IDENTIFICATION OF A PROGNOSTIC MARKER IN BREAST CANCER

BY A LECTIN FROM

HELIX POMATIA (THE ROMAN SNAIL).

by

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Helix pomatia - the Roman snail.
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IDENTIFICATION OF A PROGNOSTIC MARKER IN BREAST CANCER
BY A LECTIN FROM HELIX POMATIA
(THE ROMAN SNAIL).

The aim of this study was to investigate the potential value of *Helix pomatia* lectin binding to tissue sections as an indicator of patient prognosis in breast cancer.

Formalin-fixed, paraffin-embedded sections of a retrospective series of 373 primary breast cancers diagnosed at the Middlesex Hospital between January 1967 and June 1972 were stained by a simple immunoperoxidase technique for the binding of a lectin from the Roman snail, *Helix pomatia*.

Log-rank life table analyses revealed a highly significant correlation between *Helix pomatia* lectin binding to primary breast cancers and the clinical course of the disease (in terms of disease free survival and total survival time) over a 15-20 year follow-up period (p<0.00001).

*Helix pomatia* lectin binding was compared with other, established, prognostic markers for which we had data, including lymph node status, histological tumour grade, tumour size, patient age at diagnosis, and S-phase fraction (calculated by the technique of flow cytometry). Multivariate analysis was performed to assess the relationships of these variables.

The distribution of *Helix pomatia* lectin binding substances in crude homogenates of primary breast cancer, breast cancer metastases to lymph node, liver, lung and bone, and a range of other, normal, human tissues was investigated by the technique of S.D.S.-P.A.G.E., in conjunction with Western-style lectin electroblotting.

Attempts were made to isolate lectin-binding material from these sources by anion exchange chromatography and lectin affinity chromatography. The purity of isolated substances was analysed by a combination of S.D.S.-P.A.G.E. / Western blotting, and high performance liquid chromatography (H.P.L.C.).
The aims of this study were:

(1) To investigate the value of *Helix pomatia* lectin binding as a potential marker of prognosis in breast cancer, and to compare it with other, established, prognostic markers.

(2) To characterise the material recognised by the lectin in breast cancers, their metastases, and other human tissues.

(3) To isolate the *Helix pomatia* lectin binding material.
Identification of all breast cancers diagnosed at Middlesex Hospital 1967-1972

Collection of formalin-fixed paraffin-embedded tissues

Sections cut

Identification of breast cancer metastases

Sections stained for binding of Helix pomatia lectin

Determination of staining methodology

Sections stained

Staining evaluated

Computer storage of data

Correlation of staining with clinical follow-up/statistical analysis

Collection of fresh tissues

Isolation and characterisation of breast cancer metastasis-associated Helix-binding material

H.P.L.C.

Lectin affinity chromatography

S.D.S.-P.A.G.E. and Western style electrophoresis

Dot-blotting

Ion exchange chromatography
(1) Establishment of a patient library

The first stage in the investigation was to establish a retrospective breast cancer patient "library". 500 consecutive cases of histologically confirmed primary carcinoma of the breast were selected from the histology reports of the Middlesex Hospital of January 1967-June 1972.

The clinical progress of these patients was plotted from the date of diagnosis to January 1987 (total follow-up period 15-20 years from diagnosis).

Blocks of the original tumour, fixed in formalin and embedded in paraffin-wax, were retrieved from the hospital archives. 5μm sections were cut from the blocks for subsequent evaluation of Helix pomatia lectin binding.

(2) Determination of optimal methodology for evaluation of Helix pomatia lectin binding

Helix pomatia lectin binding to formalin-fixed, paraffin-embedded sections of breast cancer can be demonstrated by a number of histochemical techniques. The methodology for each of 6 different techniques was adjusted to give optimal staining results in terms of maximum specific lectin binding coupled with minimum non-specific background on a small selection of cases pre-selected on the basis that they fell into a particularly "good-" or "bad- prognosis" group.

(3) Helix pomatia lectin binding to the breast cancer library

The method which gave the greatest distinction in Helix pomatia lectin binding between the "good-" and "bad-prognosis" cases was then applied to 373 evaluable cases from the larger series of breast cancers in the retrospective patient library.

(4) Correlation of Helix pomatia lectin binding to patient prognosis

The presence or absence of Helix pomatia lectin binding to the primary breast cancers of the library was correlated with prognosis in terms of interval to first recurrence and overall survival time.

