td>31.2 (5.1)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>6⁺</td>
<td>33.0 (4.9)</td>
</tr>
</tbody>
</table>

Notes:

a In g, mean(±SD); body weights were determined weekly

b No body weights were determined in week 11

c All rats were necropsied in week 8
Table 5.18

Trial III: Body weights* of Female Wistar rats fed experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>5</td>
<td>32.6 (1.1)</td>
<td>55.4 (3.4)</td>
<td>83.8 (5.9)</td>
<td>103.2 (19.7)</td>
<td>120.6 (15.0)</td>
<td>136.9 (13.6)</td>
<td>155.2 (14.3)</td>
<td>163.5 (14.4)</td>
<td>173.5 (13.0)</td>
<td>179.2 (12.7)</td>
<td>188.4 (12.3)</td>
<td>196.0</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>5'</td>
<td>32.8 (1.6)</td>
<td>56.0 (4.2)</td>
<td>88.6 (6.5)</td>
<td>107.2 (8.4)</td>
<td>120.2 (11.1)</td>
<td>130.0 (11.3)</td>
<td>138.8 (18.6)</td>
<td>142.9 (28.4)</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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</tbody>
</table>

Notes:

a  In g, mean±SD; body weights were determined weekly
b  No body weights were determined in week 11
c  All rats were necropsied in week 8
Table 5.19

Trial III: Body weights* of Female Sprague Dawley rats fed experimental diets

<table>
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<th>Diet</th>
<th>No of rats</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
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<tbody>
<tr>
<td>Maintenance</td>
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<td>52.6</td>
<td>(1.7)</td>
<td>83.4</td>
<td>(3.3)</td>
<td>129.2</td>
<td>(7.9)</td>
<td>166.2</td>
<td>(13.8)</td>
<td>181.2</td>
<td>(15.6)</td>
<td>198.3</td>
<td>(17.4)</td>
<td>217.0</td>
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<td>SSD(ii)</td>
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<td>53.5</td>
<td>(1.9)</td>
<td>87.8</td>
<td>(3.6)</td>
<td>132.8</td>
<td>(5.8)</td>
<td>164.8</td>
<td>(8.4)</td>
<td>185.7</td>
<td>(10.0)</td>
<td>204.8</td>
<td>(11.3)</td>
<td>214.8</td>
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</table>

Notes:

a In g, mean(±SD); body weights were determined weekly
b No body weights were determined in week 11
c All rats were necropsied in week 11
Table 5.20
Trial III: Mean diet consumption* (absolute values) and mean body weights* of female F344 rats

<table>
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<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10 11 12 13</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>10.9 11.6 12.3 13.2 13.9 14.5 15.8 15.5 15.8 15 15.6 15.4 15.1 14.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body Weight</td>
<td>56.3 81.8 81.8 100.7 120.8 131.6 148.7 160.6 167.7 175.6 175.6 186.6 191.2 136.8</td>
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</tr>
<tr>
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<td>SSD(i)</td>
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</tr>
<tr>
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<td>Body Weight</td>
<td>52 79 79 98.4 108.6 114.8 122.1 141.3 148.6 154 154 162.3 163 121.3</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>Body Weight</td>
<td>57 80.2 80.2 100.2 111.8 121 131.3 133 132.4 139.5 139.5 146.3 139.3 116.3</td>
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</tr>
<tr>
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<tr>
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<td>49 69 69 77.2 82.1</td>
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</tr>
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<td>SSD(ii) plus VAA</td>
<td>9.5 9.8 10.8 12.3 12.5 15.2 14.2 14.2 13.7 13.7 12.4 15.1 12.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body Weight</td>
<td>51.5 74.8 74.8 95.2 114.8 127.9 143.8 153.7 159.6 167.7 167.7 174.8 181.2 129.8</td>
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</tr>
<tr>
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<td>SSD(ii)</td>
<td>10.4 10.9 11.5 13 11.9 15.4 13.8 - - - - - 12.4</td>
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</tr>
<tr>
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<td>Body Weight</td>
<td>54 78.5 78.5 95.5 111.9 120.6 132.7</td>
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</tr>
<tr>
<td>7</td>
<td>SSD(ii) plus 25% LVAD</td>
<td>9.7 10.3 10.4 10.1 10.6 11.1 11.5 11 13 12.9 13 13 12.3 11.5</td>
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</tr>
<tr>
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<td>Body Weight</td>
<td>58.6 .3 83.2 100.4 115 121.1 131.2 135.2 146.5 151.9 151.9 163 162.8 123.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>SSD(ii) plus 50% LVAD</td>
<td>10.8 11.1 11.2 11.6 10.4 10.9 11 11.7 12.6 13.6 12.8 13 11 11.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body Weight</td>
<td>55.2 82.8 82.8 101.2 111.8 120 127.2 132.2 138.3 141.6 141.6 157 152 118.7</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>SSD(ii) plus 75% LVAD</td>
<td>10.1 11.5 11.5 12.2 11.6 12.1 13.3 11.9 12.5 11.9 12.3 12.5 10.8 11.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body Weight</td>
<td>54.6 80.2 80.2 96.8 107.2 115.1 124.7 131.4 138 138.9 138.9 145 142 114.9</td>
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</tr>
</tbody>
</table>

Notes:
- Consumptions (g/rat/day) were determined continuously for periods of 7-10 days
- Body weights (in g) were determined weekly apart from weeks 5-10 in which the mean of 2-3 body weight determinations each week is tabulated. At times a single body weight result was the most appropriate for 2 consecutive diet consumptions (e.g. week 2 and 3, and 10 and 11)
- Average of mean weekly diet consumption or body weight for week 1-5, 1-7 or 1-13 respectively
### Table 5.21

**Trial III: Mean diet consumption* (relative values) of female F344 rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>Average mean value*</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Maintenance</td>
<td>193.40</td>
<td>141.60</td>
<td>150.80</td>
<td>131.30</td>
<td>114.90</td>
<td>110.20</td>
<td>106.10</td>
<td>96.70</td>
<td>94.10</td>
<td>85.40</td>
<td>88.70</td>
<td>82.70</td>
<td>78.70</td>
<td>113.40</td>
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<td>153.20</td>
<td>162.70</td>
<td>138.90</td>
<td>117.60</td>
<td>123.70</td>
<td>146.90</td>
<td>115.50</td>
<td>116.60</td>
<td>115.30</td>
<td>105.80</td>
<td>98.30</td>
<td>124.50</td>
<td>135.10</td>
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<tr>
<td>3</td>
<td>LVAD</td>
<td>190.60</td>
<td>137.80</td>
<td>151.10</td>
<td>124.10</td>
<td>108.10</td>
<td>105.20</td>
<td>84.80</td>
<td>99.90</td>
<td>112.00</td>
<td>93.20</td>
<td>90.80</td>
<td>86.20</td>
<td>84.50</td>
<td>112.90</td>
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<td>Brompton</td>
<td>194.60</td>
<td>149.30</td>
<td>156.10</td>
<td>136.90</td>
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<td>149.60</td>
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<td>184.50</td>
<td>130.30</td>
<td>144.50</td>
<td>129.30</td>
<td>108.90</td>
<td>118.60</td>
<td>98.90</td>
<td>92.50</td>
<td>88.90</td>
<td>81.50</td>
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<td>71.20</td>
<td>83.50</td>
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<tr>
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<td>193.40</td>
<td>138.50</td>
<td>147.10</td>
<td>135.80</td>
<td>106.00</td>
<td>127.60</td>
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<td>136.16</td>
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<td>SSD(ii) plus 25% LVAD</td>
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<td>123.20</td>
<td>125.30</td>
<td>100.30</td>
<td>91.90</td>
<td>91.40</td>
<td>87.40</td>
<td>81.10</td>
<td>90.60</td>
<td>84.70</td>
<td>85.40</td>
<td>79.80</td>
<td>75.30</td>
<td>98.70</td>
</tr>
<tr>
<td>8</td>
<td>SSD(ii) plus 50% LVAD</td>
<td>195.70</td>
<td>133.50</td>
<td>135.30</td>
<td>114.30</td>
<td>93.00</td>
<td>90.60</td>
<td>84.50</td>
<td>88.20</td>
<td>87.20</td>
<td>95.90</td>
<td>90.60</td>
<td>82.80</td>
<td>72.10</td>
<td>105.10</td>
</tr>
<tr>
<td>9</td>
<td>SSD(ii) plus 75% LVAD</td>
<td>185.60</td>
<td>142.80</td>
<td>143.20</td>
<td>126.40</td>
<td>108.50</td>
<td>104.80</td>
<td>106.40</td>
<td>90.70</td>
<td>90.80</td>
<td>85.90</td>
<td>88.80</td>
<td>86.20</td>
<td>75.60</td>
<td>110.40</td>
</tr>
</tbody>
</table>

**Notes:**

- **a** Consumptions (g/animal body weight/day) were determined continuously for periods of 3-7 days. At times, a single body weight determination was the most appropriate from which to calculate 2 consecutive relative consumptions (e.g. week 2 and 3, and 10 and 11).
- **b** Average of mean weekly diet consumption or body weight for week 1-5, 1-7 or 1-13 respectively
- **c** 2064 µgRE/kg of diet
Table 5.22  
Trial III:  Mean diet consumptions* (absolute values) and mean body weightsb of male F344 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean valued</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>56.00 92.40 92.40 123.60 151.40 179.90 211.70 228.20 245.50 259.00 259.60 284.40 292.80 190.60</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii) Diet consumed</td>
<td>10.20 11.60 12.60 13.60 12.70 15.10 7.70 - - - - - 11.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>59.80 87.70 87.70 112.70 132.30 145.70 139.00 - - - - - 222.00</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

a Consumptions (g/rat/day) were determined continuously for periods of 3-7 days
b Body weights (in g) were determined weekly apart from weeks 5-10 in which the mean of 2-3 body weight determinations each week is tabulated. At times a single body weight result was the most appropriate for 2 consecutive diet consumptions (e.g. week 2 and 3, and 10 and 11)
c Average of mean weekly diet consumption or body weight for week 1-7 or 1-13 respectively
Table 5.23

Trial III: Mean diet consumptions* (relative values) of male F344 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>225.00</td>
<td>151.50</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>170.00</td>
<td>132.60</td>
</tr>
</tbody>
</table>

Notes:

a Consumptions (g/kg rat body weight/day) were determined continuously for periods of 3-7 days. At times a single body weight determination was the most appropriate from which to calculate 2 consecutive relative consumptions (e.g. week 2 and 3, and 10 and 11)

b Average of mean weekly diet consumption or body weight for week 1-7 or 1-13 respectively
Table 5.24
Trial III: Mean diet consumptionsa (absolute values) and mean body weightsb of female Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>Diet consumed</td>
<td>11.30</td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>55.40</td>
<td>83.80</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>Diet consumed</td>
<td>13.30</td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>56.00</td>
<td>88.60</td>
</tr>
</tbody>
</table>

Notes:
- a Consumptions (g/rat/day) were determined continuously for periods of 3-7 days.
- b Body weights (in g) were determined weekly apart from weeks 5-10 in which the mean of 2-3 body weight determinations each week is tabulated. At times a single body weight result was the most appropriate for 2 consecutive diet consumptions (e.g. week 2 and 3, and 10 and 11).
- c Average of mean weekly diet consumption or body weight for week 1-7 or 1-13 respectively.
Table 5.25

Trial III: Mean diet consumptions* (relative values) of female Wistar rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>203.40</td>
<td>137.20</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>238.10</td>
<td>173.80</td>
</tr>
</tbody>
</table>

Notes:

a Consumptions (g/kg rat body weight/day) were determined continuously for periods of 3-7 days. At times a single body weight determination was the most appropriate from which to calculate 2 consecutive relative consumptions (e.g. week 2 and 3, and 10 and 11)

b Average of mean weekly diet consumption or body weight for week 1-7 or 1-13 respectively
Table 5.26
Trial III: Mean diet consumptions* (absolute values) and mean body weights* of female Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diet consumed</td>
<td>16.70</td>
<td>18.90</td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>83.40</td>
<td>129.20</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diet consumed</td>
<td>15.20</td>
<td>17.70</td>
</tr>
<tr>
<td></td>
<td>Body weight</td>
<td>87.80</td>
<td>132.80</td>
</tr>
</tbody>
</table>

Notes:
- a Consumption (g/rat/day) were determined continuously for periods of 3-7 days.
- b Body weights (in g) were determined weekly apart from weeks 5-10 in which the mean of 2-3 body weight determinations each week is tabulated. At times a single body weight result was the most appropriate for 2 consecutive diet consumptions (e.g. week 2 and 3, and 10 and 11).
- c Average of mean weekly diet consumption or body weight for week 1-10 or 1-13 respectively.
Table 5.27
Trial III: Mean diet consumptions* (relative values) of female Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Time (weeks)</th>
<th>Average mean value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Maintenance</td>
<td>200.60</td>
<td>145.90</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>173.40</td>
<td>133.30</td>
</tr>
</tbody>
</table>

Notes:

a Consumptions (g/kg rat body weight/day) were determined continuously for periods of 3-7 days. At times, a single body weight determination was the most appropriate from which to calculate 2 consecutive relative consumptions (e.g. week 2 and 3, and 10 and 11).

b Average of mean weekly diet consumption or body weight for week 1-10 or 1-13 respectively.
Table 5.28
Trial III: Summary of body weights and diet consumptions

<table>
<thead>
<tr>
<th>Diet</th>
<th>Rats</th>
<th>Initial body weight</th>
<th>Final body weight</th>
<th>(Week)</th>
<th>Mean absolute diet consumption</th>
<th>Mean relative diet consumption</th>
<th>(Weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>F344 (Female)</td>
<td>56.3</td>
<td>191.2</td>
<td>(13)</td>
<td>14.2</td>
<td>113.4</td>
<td>(0-13)</td>
</tr>
<tr>
<td></td>
<td>F344 (Male)</td>
<td>56.0</td>
<td>292.8</td>
<td>(13)</td>
<td>17.4</td>
<td>108.8</td>
<td>(0-13)</td>
</tr>
<tr>
<td></td>
<td>Wistar (Female)</td>
<td>55.4</td>
<td>196.0</td>
<td>(13)</td>
<td>14.7</td>
<td>114.8</td>
<td>(0-13)</td>
</tr>
<tr>
<td></td>
<td>SD (Female)</td>
<td>83.4</td>
<td>268.0</td>
<td>(13)</td>
<td>20.1</td>
<td>111.3</td>
<td>(0-13)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>F344 (Female)</td>
<td>54.0</td>
<td>132.7</td>
<td>(7)</td>
<td>12.4</td>
<td>136.1</td>
<td>(0-7)</td>
</tr>
<tr>
<td></td>
<td>F344 (Male)</td>
<td>59.8</td>
<td>139.0</td>
<td>(7)</td>
<td>11.9</td>
<td>117.4</td>
<td>(0-7)</td>
</tr>
<tr>
<td></td>
<td>Wistar (Female)</td>
<td>56.0</td>
<td>138.8</td>
<td>(7)</td>
<td>16.8</td>
<td>168.9</td>
<td>(0-7)</td>
</tr>
<tr>
<td></td>
<td>SD (Female)</td>
<td>87.8</td>
<td>230.2</td>
<td>(10)</td>
<td>17.9</td>
<td>105.9</td>
<td>(0-10)</td>
</tr>
<tr>
<td>Brompton</td>
<td>F344 (Female)</td>
<td>49.000</td>
<td>82.100</td>
<td>(5)</td>
<td>10.100</td>
<td>149.600</td>
<td>(0-5)</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>F344 (Female)</td>
<td>52.000</td>
<td>163.000</td>
<td>(13)</td>
<td>15.300</td>
<td>135.100</td>
<td>(0-13)</td>
</tr>
<tr>
<td>LVAD</td>
<td>F344 (Female)</td>
<td>57.000</td>
<td>139.300</td>
<td>(13)</td>
<td>12.400</td>
<td>112.900</td>
<td>(0-13)</td>
</tr>
<tr>
<td>SSD(ii) plus 25% LVAD</td>
<td>F344 (Female)</td>
<td>58.600</td>
<td>162.800</td>
<td>(13)</td>
<td>11.500</td>
<td>98.700</td>
<td>(0-13)</td>
</tr>
<tr>
<td>SSD(ii) plus 50% LVAD</td>
<td>F344 (Female)</td>
<td>55.200</td>
<td>118.700</td>
<td>(13)</td>
<td>11.600</td>
<td>105.100</td>
<td>(0-13)</td>
</tr>
<tr>
<td>SSD(ii) plus 75% LVAD</td>
<td>F344 (Female)</td>
<td>54.600</td>
<td>114.900</td>
<td>(13)</td>
<td>11.900</td>
<td>110.400</td>
<td>(0-13)</td>
</tr>
<tr>
<td>SSD(ii) plus VAA</td>
<td>F344 (Female)</td>
<td>51.500</td>
<td>181.200</td>
<td>(13)</td>
<td>12.900</td>
<td>108.800</td>
<td>(0-13)</td>
</tr>
</tbody>
</table>

Notes:

a  Initial mean body weight (in g) at week 1
b  Final mean body weight prior to post-mortem at week 5, week 7, week 10 or week 13
c  Average mean weekly consumption (g/rat/day) for week 0-5, 0-7, 0-10 or 0-13 respectively
d  Average mean weekly relative consumption (g/ kg rat body weight/day) for week 0-5, 0-7, 0-10 or 0-13 respectively
Table 5.29  
Trial III: Female F344 rats; plasma analysis for retinol, α-tocopherol and β-carotene<sup>a</sup>

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Post-mortem (weeks)</th>
<th>Retinol&lt;sup&gt;b&lt;/sup&gt;</th>
<th>α-tocopherol&lt;sup&gt;c&lt;/sup&gt;</th>
<th>β-Carotene&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>12</td>
<td>0&lt;sup&gt;9&lt;/sup&gt;</td>
<td>32.00±2.56 (29.20-35.80)</td>
<td>0.86±0.07 (0.78-0.94)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>Brompton</td>
<td>5</td>
<td>6&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.65±0.79 (0.00-1.60)</td>
<td>0.37±0.07 (0.29-0.45)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>56</td>
<td>8&lt;sup&gt;9&lt;/sup&gt;</td>
<td>1.37±1.27 (0.00-2.90)</td>
<td>0.03±0.01 (0.02-0.04)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>SSD(i)</td>
<td>4</td>
<td>13</td>
<td>2.90±1.25 (1.10-3.80)</td>
<td>0.33±0.09 (0.21-0.43)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>LVAD</td>
<td>3</td>
<td>13</td>
<td>3.77±2.72 (2.10-6.90)</td>
<td>0.40±0.06 (0.33-0.47)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>SSD(ii) plus VAA&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6</td>
<td>13</td>
<td>25.03±1.62 (22.30-26.80)</td>
<td>0.04±0.02 (0.00-0.06)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>75% SSD(ii); 25% LVAD</td>
<td>5</td>
<td>13</td>
<td>1.08±1.74 (0.00-4.00)</td>
<td>0.16±0.04 (0.11-0.22)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>50% SSD(ii); 50% LVAD</td>
<td>4</td>
<td>13</td>
<td>0.85±0.98 (0.00-1.80)</td>
<td>0.21±0.09 (0.13-0.29)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>25% SSD(ii); 75% LVAD</td>
<td>5</td>
<td>13</td>
<td>2.00±0.60 (1.40-2.90)</td>
<td>0.25±0.06 (0.19-0.33)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes:

a  All values are group means ±SD, with observed ranges in parentheses
b  Values expressed as µg/dl
c  Values expressed as mg/dl
d  Plasma samples were thawed and refrozen once prior to analysis; all other samples were thawed and refrozen twice prior to analysis
e  2064 µgRE/kg
Table 5.30  
Trial III: Male F344 rats; plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Post-mortem (weeks)</th>
<th>Retinol*</th>
<th>α-tocopherol*</th>
<th>β-Carotene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>5</td>
<td>13</td>
<td>60.14±7.23 (52.20-71.30)</td>
<td>0.53±0.13 (0.39-0.69)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>2</td>
<td>7</td>
<td>1.95±0.92 (1.30-2.60)</td>
<td>0.04±0.01 (0.03-0.05)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes:

a  All values are group means ±SD, with observed ranges in parentheses  
b  Values expressed as µg/dl  
c  Values expressed as mg/dl
Table 5.31
Female Wistar rats; plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Post-mortem (weeks)</th>
<th>Retinol*</th>
<th>α-tocopherol*</th>
<th>β-Carotene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>5</td>
<td>13</td>
<td>16.28±3.29</td>
<td>0.94±0.09</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(12.20-20.10)</td>
<td>(0.87-1.09)</td>
<td>(0.00-0.00)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>5</td>
<td>8</td>
<td>2.04±1.22</td>
<td>0.09±0.04</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.00-3.20)</td>
<td>(0.06-0.15)</td>
<td>(0.00-0.00)</td>
</tr>
</tbody>
</table>

Notes:

a  All values are group means ±SD, with observed ranges in parentheses
b  Values expressed as µg/dl
c  Values expressed as mg/dl
### Table 5.32

**Trial III:** Female Sprague-Dawley rats; plasma analysis for retinol, α-tocopherol and β-carotene

<table>
<thead>
<tr>
<th>Diet</th>
<th>No of rats</th>
<th>Post-mortem (weeks)</th>
<th>Retinol*</th>
<th>α-tocopherol*</th>
<th>β-Carotene*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance</td>
<td>5</td>
<td>13</td>
<td>15.96±1.43 (14.60-18.10)</td>
<td>0.96±0.11 (0.82-1.09)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
<tr>
<td>SSD(ii)</td>
<td>6</td>
<td>10</td>
<td>0.62±0.73 (0.00-1.70)</td>
<td>0.06±0.02 (0.05-0.11)</td>
<td>0.00±0.00 (0.00-0.00)</td>
</tr>
</tbody>
</table>

**Notes:**

a. All values are group means ±SD, with observed ranges in parentheses

b. Values expressed as μg/dl

c. Values expressed as mg/dl
Figure 5.10 A comparison of the response of different rat sexes, stocks and strains to the SSD(ii) vitamin A-deficient diet. This figure shows the mean body weight increases of female F344 rats (open circles), male F344 rats (closed circles), female Wistar rats (closed squares), and female Sprague-Dawley rats (open squares) fed the SSD (ii) diet.
Figure 5.10

Body weight (g) vs. Time (wk)
Table 5.33
Levels of retinol$^a$ and $\alpha$-tocopherol$^b$ in duplicate plasma samples analysed after storage for 20 months and 42 months

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Storage Time</th>
<th>Retinol</th>
<th>$\alpha$-tocopherol</th>
<th>Retinol</th>
<th>$\alpha$-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 months</td>
<td></td>
<td></td>
<td>42 months*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>19.0</td>
<td>1.0</td>
<td>17.9 (+1.1)</td>
<td>0.52 (+0.51)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>25.9</td>
<td>1.1</td>
<td>24.1 (+1.8)</td>
<td>0.79 (+0.28)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>18.1</td>
<td>1.3</td>
<td>18.5 (-0.4)</td>
<td>1.58 (+0.69)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>6.8</td>
<td>0.4</td>
<td>8.1 (-1.3)</td>
<td>0.07 (+0.31)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>0.6</td>
<td>5.4 (+0.1)</td>
<td>0.23 (+0.35)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>0.6</td>
<td>5.8 (-1.1)</td>
<td>0.30 (+0.31)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>6.7</td>
<td>0.6</td>
<td>6.7 (0.0)</td>
<td>0.32 (+0.25)</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

a  Values expressed as µg/dl

b  Values expressed as mg/dl

c  Samples taken from Trial II terminal kill (13 weeks). Samples 1-3 from female Wistar rats fed the SSD(ii) diet plus VAA (2064 µgRE/kg); samples 4-7 from female Wistar rats fed the SSD(i) diet without vitamin A supplementation

d  Figures in parentheses are values at 42 months subtracted from values at 20 months
Table 5.34
Relationship of plasma retinol levels to the presence (+ve) or absence (-ve) of reduced body weight gain and other clinical signs (eye lesions) of vitamin A deficiency in rats of different strains and sexes fed various vitamin A-deficient diets at the terminal kills in Trials I-III

<table>
<thead>
<tr>
<th>Rats</th>
<th>Post Mortem (Weeks)</th>
<th>Diets</th>
<th>Clinical Signs</th>
<th>Plasma Retinol (% of Control level)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSD(i)</td>
<td>Reduced body weight gain</td>
<td>Eye lesions</td>
</tr>
<tr>
<td>Trial I</td>
<td>Female F344</td>
<td>20</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>Female F344</td>
<td>13</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>Male F344</td>
<td>13</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>Female Wistar</td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>Female Sprague Dawley</td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>Trial III</td>
<td>Female F344</td>
<td>13</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td></td>
<td>Male F344</td>
<td>7</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td></td>
<td>Female Wistar</td>
<td>8</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td></td>
<td>Female Sprague-Dawley</td>
<td>10</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Notes:
a Control rats were fed diets containing a normal level of vitamin A. Either Maintenance diet or vitamin A-deficient diets supplemented with VAA were used.
### Table 6.1