The efficacy of Helix pomatia lectin binding as a potential prognostic marker in breast cancer
The relationship of *Helix pomatia* lectin binding to other prognostic factors was investigated by means of a Cox proportional hazards regression analysis.

(5) *Helix pomatia* lectin binding to metastases arising from primary breast cancer

The binding of *Helix pomatia* lectin binding to formalin-fixed, paraffin-embedded sections of 97 metastases arising from 46 primary breast cancers was investigated in order to determine whether expression of *Helix pomatia* lectin binding material persists in metastatic deposits.

(6) Characterisation of *Helix pomatia* lectin binding material

*Helix* lectin binding material present in fresh primary breast cancers and their metastases was separated and characterised by the technique of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (S.D.S.-P.A.G.E.) coupled with Western style electroblotting.

*Helix* lectin-binding bands were sought which might be unique to breast cancers and their metastases, and not found in normal control tissues.

(7) Isolation of *Helix* lectin binding material

*Helix* lectin binding material was isolated from homogenates of fresh primary breast cancers and their metastases, as well as samples of normal sera and normal control tissues.

The purity of isolated H.B.M. was estimated by S.D.S.-P.A.G.E and Western- style electroblotting, and by high performance liquid chromatography.
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1. INTRODUCTION

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A BRIEF HISTORY OF BREAST CANCER: IDEAS ON ITS SPREAD, AND DISCUSSION OF ITS TREATMENT.

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1.1 A BRIEF HISTORY OF BREAST CANCER, IDEAS ON ITS SPREAD, AND DISCUSSION OF ITS TREATMENT

"The story is not one of ordinary progression, but was fraught with retrogressions that are prominent even today. The horror of sepsis, the need for anaesthesia, and the wide acceptance of the incurability of cancer were prominent in delaying the development of surgery of the breast." (Lewison 1953).

1.1.1. EGYPTIAN TIMES

Breast cancer has been recognised as a disease since the times of the ancient Egyptians. The earliest known scientific writing is the "Edwin Smith Surgical Papyrus", which dates to around 3000 - 2500 B.C. (Figure 1) One of the cases described is believed to be a breast cancer. Although cauterization was employed at this time in the treatment of benign abscesses of the breast, it concludes that for breast cancer......"There is no treatment." (Translated by J.H. Breasted 1930).

Hippocrates, born around 1200 B.C., was certainly well acquainted with cancer, but in all of his very extensive writings he deals with the condition but briefly and, indeed, appears reserved regarding the advisability of any medical intervention:

"It is better to give no treatment in cases of hidden cancer; treatment causes speedy death but to omit treatment prolongs life". [Translated by W.H.S. Jones (undated)].

Hippocrates associated the origin of the tumour with the cessation of menstruation: Suppression of the menstrual discharge would lead to the engorgement of the breast and the appearance of nodules which became increasingly indurated and ultimately degenerate into "hidden" (thought to mean not yet penetrating the skin) cancers.

He implies in his writings that only in the case of an ulcerating tumour should surgery be attempted, and then perhaps as a palliative, rather than as a curative measure. In
Hippocratic times it was by no means considered unethical to simply send patients away for whom medicine had nothing to offer, and indeed, the discovery of votive offerings representing breasts excavated at sites where shrines of the Greek god of healing, Asklepios, had once stood suggest that breast diseases were amongst the ailments for which supernatural help was often sought. [Taken from the writings of J.B. Kuhn (undated)].

1.1.3. AULUS CORNELIUS CELSUS (30 B.C. - 38 A.D.)

Celsus, a Roman author, wrote an excellent survey of the contempory medicine of the day - "Of a cancer" he wrote - "There is not so great a danger of a cancer, unless it be irritated by the imprudence of the physician..."

He went on to describe what may be considered as the first clinical classification of the disease:

"Its general progress is this; first appears what the Greeks call a cacoethes, then it becomes a carcinoma without an ulcer, from that an ulcer, and from an ulcer a thymium" (A "thymium" is a growth that resembles thyme flowers and bleeds readily)

He rejected the advisability of any treatment for the latter three stages of the disease, be it by the application of caustic medicines, cautery, or actual surgery, and readily admitted the difficulty in distinguishing the curable "cacoethes" from the incurable later stages of carcinoma (Translations by W.G. Spencer 1953)

1.1.4. LEONIDES

Leonides, a surgeon of the first century A.D., operated upon patients as follows-

"I make the patient lie on her back. Then I make an incision in the sound part of the breast above the cancer and I apply cauteries until an eschar is produced that stops the bleeding. I then make another incision and cut into the deep of the breast and again sear the severed parts. This I repeat often, alternately cutting and burning in order to arrest the bleeding. For in this way the danger of haemorrhage is avoided. When the amputation is completed, I burn once again all parts until they are dry. The first cauterizations are made for the purpose of arresting the haemorrhage. The rest, however, with the intention of eradicating all remnants of the disease."