#### Trial IV: Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP* supplementation from week 8</th>
<th>Number of rats</th>
<th>Depletion period (week 0-8) Supplementation period (week 8-24)</th>
<th>Unscheduled* mortalities (week 8-24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Scheduled post-mortems (week 0-24)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA'</td>
<td>-</td>
<td>50*</td>
<td>5 5 5 5 5 5 5 5 5 5 5 5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SSD (ii)</td>
<td>-</td>
<td>35</td>
<td>5 5 5 5 5 5 5 5 5 5 5 5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>SSD (ii)</td>
<td>0.03</td>
<td>25</td>
<td>- - - - - - 4 5 5 3 -</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>SSD (ii)</td>
<td>0.15</td>
<td>25</td>
<td>- - - - - - 5 5 1 -</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>SSD (ii)</td>
<td>0.375</td>
<td>25</td>
<td>- - - - - - 3 3 5 -</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>SSD (ii)</td>
<td>0.75</td>
<td>25</td>
<td>- - - - - - 5 5 1 -</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>SSD (ii)</td>
<td>1.5</td>
<td>25</td>
<td>- - - - - - 5 5 5 5</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
<td>SSD (ii)</td>
<td>3</td>
<td>25*</td>
<td>- - - - - - 5 5 5 5 4</td>
<td>2</td>
</tr>
</tbody>
</table>

Notes:

- **a** VAP in the drinking water expressed as μgRE/rat/day assuming a consumption of 16ml/rat/day
- **b** Unscheduled mortalities due to vitamin A deficiency (killed in extremis or found dead)
- **c** VAA in the diet (2064 μgRE/kg)
- **d** 5 rats transferred to another experiment at 24 weeks
- **e** 4 rats transferred to another experiment at 24 weeks
Figure 6.1  Mean body weight increases of female F344 rats given a range of freshly-prepared concentrations of VAP twice weekly in the drinking water from week 8 (Trial IV). Concentrations of VAP were calculated assuming a water consumption of 16 ml/rat/day. Rats in Group 1 (indicated by the closed circles) were fed a control diet (SSD (ii) plus 2064 μgRE/kg VAA). The growth curves of rats in the other groups are shown as follows: Group 2, SSD (ii) with no VAP supplement (open circle); Group 3, SSD (ii) plus 0.03 μgRE VAP/rat/day (open square); Group 4, SSD (ii) plus 0.15 μgRE VAP/rat/day (closed square); Group 5, SSD (ii) plus 0.375 μgRE VAP/rat/day (open triangle); Group 6, SSD (ii) plus 0.75 μgRE VAP/rat/day (closed triangle); Group 7, SSD (ii) plus 1.5 μgRE VAP/rat/day (open diamond); Group 8, SSD (ii) plus 3.0 μgRE VAP/rat/day (closed diamond).
<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP Suppl*</th>
<th>Time (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0° 1 4 6 7 8 10 12 14 16 17 19 20 21 22 24</td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) + VAA*</td>
<td>Mean body weight</td>
<td>38.5 54.0 112.6 140.5 153.7 163.7 177.9 196.0 190.4 190.4 197.5 203.3 206.5 209.3 209.1 210.1 212.6</td>
</tr>
<tr>
<td></td>
<td>SSD(ii)</td>
<td>Mean body weight</td>
<td>34.4 52.5 105.8 126.6 136.9 145.1 154.8 150.5</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii) 0.03</td>
<td>Mean body weight</td>
<td>33.9 54.6 108.3 128.2 135.1 143.1 150.6 141.0</td>
</tr>
<tr>
<td></td>
<td>SSD(ii) 0.15</td>
<td>Mean body weight</td>
<td>33.1 55.7 111.1 130.4 136.6 142.2 151.8 140.0 138.0</td>
</tr>
<tr>
<td>3</td>
<td>SSD(ii) 0.375</td>
<td>Mean body weight</td>
<td>27.9 49.7 100.8 119.3 124.2 126.8 142.8 126.4</td>
</tr>
<tr>
<td></td>
<td>SSD(ii) 1.5</td>
<td>Mean body weight</td>
<td>33.7 66.5 109.4 129.6 136.4 137.4 147.9 161.0 154.4 153.6 144.5</td>
</tr>
<tr>
<td>4</td>
<td>SSD(ii) 3</td>
<td>Mean body weight</td>
<td>31.0 51.9 106.4 125.6 132.4 137.2 147.7 161.2 173.4</td>
</tr>
<tr>
<td>Notes:</td>
<td></td>
<td></td>
<td>a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>d</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>e</td>
</tr>
</tbody>
</table>
Figures 6.2 - 6.8

Eye lesions as clinical signs of vitamin A deficiency in rats fed the SSD (ii) diet, and the reversal of these lesions by VAP supplementation in the drinking water. Eye lesions were graded on a five-point scale (1 to 5), in increasing order of severity. Grades 1 to 3 (Figures 6.3, 6.4, and 6.5) were reversible with the administration of VAP (3.0 μgRE/rat/day) in the drinking water. Grade 4 (Figure 6.6) and Grade 5 lesions (Figure 6.7), however, were irreversible.

Figure 6.2 The normal bright, slightly bulging eye of a control rat fed the SSD (ii) diet supplemented with an adequate level of vitamin A (2064 μgRE/kg VAA).

Figure 6.3 Grade 1 eye lesions. The earliest ocular evidence of developing vitamin A deficiency in rats fed the SSD (ii) diet without supplemental vitamin A was dull, slightly sunken eyes, with one or two specks of red porphyrin pigment around the orbits (periocular porphyrin).

Figure 6.4 Grade 2 eye lesions. With increasing time on the vitamin A-deficient diet, the eyes appeared to sink further into the eye socket and more porphyrin was deposited around the orbits. In addition, there was often some hair loss (depilation) around the orbits.

Figure 6.5 Grade 3 eye lesions. Further depilation around the orbit has resulted in a complete ring of hair loss around the eye. The eye has sunk still further into the socket, as a result of which it has lost its circular shape and has begun to take on a slit-like appearance.
Figure 6.6  Grade 4 eye lesions. The eye has become slit-like in appearance. Porphyrin deposition and hair loss around the orbit have stopped.

Figure 6.7  Grade 5 lesions. The eyelids have closed. The membranes of the eye have dried out, indicative of xerophthalmia. These severe eye lesions were only seen occasionally, as those few rats which had developed Grade 4 lesions were usually killed before progressing further.

Figure 6.8  Reversal of eye lesions with VAP (3.0 μgRE/rat/day) in the drinking water. This photograph was taken 14 days after the start of VAP administration and shows reversal of the Grade 2 lesions illustrated in Figure 6.4 (the same rat is pictured in both figures). After VAP administration, the eye was once again bright and bulging, similar to that of the control animal shown in Figure 6.2.
Figure 6.8
Figures 6.9 - 6.10

Macroscopic post-mortem findings in rats with advanced vitamin A deficiency.

Figure 6.9  The appearance of the viscera at post-mortem in a young control rat which had received the SSD (ii) diet supplemented with an adequate level of vitamin A (2064 μgRE/kg of VAA). An adequate amount of fat was evident in the abdominal fat depots (arrow). The intestines appeared normal, with no evidence of gas.

Figure 6.10  The appearance of the viscera at post-mortem in a rat of similar age to that in Figure 6.9 which had died as a result of advanced vitamin A deficiency. There was a complete lack of body fat, indicating that the animal was emaciated. The intestines were devoid of ingesta and greatly distended with intestinal gas.
Figure 6.9

Figure 6.10
### Table 6.3

**Trial IV: Plasma retinol\(^a\) and α-tocopherol\(^b\) values in control rats (Group 1) and vitamin A-deficient rats supplemented with various levels of VAP in the drinking water**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP from week 8</th>
<th>Depletion</th>
<th>Scheduled post-mortems (week 0-24)</th>
<th>Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA</td>
<td>-</td>
<td>27.30(NA)(^c)</td>
<td>38.62(5.97)</td>
<td>32.8(2.06)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n(^e) retinol</td>
<td>38.62(5.97)</td>
<td>32.8(2.06)</td>
<td>0.95(0.09)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.97(NA)</td>
<td>0.95(0.09)</td>
<td>0.85(0.15)</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>-</td>
<td>25.8(NA)</td>
<td>16.8(10.03)</td>
<td>5.30(5.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n(^e) retinol</td>
<td>25.8(NA)</td>
<td>16.8(10.03)</td>
<td>5.30(5.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.97(NA)</td>
<td>0.95(0.09)</td>
<td>0.85(0.15)</td>
</tr>
<tr>
<td>3</td>
<td>SSD(ii)</td>
<td>0.03</td>
<td>n(^e) retinol</td>
<td>0.73(0.88)</td>
<td>0.32(0.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.32(0.03)</td>
<td>0.56(0.71)</td>
<td>0.40(0.02)</td>
</tr>
<tr>
<td>4</td>
<td>SSD(ii)</td>
<td>0.15</td>
<td>n(^e) retinol</td>
<td>3.00(0.61)</td>
<td>0.31(0.02)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.31(0.02)</td>
<td>0.68(0.86)</td>
<td>0.37(0.05)</td>
</tr>
<tr>
<td>5</td>
<td>SSD(ii)</td>
<td>0.375</td>
<td>n(^e) retinol</td>
<td>1.50(NA)</td>
<td>0.09(NA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.09(NA)</td>
<td>1.93(0.97)</td>
<td>0.45(0.05)</td>
</tr>
<tr>
<td>6</td>
<td>SSD(ii)</td>
<td>0.75</td>
<td>n(^e) retinol</td>
<td>1.92(1.14)</td>
<td>0.30(0.07)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.30(0.07)</td>
<td>1.58(1.00)</td>
<td>0.33(0.09)</td>
</tr>
<tr>
<td>7</td>
<td>SSD(ii)</td>
<td>1.5</td>
<td>n(^e) retinol</td>
<td>3.56(0.75)</td>
<td>0.28(0.11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.28(0.11)</td>
<td>2.04(0.50)</td>
<td>0.40(0.02)</td>
</tr>
<tr>
<td>8</td>
<td>SSD(ii)</td>
<td>3.0</td>
<td>n(^e) retinol</td>
<td>8.10(2.09)</td>
<td>0.45(0.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-toc</td>
<td>0.45(0.10)</td>
<td>0.62(0.46)</td>
<td>0.42(0.04)</td>
</tr>
</tbody>
</table>

**Notes:**

- Mean (± s.d.) retinol values expressed as μg/dl
- Mean (± s.d.) α-tocopherol values expressed as mg/dl
- In most cases, mean vitamin values are calculated from 5 separate samples, except where animals had died prior to scheduled kill or where the samples were pooled, e.g. week 0 values
- NA = Not Applicable
Figures 6.11 - 6.14

Histological appearance of the urothelium and the tracheal epithelium in control rats fed a diet containing an adequate amount of vitamin A (2064 μgRE/kg VAA), or in vitamin A-deficient rats maintained for at least 4 weeks by low levels of VAP supplementation in the drinking water.

Figure 6.11 Normal urothelium in a control rat showing three layers of transitional cells (arrow). Note that there are relatively more basal cells and intermediate cells than superficial cells. The oval-shaped nuclei of the small basal cells are oriented in a north/south direction, while those of the relatively larger superficial cells are oriented east/west.

Figure 6.12 Normal tracheal epithelium in a control rat showing the mixture of cell types characteristic of this tissue.

Figure 6.13 Hyperplastic urothelium in a vitamin A-deficient rat. The urothelium is more than 3 cells thick, consisting of small, transitional cells. Much of the epithelium is showing squamous metaplasia, where the transitional cells have changed type to become squamous cells (arrow). Some areas of squamous metaplasia are associated with focal keratinisation (open arrowhead).

Figure 6.14 A focal area of squamous metaplasia (arrow) within the tracheal epithelium of a vitamin A-deficient rat. There is evidence of inflammation (tracheitis) in the sub-epithelial tissue of the tracheal wall. Debris is present in the tracheal lumen including a sheet of shed epithelial cells, many of which are squamous cells.
Table 6.4

Grading system defining the progression of clinical signs of vitamin A deficiency in female F344 rats fed the SSD(ii) diet

<table>
<thead>
<tr>
<th>Grade</th>
<th>Clinical sign</th>
<th>Reversibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eyes dull</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eyes slightly sunken</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Specks of periocular porphyrin</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Eyes sunken</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased porphyrin deposition on hair around eyes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Eyes losing circular shape</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some hair loss (depilation) around eyes</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Eyes slit-like</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased depilation forming complete ring around eyes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hunched posture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respiratory difficulties</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Paresis</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Eyes closed and/or showing xerophthalmia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Brown staining on fur around anus (gastro-intestinal haemorrhage?)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Abdomen swollen with intestinal gas</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Emaciation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moribund</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

a  Not Reversible
In vitro investigations of the stability of VAP in dilute solution, carried out by Roche Products Limited, Dundee, Scotland (Trial Va). Solutions were left standing at room temperature (22°C) in non-actinic glassware. The vitamin A concentration was determined at various times up to 72 hours, using UV spectrophotometry.

Figure 6.15 The stability of VAP Type 100 in dilute solution over 72 hours. The percentage degradation over 72 hours of 2 concentrations of VAP in deionised water: dilution 1, starting concentration 3.0 µgRE/ml (open squares), and dilution 2, starting concentration 0.3 µgRE/ml (closed circles).

Figure 6.16 A comparison of the effects of deionised water or glass distilled water on the stability over 72 hours of VAP in Rovisol Type 100. The percentage degradation over 72 hours for VAP (starting concentration 300 µgRE/ml) dissolved in deionised water (open squares) or glass distilled water (closed circles).
Fig 6.15
Fig 6.16
Table 6.5

Trial V(a)*: The stability\textsuperscript{b} of VAP type 100 in dilute solution with deionised water over 72 hours

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Dilution 1\textsuperscript{c}</th>
<th>Dilution 2\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µgRE/ml UVmax(nm)</td>
<td>µgRE/ml UVmax(nm)</td>
</tr>
<tr>
<td>0</td>
<td>3.07 326</td>
<td>0.28 328</td>
</tr>
<tr>
<td>12</td>
<td>2.26 325</td>
<td>0.17 314</td>
</tr>
<tr>
<td>24</td>
<td>2.23 325</td>
<td>0.19 314</td>
</tr>
<tr>
<td>36</td>
<td>1.79 314</td>
<td>0.14 314</td>
</tr>
<tr>
<td>48</td>
<td>1.78 314</td>
<td>0.16 314</td>
</tr>
<tr>
<td>72</td>
<td>1.46 314</td>
<td>0.11 300</td>
</tr>
</tbody>
</table>

Notes:

a Experiment carried out by Roche Products Ltd
b VAP measured at intervals throughout a 72 hour period by UV spectrophotometry, in dilute solutions of VAP type 100 in deionised water left at room temperature in non-actinic glassware
c VAP type 100 diluted to give an initial VAP concentration of 3.0 µgRE/ml
d VAP type 100 diluted to give an initial VAP concentration of 0.3 µgRE/ml
Table 6.6
Trial V(a)°: The effect of deionised water or glass distilled water upon the stability° of vitamin A palmitate in solutions of Rovisol Type 100°

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Deionised water</th>
<th>Glass distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µgRE/ml</td>
<td>UVmax(nm)</td>
</tr>
<tr>
<td>0</td>
<td>3.13</td>
<td>328</td>
</tr>
<tr>
<td>12</td>
<td>3.02</td>
<td>328</td>
</tr>
<tr>
<td>24</td>
<td>3.03</td>
<td>328</td>
</tr>
<tr>
<td>36</td>
<td>2.84</td>
<td>328</td>
</tr>
<tr>
<td>48</td>
<td>2.81</td>
<td>328</td>
</tr>
<tr>
<td>72</td>
<td>2.6</td>
<td>327</td>
</tr>
</tbody>
</table>

Notes:
a Experiment carried out by Roche Products Ltd
b VAP measured at intervals throughout a 72 hour period by UV spectrophotometry, in solutions of Rovisol Type 100 in either deionised water or glass distilled water left at room temperature in non-actinic conditions
c Rovisol type 100 was made up in deionised water or glass distilled water to an initial concentration with respect to VAP of 300 µgRE/ml. Each solution was diluted 1:100 immediately prior to each vitamin A determination
Table 6.7

Trial Vb: Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP from day 47 (µgRE/ml)</th>
<th>Number of rats</th>
<th>Scheduled post-mortems (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>57* 58 59 60 61 63 64</td>
</tr>
<tr>
<td>1</td>
<td>SSD (ii) plus VAA[^a]</td>
<td>0</td>
<td>12</td>
<td>3 - - 3 - 3 3</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>0.1875[^d]</td>
<td>18</td>
<td>3 3 3 3 3 3 -</td>
</tr>
</tbody>
</table>

Notes:

a Water dispersible VAP administered continuously in the drinking water from day 47. At each change of drinking water on days 47, 50, 54, 57 and 61, fresh VAP was prepared to give an initial concentration of 0.1875 µgRE/ml vitamin A.

b Rats killed immediately before the administration of fresh VAP on day 57.

c Rats killed immediately before the administration of fresh VAP on day 61.

d VAA in the diet (2064 µgRE/kg).

e Assuming no VAP breakdown and a consumption of 16 ml of water/rat/day, this concentration of VAP would provide 3.0 µgRE vitamin A/rat/day.
Figure 6.17  Mean body weight increases for rats in Trial Vb. Rats in Group 1 (open squares) were fed the SSD (ii) diet supplemented with an adequate amount of vitamin A (2064 μgRE/kg VAA). The animals in Group 2 (closed circles) received unsupplemented SSD (ii) diet until week 7 (day 47), after which time they were supplemented with VAP in the drinking water to give a daily intake of 3.0 μgRE/rat, assuming no VAP breakdown and water consumption of 16 ml/day.

Figure 6.18  The effect of fluctuating VAP intake (caused by VAP breakdown) on the plasma retinol values of vitamin A-deficient rats in Trial Vb. The mean (± sd) plasma retinol values for control rats in Group 1 (open circles), or for vitamin A-deficient rats in Group 2 (closed squares) over a 7-day period of VAP administration (from the end of week 8 to the beginning of week 10). The times of fresh VAP administration are shown by the arrows. Rats in Group 1 were fed the SSD (ii) diet supplemented with an adequate amount of vitamin A (2064 μgRE/kg VAA). Animals in Group 2 were fed unsupplemented SSD (ii) diet until week 7 (day 47), after which time they were supplemented with VAP in the drinking water to give a daily intake of 3.0 μgRE/rat, assuming no VAP breakdown and water consumption of 16 ml/day.
Figure 6.18

Plasma retinol (µg/dl)

Time (h)

VAP
Table 6.8

Trial V(b): Group mean body weights (g) of rats fed a control diet (Group 1) or a vitamin A-deficient diet supplemented with low-level VAP in the drinking water (Group 2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP supplementation (µgRE/ml)</th>
<th>Weeks on diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0^3</td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA^1</td>
<td>0</td>
<td>Mean body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n)</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>0.1875</td>
<td>Mean body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n)</td>
</tr>
</tbody>
</table>

Notes:

a  VAP administered in the drinking water from day 47 (week 7)
b  Body weights determined on day 0 after starvation for 18 hours and immediately prior to the administration of the experimental diets
c  VAA in the diet (2064 µgRE/kg)
Table 6.9

Macroscopic findings at post-mortem of rats fed a control diet (Group 1) or a vitamin A-deficient diet supplemented with low-level VAP in the drinking water (Group 2)

<table>
<thead>
<tr>
<th>Day of post-mortem</th>
<th>Group</th>
<th>Rat no.</th>
<th>Vit A def grade</th>
<th>General appearance</th>
<th>Fat deposits</th>
<th>GI contents</th>
<th>Gas</th>
<th>Haem</th>
</tr>
</thead>
<tbody>
<tr>
<td>57</td>
<td>1^s</td>
<td>1</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2^s</td>
<td>16</td>
<td>3</td>
<td>Thin, eyes slit like and showing depilation and periocular porphyrin</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20^c</td>
<td>4</td>
<td>Very thin, eyes closed, fresh blood on fur of lower abdomen</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0</td>
<td>Slightly thin otherwise normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>58</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>Slightly untidy coat, eyes slightly sunken</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>3</td>
<td>Slightly thin, eyes becoming slit-like with depilation and periocular porphyrin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>4</td>
<td>Thin, eyes almost closed, much depilation, some periocular porphyrin</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>Slightly thin, periocular porphyrin, severe depilation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>27</td>
<td>0</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13</td>
<td>0</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>1</td>
<td>Eyes slightly sunken</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32</td>
<td>1</td>
<td>Specks of periocular porphyrin and some depilation, untidy coat</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>61</td>
<td>2</td>
<td>17</td>
<td>0</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td>2</td>
<td>Thin, some depilation, untidy coat</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>0</td>
<td>Normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63</td>
<td>1</td>
<td>5</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>0</td>
<td>Slightly thin, otherwise normal</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34</td>
<td>2</td>
<td>Specks of periocular porphyrin and also much depilation</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>38</td>
<td>0</td>
<td>Slightly thin with slightly untidy coat. Yellow staining of fur in urino-genital area</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>0</td>
<td>Normal</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Key to Table 6.9

Trial Vb: Macroscopic findings at post-mortem of rats fed a control diet (Group 1) or a vitamin A deficient diet supplemented with low-level VAP in the drinking water (Group 2)

a Severity of clinical signs of vitamin A deficiency (see Table 6.4 for grading system)
b Immediately prior to post-mortem examination
c Amount of fat in abdominal fat stores [absent (-), negligible (+), moderate (++)], abundant (+++)]
d Presence (+) or absence (-) of ingesta in G.I. tract
e Gas in G.I. tract [absent (-), mild (+), moderate/severe (+++)]
f Intestinal haemorrhage [absent (-), mild (+), moderate/severe (++)]
g Rats fed SSD(ii) diet plus VAA to give a total of 2064 μgRE/kg vitamin A
h Rats fed SSD(ii) diet alone until day 47, when they were supplemented with VAP in the drinking water. Freshly prepared VAP gave a vitamin A concentration of 0.1875 μgRE/ml
i This rat was found dead on day 57. It was subjected to a post-mortem examination 20 minutes after dying.
Table 6.10

Trial V(b): Mean absolute liver weight (g) and mean liver weight relative to body weight (g liver weight/kg rat body weight) of rats fed a control diet (Group 1) or a vitamin A deficient diet supplemented with VAP* (Group 2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Scheduled post-mortems (day)</th>
<th>57</th>
<th>58</th>
<th>59</th>
<th>60</th>
<th>61</th>
<th>63</th>
<th>64</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mean body wt</td>
<td>169.000</td>
<td>-</td>
<td>-</td>
<td>188.667</td>
<td>-</td>
<td>178.000</td>
<td>181.000</td>
</tr>
<tr>
<td></td>
<td>Mean liver weight (sd)</td>
<td>5.876 (0.242)</td>
<td>-</td>
<td>-</td>
<td>7.478 (0.239)</td>
<td>-</td>
<td>6.653 (0.114)</td>
<td>6.647 (0.440)</td>
</tr>
<tr>
<td></td>
<td>Mean liver weight/kg body wt</td>
<td>34.781 (1.677)</td>
<td>-</td>
<td>-</td>
<td>39.639 (0.359)</td>
<td>-</td>
<td>37.383 (0.495)</td>
<td>36.714 (2.084)</td>
</tr>
<tr>
<td>2</td>
<td>Mean body wt</td>
<td>105.000</td>
<td>115.667</td>
<td>135.667</td>
<td>147.667</td>
<td>122.333</td>
<td>143.667</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mean liver weight (sd)</td>
<td>3.945 (NA)</td>
<td>4.304 (0.853)</td>
<td>5.397 (0.517)</td>
<td>5.824 (0.784)</td>
<td>4.663 (0.722)</td>
<td>5.993 (0.911)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Mean liver weight/kg body wt</td>
<td>37.610 (NA)</td>
<td>37.211 (1.672)</td>
<td>39.793 (3.723)</td>
<td>39.413 (1.995)</td>
<td>38.496 (2.698)</td>
<td>41.597 (2.505)</td>
<td>-</td>
</tr>
</tbody>
</table>

Notes:

a Mean values were calculated from 3 rats/group at each kill, except on day 57 when livers were obtained from only 2 rats in Group 2

b VAP administered in the drinking water from day 47. At each change of drinking water on day 47, 50, 54, 57 and 61, fresh VAP was prepared to give an initial concentration of 0.1875 µgRE vitamin A/ml

c Not applicable (on day 57, values were obtained for 2 livers only from Group 2)
Table 6.11