Although most doctors of the time had a fairly nihilistic attitude toward cancer, Leonides actually performed courageous surgery distinguishing between operable and inoperable cases. He was the first to describe inversion of the nipple as a clinical indication of malignancy.
Leonides likened the firm adherence of the tumour to underlying structures with the tenacity of the claws of the crab. [Translation by F. Adams (undated)].

1.1.5. GALEN (131 - 203 A.D.)

Galen, a remarkable scholar, left a vast and comprehensive literary legacy of nearly one hundred books dealing with every aspect of contemporary medicine - an ambitious effort to summarise the entire medical knowledge of the time, the authority of which stood unquestioned well into the sixteenth century.

Galenic pathology was based upon the concept of "humoralism" which originated in the ideas of Hippocrates, and persisted unchallenged for centuries. Good health, it was believed, depended upon the perfect equilibrium of four cardinal humors - blood, yellow bile, black bile, and phlegm.

Carcinoma arose from a congestion of black bile rendering the blood thick and dark, and its accumulation within the tissues gave rise to tumours. In younger women, menstruation represents a monthly purging of noxious substances from the entire system - but after menopause, black bile accumulates within the breasts and may give rise to cancers. Black bile of a "sharp" nature may lead to the formation of a tumour that ulcerates the skin.

Treatment of breast cancer could either be conservative - dispersal of the offending noxious black bile by diet, and by purging and bleeding the patient, plus the use of caustics - in the case of fairly early cancer, or operative.

In spite of the dangers of haemorrhage, blood vessels were not immediately sealed for fear of trapping evil black bile, which, according to humoralist doctrine, it was considered prudent to expel.

Galen too drew a likeness between the dilated veins (full of black bile) frequently seen surrounding a breast tumour and the shape of the crab after which the disease was named. [Taken from the writings of G.C. Kuhn (undated)].

1.1.6. THE MIDDLE AGES

The Middle Ages, with the prevailing illiteracy and the population scattered between isolated peasant communities, was a period of stagnation as far as scientific and medical progress was concerned. Few original contributions were made during these years and what medical knowledge existed was the province of the literate men of the church, where, in monastic libraries, such documents as remained from earlier times were read, copied, summarised, and preserved in anthologies. Primitive, popular medicine was practised by
monks, and what elementary surgery existed was performed not by the clergy, but by ignorant empirics.

In Roman times when scientific medicine proved inadequate, the sick turned to their healing god, Asklepios. Now in an age of Christianity, reports flourished of miraculous cures as people, in the absence of any earthly aid, turned to religion. Several Saints are associated with diseases of the breast, the best known of whom is Saint Agatha, whose martyrdom has been frequently depicted by artists.

1.1.7. THE LEGEND OF SAINT AGATHA

Agatha, true to her Christian ideals, resisted the amorous advances of the Roman governor Consul Quintanius. Outraged, he ordered that her breasts be destroyed - a number of colourful accounts of her ordeal exist in which her breasts are variously hacked, torn, or shorn off with hot iron shears, and a great many sixteenth and seventeenth century paintings exist depicting her martyrdom (figure 3). Agatha bore her suffering with equanimity, and later Saint Peter and Christ are said to have visited her in prison and restored the severed flesh. Quintanius then had her burnt naked upon a pyre fed by glowing coals, and she died on February 5th, which has since been celebrated as Saint Agatha’s day (Lewison 1950; Schechter and Swan 1962).

1.1.8. THE RENAISSANCE

From the middle of the fifteenth century, a cultural revolution took place in Europe heralding a new era in scientific and artistic thought.

Although Galenic humoralism still dominated medical thought, anatomy gained increasing popularity, and as a result of the wars of the sixteenth and seventeenth century, surgery became an increasingly sophisticated discipline.

Marcus Aurelius Severinus (1580 - 1656), for example, was one of the first surgeons to routinely remove enlarged axillary lymph nodes at the same time as performing radical surgery for breast cancer (Cooper 1941). Andreas Vesalius, Professor at Padua during the early Renaissance in Italy, practised wide local excisions and, unusually for his time, stemmed bleeding vessels by means of ligatures. He was one of the first surgeons to oppose Galen's doctrines, and his anatomy book - published in 1543 - marks the beginning of modern anatomy (Cooper 1941).