Trial V(b): Mean (sd) plasma retinol values* at post-mortem (day 57 - 64) of rats fed a control diet (Group 1) or a vitamin A-deficient diet supplemented with low-level VAP in the drinking water^ (Group 2)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP supplementation (µgRE/ml)</th>
<th>Number of rats</th>
<th>Mean (sd) plasma retinol values at post-mortem (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>57</td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA</td>
<td>0</td>
<td>12</td>
<td>25.60 (0.62)</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>0.1875</td>
<td>17</td>
<td>1.75 (0.21)</td>
</tr>
</tbody>
</table>

Notes:

a All values expressed as µg/dl
b Mean values were calculated from 3 rats/group at each kill, except on day 57 when plasma samples were obtained from only 2 rats in group 2.
c Drinking water was changed on day 57 and day 61, when fresh VAP was given to all rats in Group 2 except those killed on these days. Fresh VAP was prepared to give an initial vitamin A concentration of 0.1875 µgRE/ml.
Table 6.12

Trial Vb: Mean values for absolute* and relative^ water consumption (and estimated average VAP* intake). Water consumptions calculated from data obtained from 8 cages of rats, the number of animals per cage varying from 1 to 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>VAP* supplementation (µgRE/ml)</th>
<th>Absolute consumption (µgRE)</th>
<th>Mean body weight (g)</th>
<th>Relative consumption</th>
<th>Estimated VAP intake (µgRE/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SSD(ii) + VAA</td>
<td>0</td>
<td>22.6 (3.5)</td>
<td>152.6</td>
<td>201.7 (31.6)</td>
<td>0.1875</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii)</td>
<td>0.1875</td>
<td>16.0 (0.7)</td>
<td>121.4</td>
<td>163.8 (7.6)</td>
<td>45.59</td>
</tr>
</tbody>
</table>

Notes:

a Values expressed as weight (g) of water consumed/rat/day
b Values expressed as weight (g) of water consumed per day relative to body weight (g/kg rat body weight)
c Average VAP intake for each 3 or 4 day consumption period expressed as µgRE/rat/day. Values were estimated using the VAP degradation curve (Fig. 6.15, closed circles) and the absolute water consumption. (For the method of estimation, refer to Appendix 2)
d VAP administered in the drinking water on day 47, 50, 54, 57 and 61. At each change of drinking water fresh VAP was prepared to give an initial concentration of 0.1875 µgRE/ml
e Consumptions were determined for periods of 3 or 4 days. No consumptions were determined between days 22-33-36 and 40-44.
f Nearest mean body weight values to each consumption determination. At times a single body weight value was the most appropriate to use in the calculation of several relative consumption variables (e.g. days 44-47, 47-50 and 50-54)
g High value due to leakage of water bottle
h Not Done

Values expressed as weight (g) of water consumed/day
Values expressed as weight (g) of water consumed per day relative to body weight (g/kg rat body weight)
Average VAP intake for each 3 or 4 day consumption period expressed as µgRE/rat/day. Values were estimated using the VAP degradation curve (Fig. 6.15, closed circles) and the absolute water consumption. (For the method of estimation, refer to Appendix 2)
VAP administered in the drinking water on day 47, 50, 54, 57 and 61. At each change of drinking water fresh VAP was prepared to give an initial concentration of 0.1875 µgRE/ml
Consumptions were determined for periods of 3 or 4 days. No consumptions were determined between days 22-33-36 and 40-44.
Nearest mean body weight values to each consumption determination. At times a single body weight value was the most appropriate to use in the calculation of several relative consumption variables (e.g. days 44-47, 47-50 and 50-54)
High value due to leakage of water bottle
Not Done
Table 6.13
Trial VI: Experimental Design

<table>
<thead>
<tr>
<th>Group</th>
<th>No. rats</th>
<th>Diet</th>
<th>BC* from day 54</th>
<th>Scheduled post-mortems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>day 90</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>SSD(ii) plus VAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ BC</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>SSD(ii) plus VAA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>SSD(ii)</td>
<td>+ BC</td>
<td>5</td>
</tr>
</tbody>
</table>

Notes:

a  BC (6mM/kg of diet)

b  VAA (2064 µgRE/kg)
Figure 6.19  Mean body weight increases for rats in Trial VI. From the beginning of the trial, Group 1 (open circles) and Group 2 (closed squares) were fed the SSD (ii) diet supplemented with an adequate amount of vitamin A (2064 μgRE/kg VAA), while Group 3 (open triangles) was given the unsupplemented SSD (ii) diet. From week 8, BC (6mM/kg) was administered in the diet to Groups 1 and 3, but not to Group 2.
Figure 6.19
Table 6.14
Trial VI: Group mean body weights (g) of rats fed a control diet (SSD(ii) plus VAA), or the same diet and given BC from day 54 (week 8), or a vitamin A-deficient diet (SSD(ii) alone) and given 6 mM/kg dietary BC from day 54 (week 8)

| Group | Diet | BC from day 54 | Weeks on diet
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA</td>
<td>+BC</td>
<td>Mean body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n)</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii) plus VAA</td>
<td>-</td>
<td>Mean body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n)</td>
</tr>
<tr>
<td>3</td>
<td>SSD(ii)</td>
<td>+BC</td>
<td>Mean body weight</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sd.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n)</td>
</tr>
</tbody>
</table>

Notes:

a Body weights determined on day 54, immediately prior to the administration of BC in the diets
b VAA (2064 µgRE/kg diet)
c One rat died after excess anaesthetic prior to being photographed
Table 6.15

Trial VI: Plasma analyses\(^a\) for retinol\(^b\), BC\(^c\) and \(\alpha\)-tocopherol\(^d\) of rats fed a control diet (SSD(ii) plus VAA), the same diet and given 6mM/kg BC from day 54 (week 8), or a vitamin A-deficient diet (SSD(ii) alone) and given 6mM/kg dietary BC from day 54 (week 8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>BC from day 54</th>
<th>Plasma analyses</th>
<th>day 90</th>
<th>day 140</th>
<th>α-toc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Retinol</td>
<td>Retinol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BC</td>
<td>BC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α-toc</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>SSD(ii) plus VAA</td>
<td>+BC</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>22.51±1.83</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(18.70-24.70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>142.00±52.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(80.00-248.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.96±0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.79-1.11)</td>
</tr>
<tr>
<td>2</td>
<td>SSD(ii) plus VAA</td>
<td>-</td>
<td>25.94±1.55</td>
<td>0.00±0.00</td>
<td>0.91±0.12</td>
<td>26.44±3.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(24.40-27.70)</td>
<td>(0.00-0.00)</td>
<td>(0.79-1.10)</td>
<td>(22.30-31.10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00±0.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.00-0.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.85±0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.72-1.03)</td>
</tr>
<tr>
<td>3</td>
<td>SSD(ii)</td>
<td>+BC</td>
<td>24.05±1.95</td>
<td>189.88±17.49</td>
<td>1.03±0.06</td>
<td>24.06±4.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(21.50-25.90)</td>
<td>(169.00-206.0)</td>
<td>(0.95-1.08)</td>
<td>(20.00-30.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(133.00-452.50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.86-1.28)</td>
</tr>
</tbody>
</table>

Notes:

a All values expressed as the mean ± sd, with the ranges in parentheses
b Retinol values expressed as µg/dl
c BC values expressed as µg/dl
d \(\alpha\)-tocopherol values expressed as mg/dl
Qualitative evidence suggestive of the absorption of unconverted BC into the body tissues of rats fed a high dietary level of BC (6mM/kg).

Figure 6.20 The normal appearance of the viscera of a control rat fed the SSD (ii) diet supplemented with an adequate amount of vitamin A (2064 μgRE/kg VAA). The abundant body fat is pale white, while the liver (see Figure 6.21), is a dark maroon colour.

Figure 6.21 The normal appearance of a freshly excised liver from a control rat fed the SSD (ii) diet supplemented with an adequate amount of vitamin A (2064 μgRE/kg of VAA). The liver is dark maroon in colour.

Figure 6.22 The appearance of the viscera of a rat fed a high dietary level of BC (6mM/kg). The abundant body fat is yellow/orange and the liver is orange-red in colour, suggesting that unconverted BC had been absorbed into the body tissues.

Figure 6.23 The appearance of a freshly excised liver from a rat fed a high dietary level of BC (6mM/kg). The liver is orange-red in colour and is paler (less maroon and more orange) than the livers from control rats (Figure 6.21), suggesting the presence of substantial amounts of unconverted BC in the hepatic tissues.
Figure 7.1 A typical bladder map from Trial VII, showing each half of a longitudinally bisected urinary bladder from a rat treated with a total dose of 700 mg BBN. The map shows the relative size and the approximate shape and location in the bladder of the macroscopic lesions observed by eye and under the low power of a dissecting microscope. In this case the lesions range from two large masses (A and B), two medium-sized masses (C and D), several small creamy white masses (E, F, G, H and I), and two very small bleb-like areas (J and K). The surface of the large mass (A) is vascularised, while that of the medium-sized mass (D) shows distinct areas of haemorrhage (shading).
Figure 7.1

Bladder Dome

Left

Bladder Trigone

Right

Approximate scale

5mm
Table 7.1
Summary of diagnostic criteria for rat urothelium

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Three cell layers thick. Mitoses very rare. Small basal cells, larger intermediate cells and very much larger frequently multinucleate superficial cells, with their nuclei orientated parallel to the luminal surface.</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Focal or diffuse areas of 4 or more epithelia cell layers. Cells often immature, i.e. basal-type, but otherwise well differentiated with normal polarity. Growth pattern may be flat, or papillary and/or nodular. Papillary hyperplasia further subdivided into 2 groups: early lesions consisting of a single papillary fold of urothelium and larger, more complex lesions comprising many unbranched papillary processes (Pap. Hyp. I), or discrete, exophytic lesions exhibiting complex branching of papillary processes often with fibrovascular cores extending into the individual processes (Pap. Hyp. II)</td>
</tr>
</tbody>
</table>

Invasive Carcinomas

- (a) Growth pattern
  - Papillary and/or nodular, adenomatous, solid or disseminated
- (b) Cell type
  - Transitional, squamous, mucous or undifferentiated (anaplastic)
- (c) Cytological characteristics
  - Low-grade, well differentiated, to high-grade, poorly differentiated and/or dysplastic
- (d) Depth of invasion (WHO classification)
  - P1a, into the stromal core of the stalk of the papillary lesions
  - P1b, into the sub-epithelial lamina propria
  - P2, into the superficial bladder muscle layers
  - P3, into the deep bladder muscle layers and extending to the peritoneal surface
  - P4, to adjacent or distant organs, by local or metastatic spread

Notes:

a Adapted from the diagnostic criteria of Hicks et al (1982)
Table 7.2

Haematuria determined in urine samples collected 32-35 weeks and 41 weeks after BBN dosing and macroscopic and microscopic assessment of urinary bladder tumours at 42 weeks and 50 weeks after BBN dosing respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Rat number</th>
<th>Determination of Haematuria</th>
<th>Assessment of Bladder Tumours at Post-mortem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>32-35 weeks after BBN¹</td>
<td>41 weeks after BBN¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>week 1</td>
<td>week 2</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>1</td>
<td>++++++</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2</td>
<td>++++++</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>4</td>
<td>++</td>
<td>-ve</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>5</td>
<td>++</td>
<td>-ve</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>6</td>
<td>+</td>
<td>-ve</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>7</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Vitamin A-deficient</td>
<td>1</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

Notes:

a Animals were given different BBN dosages. Group 1 (control diet) were given 7 weekly aliquots of 50 mg (rats 1-3; a total dose of 350 mg BBN), or 7 weekly aliquots of 100 mg (rats 4-7; a total dose of 700 mg BBN). The single Group 2 animal (vitamin A-deficient) received 5 weekly aliquots of 100 mg (a total BBN dosage of 500 mg).

b Haematuria was determined semi-quantitatively using 'Clinistest Labstix' (Ames Laboratories) testing strips. Determinations were made using a 6-point scale: -ve, negative; +, non-haemolysed trace; ++, haemolysed trace; ++++, small; ++++, medium; +++++, large.

c Haematuria determined in urine samples collected from 8 rats metabolized overnight, once each week for 4 weeks.

d Haematuria determined in urine samples collected from 7 rats metabolized overnight on one occasion.

e Macroscopic examination of urinary bladder tumours carried out by eye and under a dissecting microscope.

f Microscopic examination of urinary bladder tumours using light microscopy (paraffin histology). Histological diagnoses are presented for the most severe lesion in each bladder only. Abbreviations used for various histological diagnoses are: Pap. Hyp. I, papillary hyperplasia; Pap. Hyp. II, papillary hyperplasia showing complex branching of papillary processes and often with fibrovascular cores extending into each papillary process; TCC(P1a), transitional cell carcinoma with invasion of the stromal core of papillary lesions; TCC(P1b), transitional cell carcinoma with invasion of the lamina propria of the bladder wall.
Table 7.3

**Trial VIII:** Experimental design to evaluate the anti-cancer activity of dietary BC against carcinogen-induced urinary bladder cancer in vitamin A-deficient and vitamin A-normal rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of rats</th>
<th>Diet from week 0</th>
<th>Dietary supplement from week 7</th>
<th>Weekly dosing (week 15-19)</th>
<th>No of rats in terminal kill (week 54-57)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>Basal diet +VAA</td>
<td>+BC</td>
<td>BBN</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>Basal diet +VAA</td>
<td>-</td>
<td>BBN</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>Basal diet</td>
<td>+BC</td>
<td>BBN</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>Basal diet</td>
<td>+VAP</td>
<td>BBN</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>65</td>
<td>Basal diet +VAA</td>
<td>+BC</td>
<td>Vehicle</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>Basal diet +VAA</td>
<td>-</td>
<td>Vehicle</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>Basal diet</td>
<td>+BC</td>
<td>Vehicle</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>33</td>
<td>Basal diet</td>
<td>+VAP</td>
<td>Vehicle</td>
<td>32</td>
</tr>
</tbody>
</table>

Notes:

a) Number of rats present in each group at the start of the trial (week 0). Between week 0 and week 55, the following numbers of rats from groups 3 and 4 were subject to interim necropsies: 0 weeks; Group 3 - 6 rats, Group 4 - 6 rats; 7 weeks, Group 3 - 5 rats, Group 4 - 5 rats; 15 weeks, group 3 - 4 rats, Group 4 - 4 rats; 44 weeks, Group 4 - 5 rats; 49 weeks, Group 4 - 5 rats.

b) The basal diet fed to all groups throughout the trial was the SSD(ii) vitamin A-deficient diet. From week 0 to the end of the trial, the basal diet fed to Groups 1, 2, 5 and 6 was supplemented with VAA (2064 μgRE/kg).

c) At week 7, rats in Groups 3, 4, 7 and 8 fed the unsupplemented basal diet demonstrated evidence of vitamin A deficiency.

d) Five weekly doses of BBN (total dose, 63mg), or carcinogen-vehicle (30% v/v ethanol), were administered orally.

e) To avoid artefactual time-related differences in tumour incidence between groups, rats from each group were killed throughout the period of the terminal kill (week 54 - week 57).

f) From week 7 until the end of the trial, the basal diet of Groups 1, 3, 5 and 7 was supplemented with BC (3 mM/kg).

g) To maintain vitamin A-deficient rats in Groups 4 and 8 in a deficient but otherwise healthy condition, freshly prepared VAP (0.1875 μgRE/ml) was administered twice weekly in the drinking water.
### Table 7.4
Trial VIII: Number and times of scheduled and unscheduled deaths

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Dietary supplement from week 7</th>
<th>Treatment</th>
<th>No of rats at start (wk.1)</th>
<th>Scheduled deaths</th>
<th>Unscheduled deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Basal diet + VAA</td>
<td>+BC</td>
<td>BBN</td>
<td>32</td>
<td>wk7 26 wk15 5</td>
<td>Dosing accidents</td>
</tr>
<tr>
<td>2</td>
<td>Basal diet + VAA</td>
<td>-</td>
<td>BBN</td>
<td>32</td>
<td>wk44 26</td>
<td>Other deaths</td>
</tr>
<tr>
<td>3</td>
<td>Basal diet</td>
<td>+BC</td>
<td>BBN</td>
<td>48</td>
<td>wk49 28</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Basal diet</td>
<td>+VAF</td>
<td>BBN</td>
<td>65</td>
<td>wk55-58 33</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Basal diet + VAA</td>
<td>+BC</td>
<td>Vehicle</td>
<td>31</td>
<td>wk1 28</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Basal diet + VAA</td>
<td>-</td>
<td>Vehicle</td>
<td>31</td>
<td>wk4 28</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Basal diet</td>
<td>+BC</td>
<td>Vehicle</td>
<td>33</td>
<td>wk34 28</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Basal diet</td>
<td>+VAP</td>
<td>Vehicle</td>
<td>33</td>
<td>wk41 32</td>
<td></td>
</tr>
</tbody>
</table>

### Notes:

1. In addition to the number of rats shown here, a further 12 animals (18-21 days of age on arrival, 6 for Group C and 6 for Group 4) were purchased at a later date, acclimatised in a similar manner and killed at wk1 to provide baseline data for bladder histology, and plasma retinol and BC levels.

2. Scheduled kills at the times shown. All rats were subject to necropsy and sampled for plasma analyses and histology.

3. The terminal kill was carried out over a 3 week period (wk 55-58). To avoid artefactual time-related differences in tumour incidence between groups, rats from each group were killed throughout the 3 week period.

4. Rats which died during or immediately following dosing with carcinogen or vehicle; these deaths were attributed to accidental dosing into the lungs or overdose of CO₂ during anaesthesia. None of these animals were subject to a necropsy.

5. Animals which were killed in extremis before the terminal kill: a, found dead, wk 53 (monocytic leukaemia); b, found dead wk 43, (monocytic leukaemia); c, 2 killed in extremis - 1 in wk 34 (Sarcoma close to spinal cord), 1 killed in wk 41 (respiratory distress, no gross abnormality detected); d, 1 found dead, wk 7 (clinical signs of vitamin A deficiency - grade 5), 1 killed in extremis wk 50 (swollen adrenals, phaeochromocytoma).
Figure 7.2  Mean body weight increases for the carcinogen-dosed groups in Trial VIII. From the beginning of the trial until week 7, Group 1 (open circles) and Group 2 (closed circles) were fed the basal diet (SSD ii) supplemented with an adequate level of vitamin A (2064 µgRE/kg VAA), while Group 3 (pale squares) and Group 4 (dark squares) were given the unsupplemented basal diet. At week 7, when plasma retinol values in rats from Groups 3 and 4 were 10% of control values, BC (3mM/kg) was administered to Groups 1 and 3, while supplemental VAP (3 µgRE/rat/day) was administered to rats in Group 4. Between weeks 15-19, 5 weekly aliquots of BBN (total dose 635 mg/rat) were given. Animals were killed 38 weeks after the final dose of carcinogen.

Figure 7.3  Mean body weight increases for the vehicle-dosed groups in Trial VIII. From the beginning of the trial until week 7, Group 5 (open circles) and Group 6 (closed circles) were fed the basal diet (SSD ii) supplemented with an adequate level of vitamin A (2064 µgRE/kg VAA), while Group 7 (pale squares) and Group 8 (dark squares) were given the unsupplemented basal diet. At week 7, when plasma retinol values in rats from Groups 7 and 8 were 10% of control values, BC (3mM/kg) was administered to Groups 5 and 7, while supplemental VAP (3 µgRE/rat/day) was administered to rats in Group 8. Between weeks 15-19, 5 weekly aliquots of BBN-vehicle (30% ethanol) were given. Animals were killed 38 weeks after the final dose of vehicle.
Figure 7.2

Body weight (g)

Time (wk)

BBN
Table 7.5

Trial VIII: Group mean body weights of the BBN-dosed rats (Groups 1-4)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Dietary supplement from week</th>
<th>Time (week)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>5</td>
<td>7</td>
<td>11</td>
<td>12</td>
<td>14</td>
<td>34</td>
<td>37</td>
</tr>
<tr>
<td>1</td>
<td>Basal diet + VAA*</td>
<td>BC*</td>
<td>Mean</td>
<td>29.5</td>
<td>117.0</td>
<td>142.3</td>
<td>168.5</td>
<td>171.7</td>
<td>178.1</td>
<td>230.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>6.3</td>
<td>10.0</td>
<td>8.4</td>
<td>8.5</td>
<td>7.9</td>
<td>8.5</td>
<td>11.7</td>
</tr>
<tr>
<td>2</td>
<td>Basal diet + VAA*</td>
<td>-</td>
<td>Mean</td>
<td>32.5</td>
<td>124.1</td>
<td>149.8</td>
<td>176.1</td>
<td>179.8</td>
<td>189.3</td>
<td>233.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>5.6</td>
<td>9.8</td>
<td>10.4</td>
<td>9.8</td>
<td>9.1</td>
<td>9.2</td>
<td>12.8</td>
</tr>
<tr>
<td>3</td>
<td>Basal diet</td>
<td>BC*</td>
<td>Mean</td>
<td>26.6</td>
<td>100.7</td>
<td>116.6</td>
<td>165.6</td>
<td>171.5</td>
<td>180.3</td>
<td>227.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>1.9</td>
<td>10.1</td>
<td>9.8</td>
<td>9.5</td>
<td>10.2</td>
<td>10.2</td>
<td>14.4</td>
</tr>
<tr>
<td>4</td>
<td>Basal diet</td>
<td>VAP*</td>
<td>Mean</td>
<td>26.5</td>
<td>100.4</td>
<td>114.8</td>
<td>150.2</td>
<td>157.7</td>
<td>167.4</td>
<td>224.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SD</td>
<td>3.0</td>
<td>12.4</td>
<td>11.4</td>
<td>10.2</td>
<td>10.7</td>
<td>10.3</td>
<td>11.7</td>
</tr>
</tbody>
</table>

Notes:

a  VAA (2064 µgRE/kg of diet)

b  BC (6 mM/kg of diet)

c  VAP administered twice weekly in the drinking water. At each change of drinking water, each VAP was prepared to give an initial concentration of 0.1875 µgRE/ml
Table 7.6

Trial VIII: Group mean body weights of the vehicle-dosed rats (Groups 5-8)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Dietary supplement from week 7</th>
<th>Time (week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<tr>
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</tr>
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<td>Mean</td>
</tr>
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<td>VAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mean</td>
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<tr>
<td></td>
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<td>SD</td>
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</tbody>
</table>

Notes:

a. VAA (2064 μgRE/kg of diet)

b. BC (6 mM/kg of diet)

c. VAP administered twice weekly in the drinking water. At each change of drinking water, each VAP was prepared to give an initial concentration of 0.1875 μgRE/ml
Table 7.7

Trial VIII: Results for plasma retinol analyses in Groups 3 and 4 throughout the experiment

<table>
<thead>
<tr>
<th></th>
<th>Week 0*</th>
<th>Week 7*</th>
<th>Week 15*</th>
<th>Week 44*</th>
<th>Week 49*</th>
<th>Week 54-57*</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>X</td>
<td>sd</td>
<td>n</td>
<td>X</td>
<td>sd</td>
</tr>
<tr>
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<td>46.7</td>
<td>6.21</td>
<td>5</td>
<td>3.81</td>
<td>3.90</td>
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<tr>
<td>Group 4</td>
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<td>50.34</td>
<td>6.96</td>
<td>5</td>
<td>3.63</td>
<td>3.39</td>
</tr>
</tbody>
</table>

Notes:

a Results expressed as μg/dl of plasma

b Plasma samples from rats killed at week 0 after acclimatisation on normal maintenance diet and immediately prior to the administration of the vitamin

A-deficient basal diet

c Plasma samples from rats killed at week 7 immediately prior to the administration of 3 mM/kg dietary BC to Group 3 and supplemented VAP

(0.1875 μgRE/ml) in the drinking water to Group 4

d Plasma samples from rats killed at week 15 immediately prior to the start of carcinogen dosing

e Plasma samples from interim kills (Group 4 only), 25 weeks after the end of the carcinogen dosing

f Plasma samples from interim kills (Group 4 only), 30 weeks after the end of the carcinogen dosing

g Plasma samples from the terminal kill, 35 - 38 weeks after the end of carcinogen dosing
Table 7.8

Trial VIII: Results for plasma α-tocopherol analyses* in Groups 3 and 4 throughout the experiment

<table>
<thead>
<tr>
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<th>Week 0*</th>
<th>Week 7*</th>
<th>Week 15*</th>
<th>Week 44*</th>
<th>Week 49*</th>
<th>Week 54-57*</th>
</tr>
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<tbody>
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<td>sd</td>
<td>n</td>
<td>x</td>
<td>sd</td>
</tr>
<tr>
<td>Group 3</td>
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<tr>
<td>Group 4</td>
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<td>0.07</td>
<td>5</td>
<td>0.34</td>
<td>0.11</td>
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</table>

Notes:

a Results expressed as mg/dl of plasma
b Plasma samples from rats killed at week 0 after acclimatisation on normal maintenance diet and immediately prior to the administration of the vitamin A-deficient basal diet
c Plasma samples taken from rats killed at week 7 immediately prior to the administration of 3 mM/kg BC to Group 3 and supplemented V A P (0.1875 μgRE/ml) in the drinking water to Group 4
d Plasma samples from rats killed at week 15 immediately prior to the start of carcinogen dosing
e Plasma samples from interim kills (Group 4 only), 25 weeks after the end of the carcinogen dosing
f Plasma samples from interim kills (Group 4 only), 30 weeks after the end of the carcinogen dosing
g Plasma samples from the terminal kill, 35 - 38 weeks after the end of carcinogen dosing
Table 7.9