Bizarre remedies still flourished, even from eminent medical men such as Ambroise Pare (1510 - 1590), surgeon to royalty during the mid-sixteenth century, who recommended the application of a puppy or kitten sliced lengthwise whilst still living and applied to the affected area. When the body cooled, it was replaced by a fresh one (Battus 1952)! It is interesting that as recently as 1924, an Amsterdam quack was convicted of having applied Pare's remedy (Haddow 1936).

Around this time, much ingenuity went into the design of instruments and techniques which would most effectively facilitate a fast as well as thorough operation. Johann Schultes (1595 - 1645), for example, a German surgeon from Ulm, is best remembered for his "Method of Scultetus" (figure 4 - overleaf), published in 1655, in which cords or ligatures passed through the flesh of the breast allowed rapid amputation by allowing the organ to be pulled away from the chest wall during the operation (Scultetus 1665).

The writings of Gabrielle Falloppio (1523 - 1562) - one of the most eminent medical men of his age, who held the Chairs of anatomy, surgery and botany at the University of Padua - illustrate just how little medical theory had evolved since the time of Galen. He sites the origin of carcinoma as lying in the accumulation of sluggish black bile. Quiet tumours should be anointed with soothing balms, ulcerating lesions treated with surgery according to Leonides principles, and, if all else fails, "tunc rogandus est Deus, ut vita aegum privet" (then the Lord should be asked to take the patient's life) (Fallopio 1602).

Fortunately, during the seventeenth century, ideas began to evolve which would at last challenge the ancient Galenic traditions. Friedrich Hoffman (1660-1742), for example,
suggested that cancer would arise as a result of mechanical blockage of the lymphatics. Francois De La Boe Sylvius (1614-1672) was also much impressed by the newly discovered lymphatic system. Black bile was abandoned to be replaced by lymph (King 1970).

It is interesting that although swollen axillary lymph nodes are frequently cited in the literature of the time, post mortem findings generally omit any mention of visceral involvement. A great deal of ignorance remained regarding the disease process, with most authorities attributing death to some undefined "cancer poison". Breast cancer was also generally believed to be contagious with sufferers, for this reason, often barred from public hospitals (DeMoulin 1983).

In order to facilitate a swift as well as thorough operation, many surgeons simply swept the breast off the thoracic wall with a deft stroke of the razor, and "cure" was defined simply as successful healing of the resulting gaping wound. A number of detailed case histories remain from this period, but little mention is made of late results.

FIGURE 4: The "Method of Scultetus". Leather thongs were sutured through the breast and used for traction as the breast was swiftly severed. The gaping wound was then cauterized. (Reproduced from Ariel I.M. and Cleary J.B.(eds). 1986 Breast cancer: Diagnosis and treatment. Publ: McGraw-Hill Book Company).
1.1.9. THE EIGHTEENTH CENTURY - "THE AGE OF ENLIGHTENMENT".

With the development of increasingly sophisticated surgical instruments, steady improvement in operative technique, and clinical diagnosis based upon accurate anatomical knowledge, the eighteenth century was "the age of enlightenment" as far as surgery was concerned. More surgeons were becoming medically qualified, and teaching was based upon increasingly accurate scientific principles.

Clinical examination was confined to breast and axilla - medical students were taught to note size, shape, consistency, and mobility of the lump itself; condition of the overlying skin; and the presence or absence of swellings in the axilla. The same could be said of the training of medical students today.

The use of the cautery had declined in popularity as a treatment for breast cancer and medical opinion generally favoured surgery - and surgery at an early stage when the entire diseased area could still be readily excised. Medical opinion appeared divided, as it remains today, as to the extent of the operation required. Contemporary reports of mastectomies being completed in less than two minutes would suggest that the ancient practice of simply slicing the breast away from the chest wall to leave a gaping wound was still in use, but many surgeons increasingly favoured slightly more delicate techniques by which sufficient healthy skin was retained as to allow primary closure of the scar with sutures or with adhesive plaster.

The work of the French surgeon, Jean-Louis Petit (1674 - 1750), of the French Academy of Surgery and a fellow of the English Royal Academy, heralded a new era in mammary surgery. He practiced a wide excision of the tumour and believed that because the roots of the cancer lay within the enlarged lymphatic glands of the axilla that these should be meticulously removed, along with any doubtful tissue in the pectoral fascia and muscle. He tried to avoid actually cutting into the tumour itself during the operation and, if at all practicable, preserved the nipple and an ample amount of healthy skin to facilitate wound closure (Power 1934).