Trial VIII: Results for plasma BC analyses in Groups 3 and 4 throughout the experiment

<table>
<thead>
<tr>
<th></th>
<th>Week 0&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Week 7&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Week 15&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Week 44&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Week 49&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Week 54-57&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
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<td>x</td>
<td>sd</td>
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<td></td>
<td></td>
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<td>6</td>
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<td>0.00</td>
<td>5</td>
<td>0.00</td>
<td>0.00</td>
<td>4</td>
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<tr>
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<td></td>
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<tr>
<td>6</td>
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<td>0.00</td>
<td>5</td>
<td>0.00</td>
<td>0.00</td>
<td>4</td>
</tr>
</tbody>
</table>

Notes:

a Results expressed as µg/dl of plasma
b Plasma samples from rats killed at week 0 after acclimatisation on normal maintenance diet and immediately prior to the administration of the vitamin A-deficient basal diet
c Plasma samples taken from rats killed at week 7 immediately prior to the administration of 3mM/kg dietary BC to Group 3 and supplemented VAP (0.1875 µgRE/ml) in the drinking water to Group 4
d Plasma samples from rats killed at week 15 immediately prior to the start of carcinogen dosing
e Plasma samples from interim kills (Group 4 only), 25 weeks after the end of the carcinogen dosing
f Plasma samples from interim kills (Group 4 only), 30 weeks after the end of the carcinogen dosing
g Plasma samples from the terminal kill, 35 - 38 weeks after the end of carcinogen dosing
Table 7.10

Trial VIII: Results of analyses for retinol*, BC^b and α-tocopherol^c at the terminal post-mortems

<table>
<thead>
<tr>
<th>Group</th>
<th>Retinol</th>
<th>BC</th>
<th>α-tocopherol</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>x</td>
<td>sd</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
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<td>2</td>
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</tr>
<tr>
<td>4</td>
<td>33</td>
<td>24.78</td>
<td>2.61</td>
</tr>
<tr>
<td>5</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.03</td>
<td>3.12</td>
</tr>
<tr>
<td>6</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.17</td>
<td>2.94</td>
</tr>
<tr>
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</tr>
<tr>
<td>8</td>
<td>31</td>
<td>24.66</td>
<td>3.75</td>
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</table>

Notes:

a Results expressed as μg/dl of plasma
b Results expressed as μg/dl of plasma
c Results expressed as mg/dl of plasma
d The number of samples in this group was reduced due to the omission of a dubious value
Figure 7.4  Mean (± sd) relative urinary bladder weights (g/1000g rat body weight) of bladders from all groups at the terminal kill, 38 weeks after the final dose of BBN in Trial VIII: Group 1 (open circle), Group 5 (closed circle), Group 2 (open square), Group 6 (closed square), Group 3 (open triangle), Group 7 (closed triangle), Group 4 (open diamond), Group 8 (closed diamond). No statistically significant differences were detected (p > 0.05, ANOVA).

Figure 7.5  Mean (± SD) tumour volumes (mm³) of urinary bladders from the carcinogen-dosed groups at the terminal kill, 38 weeks after the final dose of BBN in Trial VIII: Group 1 (open circle), Group 2 (closed circle), Group 3 (open square), Group 4 (closed square). In addition, the mean (± sd) tumour volume is shown (2*) of the bladders in Group 2 without 1 outlier, an animal with a very large tumour. No statistically significant differences were detected (p > 0.05, ANOVA).

Figure 7.6  The percentage distribution of total tumour volumes for each bladder in each BBN-dosed group, based on log_{10} subdivisions of the tumour volumes in each BBN group. The tumour volumes of all bladders in Groups 1-4 were subdivided according to whether they fell within the log_{10} ranges (mm³): 0-0.1; 0.1-1.0; 1.0-10; 10-100; 100-1000. The percentage incidence of tumour volumes in each log_{10} range for each dietary group are shown in the form of histogram plots as follows: Figure 7.6A, Group 1; Figure 7.6B, Group 2; Figure 7.6C, Group 3; Figure 7.6D, Group 4.
Relative bladder weight (g/1000g body weight)

Figure 7.4
Figure 7.5
### Table 7.11

**Trial VIII:** Summary of the histological findings in the urinary bladder at the terminal post-mortems

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Dietary supplement from week 7</th>
<th>Number of rats</th>
<th>Histology of urothelium (number of rats (%))</th>
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<tr>
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<td>Normal</td>
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<tr>
<td>1</td>
<td>Basal diet + VAA*</td>
<td>BC(^b)</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Basal diet + VAA*</td>
<td>-</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Basal diet</td>
<td>BC(^b)</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Basal diet</td>
<td>VAP(^c)</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Basal diet + VAA*</td>
<td>BC(^b)</td>
<td>28</td>
<td>28</td>
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<td>6</td>
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<td>-</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>7</td>
<td>Basal diet</td>
<td>BC(^b)</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>8</td>
<td>Basal diet</td>
<td>VAP(^c)</td>
<td>32</td>
<td>31</td>
</tr>
</tbody>
</table>

**Notes:**

- VAA (2064 µgRE/kg)
- BC (6mM/kg of diet)
- VAP administered twice weekly in the drinking water. At each change of drinking water, fresh VAP was prepared to give an initial concentration of 1.875 µgRE/ml.
- A calculus was present in this bladder. The entire urothelium demonstrated massive diffuse papillary hyperplasia (papillomatosis)
Figures 7.7 - 7.8

Typical macroscopic appearance of the urinary bladder from rats treated with vehicle (Figure 7.7) or BBN (Figure 7.8) in Trial VIII. The bladders are bisected longitudinally and oriented with the anterior end (dome) at the upper edge of the photographs. The magnification is approximately 3 times the size of a fully inflated bladder.

Figure 7.7 Macroscopic appearance of the urinary bladder from a vehicle-treated rat. The luminal surface of the bladder is smooth and there are no visible lesions.

Figure 7.8 Macroscopic appearance of the urinary bladder from a BBN-treated rat. In the right half of the bladder, 3 moderately large exophytic lesions are extending into the bladder lumen.
Figures 7.9 - 7.47

Representative histology of rats treated with BBN or BBN-vehicle in Trial VIII. All the bladder tumours and other proliferative urothelial lesions shown are from different animals. The full range of bladder lesions from simple flat hyperplasia to TCC P1b was observed in all 4 BBN-treated groups.

All photomicrographs are of sections stained with haematoxylin and eosin (H&E)
Urine bladder carcinomas were TCC. A few TCC demonstrated invasion into the lamina propria of the bladder wall (Figures 7.9 - 7.12). However, most malignant tumours were papillary or papillary/nodular lesions with invasion confined to the stromal core of the stalk (Figures 7.13 - 7.14).

Figure 7.9 A papillary/nodular TCC showing invasion of malignant transitional cells into the lamina propria of the bladder wall (Plb). The invasive cells are growing in cords (arrow) extending almost to the superficial muscle.

Figure 7.10 Higher magnification of the area indicated by the arrow in Figure 7.9. The cords of invasive cells are showing a tendency towards a cystic growth pattern.

Figure 7.11 A large papillary TCC Plb with extensive invasion of the lamina propria by malignant cells growing in a cystic pattern (arrow).

Figure 7.12 Higher magnification of the invasive cells indicated by the arrow in Figure 7.11.

Figure 7.13 A papillary/nodular TCC showing invasion by malignant cells of the stromal core of the stalk (arrow).

Figure 7.14 Higher magnification of the invasive cells indicated in the arrow in Figure 7.13. The malignant cells (arrow) are growing in a slightly cystic pattern and appear paler than the more darkly stained cells of the epithelium adjacent to the lesion.
Further examples of papillary or papillary/nodular TCC with invasion confined to the stromal core of the lesion (Figures 7.15 - 7.17) and also an example of a TCC Pla showing a solid growth pattern (Figure 7.18).

Figure 7.15 A large papillary TCC Pla showing extensive invasion (arrow) of the stromal core.

Figure 7.16 Higher magnification of the area indicated by the arrow in Figure 7.15. The invasive cells are poorly differentiated and are growing in many small clusters or cords (arrows) disseminated throughout the stromal core.

Figure 7.17 A small, slightly raised papillary/nodular TCC Pla. The arrow indicates an area of invasive cells. These are stained differently to the surrounding tissue and are growing in a slightly cystic pattern.

Figure 7.18 A small TCC showing a solid growth pattern. The arrow indicates the area shown subsequently at higher magnification in Figures 7.19 - 7.20.
Figure 7.17

A T.C.E. Plot showing a specific pattern is indicated by the large arrow. The plot appears greatly distorted due to anaphylactic shock and necrotic tissue. Very little expected tissue could be observed at the base of the lesion.

Figure 7.18
Figures 7.19 - 7.22

Further examples of TCC P1a showing a solid growth pattern.

**Figure 7.19**  Higher magnification of the area indicated by the arrow in Figure 7.18. The malignant cells are slightly dysplastic; they are larger and stained more palely than normal urothelial cells.

**Figure 7.20**  This figure is a higher magnification of the area shown in Figure 7.19. Malignant cells appear to be growing in small, tight clusters (arrow), some of which are adjacent to the walls of 2 blood vessels (V).

**Figure 7.21**  A TCC P1a showing a solid growth pattern is indicated by the large arrow. The cells appear palely stained (due to eosinophilic staining) and are dysplastic. Very little stromal tissue (small arrows) remains at the base of the lesion.

**Figure 7.22**  An inverted papillary TCC P1a with a solid growth pattern. An area of invasion is shown by the arrow.
Figures 7.23 - 7.25

Examples of lesions showing squamous metaplasia (Figures 7.23 - 7.24) or a glandular/cystic growth pattern (Figure 7.25).

Figure 7.23 Papillary hyperplasia (II) showing an area of squamous metaplasia with keratin formation. The squamous cells (arrow) are larger and palely stained in comparison to the surrounding transitional cells. [This lesion, and that shown in Figure 7.24, would be classified as a benign tumour (papilloma) by some classification systems, but was included with the hyperplasias in Trial VIII to distinguish it from malignant tumours].

Figure 7.24 Papillary hyperplasia (II) showing areas of early squamous metaplasia (arrow) without keratin formation.

Figure 7.25 A large TCC Pla with an extensive area showing a glandular/cystic growth pattern (arrow).
Figure 7.25
Examples of simple papillary hyperplasia (Pap. Hyp. I) (Figures 7.26 - 7.27), nodular hyperplasia (Figure 7.28) and simple hyperplasia (Figure 7.29).

**Figure 7.26** Simple papillary hyperplasia. The hyperplastic urothelium (about 10 cells thick) has formed a single leaf-like fold extending into the lumen of the bladder. A portion of lamina propria has been drawn into the fold forming the stromal core of the lesion. The hyperplastic cells are well differentiated and have a similar appearance to the normal epithelium adjacent to the lesion.

**Figure 7.27** Higher magnification of the lesion shown in Figure 7.26.

**Figure 7.28** Nodular hyperplasia. The lesion is tending to grow downwards into the bladder wall, rather than extending into the lumen.

**Figure 7.29** Simple hyperplasia. This lesion is classified as mild simple hyperplasia, as there are 4-6 cell layers rather than the usual 3 found in normal urothelium. Similar lesions with 6-8 cell layers would be classified as moderate simple hyperplasia, while those with 8-10+ cells layers would be marked or severe simple hyperplasia.
Figure 7.28

Figure 7.29

The histology appears similar to that in Figures 7.30 and 7.31. The bladder was probably more fully effaced with fixation. The upper layers were more evident in the superficial cells on the basement membrane. This is the upper limit of these cells in comparison to the underlying cells.

in this bladder at post-mortem, and irritation of the urothelium by this is the likely cause of the extensive proliferative reaction.
Figures 7.30-7.34

Representative sections of normal bladder from each of the 4 vehicle-treated groups at the terminal kill (Figures 7.30-7.33). Also presented is a section from the single vehicle-treated animal that did not have a normal bladder at the terminal kill (Figure 7.34).

Figure 7.30 Normal appearance of the urothelium in a rat from Group 5 fed a diet containing an adequate level of vitamin A (2064 µg/kg VAA) plus 6 mM/kg BC from week 7. The urothelium consists of 3 layers of normal cells.

Figure 7.31 Normal appearance of the urothelium in a rat from Group 6 fed a diet containing an adequate level of vitamin A (2064 µg/kg VAA). The urothelium consists of 3 layers of normal cells. The urothelium appears thinner than in Figure 7.30 because this bladder was probably more fully inflated with fixative. There are fewer nuclei in the superficial cells on the luminal surface, due to the larger size of these cells in comparison to the underlying cells.

Figure 7.32 Normal appearance of the urothelium in a rat from Group 7 fed a vitamin A-deficient diet until week 7 and thereafter with the same diet supplemented with 6 mM/kg BC. The urothelium has a similar appearance to that shown in Figure 7.31.

Figure 7.33 Normal appearance of the urothelium in a rat from Group 8 fed a vitamin A-deficient diet until week 7 and thereafter supplemented with low-level VAP (3.0 µg/rat/day) in the drinking water. The urothelium is normal.

Figure 7.34 Extensive papillary hyperplasia (papillomatosis) in the bladder of a rat from Group 8 fed a vitamin A-deficient diet until week 7 and thereafter supplemented with low-level VAP (3.0 µg/rat/day) in the drinking water. A calculus was found in this bladder at post-mortem, and irritation of the urothelium by this is the likely cause of the extensive proliferative reaction.
Figure 7.30

Figure 7.31
Figure 7.34

The normal appearance of the kidney in a rat from Group 1 killed at the end of Trial VIII (week 12). The normal variants of epithelial and stromal cells are present in the renal epithelium (Detors).

Figure 7.35

The normal appearance of the kidney in a rat from Group 4 killed at the end of Trial VIII (week 12). The renal tissue is free from the accumulation of crystalline deposits associated with nephrolithiasis and from the degenerative changes of nephropathy.
Figures 7.35 - 7.37

Histology of the bladder (Figure 7.35), trachea (Figure 7.36) and kidney (Figure 7.37) in rats from Groups 3 and 4 at week 0.

Figure 7.35  The normal appearance of the urothelium in a rat from Group 4 killed at the start of Trial VIII (week 0).

Figure 7.36  The normal appearance of the trachea in a rat from Group 4 killed at the start of Trial VIII (week 0). The normal variety of ciliated and secretory cells are present in the tracheal epithelium (arrow).

Figure 7.37  The normal appearance of the kidney in a rat from Group 4 killed at the start of Trial VIII (week 0). The renal tissue is free from the accumulation of crystalline deposits associated with nephrocalcinosis and from the degenerative changes of nephropathy.
Figure 7.37

The kidney from a rat killed at the top and half of Test VII demonstrating proteinuria (C) and tubular degeneration indicative of nephrotoxicity (below).
Figures 7.38-7.39

The typical histological appearance of rat kidney at the terminal kill.

Figure 7.38  The kidney from a rat killed at the terminal kill of Trial VIII showing dark areas of crystalline deposits (nephrocalcinosis). Positive staining with von Kossa stain (not shown) demonstrated that these deposits contained calcium.

Figure 7.39  The kidney from a rat killed at the terminal kill of Trial VIII demonstrating protein casts (C) and tubular degeneration indicative of nephropathy (arrow).
Figure 7.38

Figure 7.39
Figures 7.40-7.43

The bladder (Figure 7.40) and trachea (Figure 7.41) from rats in Group 4 killed at week 7, the bladder from a rat killed at week 15 (Figure 7.42), and at the interim kill 30 weeks after the last dose of BBN (Figure 7.43).

Figure 7.40  The appearance of the bladder in a rat from Group 4 killed at week 7 when the animals in this group were vitamin A-deficient. The urothelium is normal.

Figure 7.41  The appearance of the trachea in a rat killed at week 7 when the animals in this group were vitamin A-deficient. A small focus of squamous metaplasia (arrow) is present in the tracheal epithelium. The squamous cells have replaced the normal epithelial cells of the tracheal epithelium (Figure 7.36).

Figure 7.42  Urothelium of a rat killed at week 15, the time of the first dose of BBN. The urothelium appears normal.

Figure 7.43  Papillary hyperplasia (II) in a rat from Group 4 killed 30 weeks after the last dose of carcinogen. Multiple stromal stalks (arrows) are present in this lesion indicating that it is not a simple hyperplasia. No carcinomas were present in the 5 animals of this interim kill.
Figure 7.40

Figure 7.41
Figure 7.42

Figure 7.43
Figures 7.44-7.47
Incidental neoplastic lesions found in animals from Trial VIII.

Figure 7.44  Blood smear from a rat with monocytic leukaemia, a common congenital disorder of F344 rats. This leukaemia is characterised by large malignant monocytic cells (arrows).

Figure 7.45  Liver from the same leukaemic rat represented in Figure 7.44. Many leukaemic cells can be seen in the hepatic sinusoids (arrow).

Figure 7.46  Malignant schwanoma found adjacent to the spine

Figure 7.47  Adenocarcinoma of the mammary gland found on the neck.
Figure 7.46

Figure 7.47
APPENDIX 1

COMPOSITION OF DIETS

a) VITAMIN A-DEFICIENT DIETS

(i) Low Vitamin A Diet (LVAD). Commercially prepared from natural products and supplied by SDS Ltd, Witham, Essex.

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<th>Ingredient</th>
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<td>Wheat Bulk</td>
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<tr>
<td>Comflower Snowflake</td>
<td>25%</td>
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<td>Danpro A</td>
<td>10%</td>
</tr>
<tr>
<td>Oat Groats</td>
<td>15%</td>
</tr>
<tr>
<td>Standard Whey</td>
<td>2.5%</td>
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<tr>
<td>Unextracted Yeast</td>
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</tr>
<tr>
<td>Vitamin and Mineral Premix</td>
<td>5%</td>
</tr>
</tbody>
</table>

Vitamin A analysis (performed by supplier): 38 µgRE/kg vitamin A

Calorific value (metabolisable energy): 11.09 MJ/kg

Notes

1 Whole Oats consist of about 60% carbohydrate, 11% protein, 4.5% fat, 10% fibre, 5% ash, and about 10% moisture.
2 Wheat Bulk consists of 70% carbohydrate, 11% protein, 2% fat, 3% fibre, 2% ash, and 12% moisture.
3 Comflower Snowflake is pure isolated corn (maize) starch consisting of 100% carbohydrate.
4 Danpro A is a low fat/high protein isolate extracted from Soya protein.
5 Oat Groats consist of oats with the outer husk removed, but with the endosperm intact.
6 Standard Whey is skimmed milk consisting of 12% protein, 69% carbohydrate (all sugars), 7-9% fat, and 10-12% ash.
7 This diet was calculated to contain 60-120 µgRE/kg, but when analysed it was found to contain 38 µgRE/kg. Calculated vitamin A values can vary by ± 100%.

Protein - Casein (Vitamin and Fat-Free) 20%
Carbohydrate - Cornflower Snowflake 70%
Fat - Safflower Oil 5%
Vitamin and Mineral Premix (Vitamin A-Free) 5%

Vitamin A analysis (performed by the supplier): less than the threshold limit of detection (< 15 µgRE vitamin A).
Calorific value (metabolisable energy): 13.38 MJ kg.

(iii) Semi-Synthetic Vitamin A-Free Diet (Brompton Diet). A laboratory-prepared diet designed by Dr. A. Wise and used by Dr. P. Jeffrey and Dr. P. Shields at the Brompton Hospital, London (Shields and Jeffrey 1987).

Protein - Casein (Vitamin and Fat Free) 19.8%
Carbohydrate - Rice Starch 69.8%
Fat - Safflower Oil 5%
Vitamin and Mineral Premix (Vitamin A-Free) 5.4%

Vitamin A analysis: not done, but designed to be virtually free of trace amounts of vitamin A.


Protein - Casein (Vitamin and Fat Free) 19.8%
Carbohydrate - Rice Starch 69.8%
Fat - Safflower Oil 5%
Vitamin and Mineral Premix (Vitamin A-Free) 5.4%

Estimation of vitamin A content (performed by the supplier): less than the threshold limit of detection (< 15 µgRE vitamin A).
Calorific value (metabolisable energy): not known.
MAINTENANCE DIET

Rat and Mouse Modified Expanded Maintenance Diet No. 1. (Manufactured and supplied by SDS Ltd, Witham, Essex.)

This diet is a standard maintenance diet, used in ground or pelleted form, with an estimated vitamin A content of 1500 µgRE/kg, mainly in the form of supplemental VAP. However, calculated dietary vitamin A values can be up to 50% inaccurate. Analyses of 11 previously used batches of this diet demonstrated a wide variability in the amount of vitamin A present, with values ranging from about 900 µgRE/kg to 1890 µgRE/kg and a mean value of 1216.7 µgRE/kg. Based on the mean vitamin A content, and assuming that an adult healthy rat consumes about 15 g of diet per day, this diet provided each animal with about 18.25 µgRE/day. Calorific value (metabolisable energy): 10.3 MJ/kg.
APPENDIX 2

METHOD FOR ESTIMATING VAP INTAKE

VAP was routinely administered to vitamin A-deficient rats twice weekly in the drinking water. Freshly prepared VAP was made up to a concentration of 0.1875 μgRE/ml each time the drinking water was replenished every 3 or 4 days. Assuming a consumption of 16 ml/rat/day, this concentration of VAP would provide a total of 3 μgRE/rat/day.

VAP in dilute solution was found to be unstable. In Trial V(a) this instability was quantitated over a 72 hour period for VAP at a starting concentration of 0.3 μgRE/ml. Aliquots of this VAP solution were assayed for vitamin A by UV spectrophotometry. The results of this investigation allowed a degradation curve to be constructed (Figure 6.15, dilution 2). From this plot, the amount of undegraded vitamin A in the solution was determined/estimated at intervals of 12 hours up to a total of 96 hours: 12h, 60.7%; 24h, 67.9%; 36h, 50%; 48h, 57.1%; 60h, 48%; 72h, 39.3%; 84h, 30%; 96h, 25%.

VAP intake over each 3 or 4 day period was estimated from the absolute water consumption (g water consumed /rat/day) for that period, and the values derived from the VAP degradation curve (Figure 6.15, dilution 2) for the percentage of vitamin A remaining midway through each day of the consumption period (i.e. for a 4 day consumption, 12h, 36h, 60h, 84h).

For example, assuming a mean consumption of 16 ml /rat/day over a 4 day period, the mean VAP intake is estimated as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Calculation</th>
<th>VAP Intake (μgRE/rat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>$16 \times 0.1875 \times \frac{61}{100}$</td>
<td>1.82 μgRE/rat</td>
</tr>
<tr>
<td>Day 2</td>
<td>$16 \times 0.1875 \times \frac{50}{100}$</td>
<td>1.50 μgRE/rat</td>
</tr>
<tr>
<td>Day 3</td>
<td>$16 \times 0.1875 \times \frac{48}{100}$</td>
<td>1.44 μgRE/rat</td>
</tr>
<tr>
<td>Day 4</td>
<td>$16 \times 0.1875 \times \frac{30}{100}$</td>
<td>0.90 μgRE/rat</td>
</tr>
</tbody>
</table>

The total VAP intake for the 4 day period = 1.82 + 1.50 + 1.44 + 0.90 = 5.66 μgRE/rat
APPENDIX 3

SAFETY AND DECONTAMINATION PROTOCOLS FOR CARCINOGEN DOSING PROCEDURES

The preparation and administration of BBN, and the subsequent disposal of waste materials and decontamination of reusable equipment, were conducted under safety protocols authorised by the site Carcinogen Safety Officer. BBN administration was carried out in a room specifically designated as a carcinogen handling room. Personnel were required to wear disposable protective clothing including gowns, plastic aprons, overshoes and 2 pairs of plastic gloves, the gloves next to the skin being externally dusted with talcum powder. As an extra precaution, face masks and visors were also worn. Animals were anaesthetised and dosed in a Class 1 fume cupboard, which had been lined previously with a single layer of 'Benchcote' absorbent covering paper, 2 layers of paper tissue and 2 layers of aluminium foil. Any items of reusable equipment in the fume cupboard, such as the perspex anaesthesia box, and a plastic tray for holding fresh syringes etc., were also covered in 2 layers of foil. When dosing was completed, this lining material and all other waste (unused BBN, used syringes, gowns, overshoes, masks and gloves, disposable cage bottoms with animal excreta) was removed into double plastic bags marked with carcinogen hazard tape and incinerated. The gavage needle and gag were decontaminated by immersion in a molar solution of sodium hydroxide. After removal of any covering foil, reusable equipment including the anaesthesia box, stainless steel cage tops, decontaminated gavage needle and gag, food containers and water bottles with their stoppers were thoroughly washed and rinsed in an industrial washing machine.