In addition to changes in surgical practice, the "age of enlightenment" also saw a number of advances in the understanding of the aetiology of breast cancer. The menopause and menstrual disorders, emotional disturbances, a family disposition, childlessness, the consumption of alcohol, lack of exercise, and late nights were amongst the factors thought to contribute to a susceptibility to the disease.

It is in the scientific writings of the eighteenth century that the first definite references to distant metastases appear - many authors, for example, reporting visceral involvement at post mortem. de Gorter (1735) suggested that cancerous matter could be transported to
distant sites via the circulatory system, and interest was also shown in the possibility of lymphatic dissemination.

In 1740 the first hospital devoted exclusively to cancer patients was established in Reims. It held twelve beds (Bandaline 1933). In 1792 private charity financed the opening of a free cancer charity ward at the Middlesex Hospital in London - the first in Britain (figure 5): The aims of the Middlesex ward were twofold - firstly to care for the patients; and secondly to study the natural history of the disease in long-term stay patients, in the hope of developing more effective treatment (Bandaline 1933).

**FIGURE 5:** Cancer ward - the first in Britain - at the Middlesex Hospital, London. Founded in 1745 as a result of a private charity established by Samuel Whitbread. It originally held 12 beds, but the number had increased to 26 by the year 1856. [Reproduced from Robbins G.F. (ed) 1984 Silvergirl's surgery of the breast. Publ: Silvergirl Inc., Austin, Texas]

1.1.10. THE EARLY PART OF THE NINETEENTH CENTURY - A PERIOD OF PESSIMISM.

The early part of the nineteenth century was marked by great pessimism regarding the treatment of breast cancer. The meticulous and extensive surgery initiated during the eighteenth century was found all too frequently to result in equally extensive infection; and even when sepsis was avoided, radical cures were all too rare.
Sir James Paget (1814 - 1899), Surgeon at St Bartholomew's Hospital in London, for example, regarded the disease as hopeless and simply laid down criteria by which to distinguish those patients whose suffering would be alleviated to some extent by surgery from those in whom it would only add to their problems (He reported an operative mortality rate of 10% in a series of 235 cases of breast cancer.) (Paget 1853).

John Brown (1819 - 1862) in his classic story "Rab and his friends" gives a descriptive account of a mastectomy operation performed by Syme in Edinburgh in 1821 upon a woman called Alie. The story gives us an impression of what such an operation would entail for the patient during this period - still prior to the availability of anaesthesia and antiseptic procedure. The operation would have taken place in a semi-circular wooden amphitheatre surrounded by a tiered gallery packed with medical students, as well as the patient's husband, and their dog, Rab.

"Alie stepped up on a seat, and laid herself on the table as her friend the surgeon told her, shut her eyes, rested herself on me and took my hand. The operation was begun at once. It was necessarily slow and chloroform - one of God's best gifts to his suffering children - was then unknown. The surgeon did his work. The pale face showed its pain but was still and silent.

It is over; she is dressed, steps down gently and decently from the table, then turning to the surgeon and students she curtsies, and in a low clear voice begs their pardon if she has behaved ill."

The account goes on to describe how the patient subsequently developed a septic infection and died (Quoted by Power 1934).

1.1.10.1. Compression

An alternative to the agonies and uncertain outcome of surgery was proposed by Samuel Young, who suggested treating breast tumours by a method of "compression" (Hanevald 1979), which - presumably - worked by depriving the tumour of its blood supply. It rapidly became a popular treatment for cancer of the breast in this country.

In France, the anatomist and clinician Joseph Claude Anthelme Recamier (1774 - 1856), physician to the Hotel Dieu in Paris began to treat cancer patients by compression bandaging and was soon claiming spectacular results. In 1827 he described six complete cures from a series of twenty-one patients (Recamier 1827).
1.1.10.2. The aetiology of the disease

During the early part of the nineteenth century the concept of "diathesis" (predisposition, tendency) stepped in to fill the gap left by the demise of the old humoral pathology as an explanation for the cause and spread of cancer.

Boyer, for example, noting that patients susceptible to cancer of the breast were generally those of an irritable disposition, a bilious constitution, and a dejected, melancholy character, concluded that it was these characteristics which rendered the woman susceptible to the disease and became one of the first writers to propose the soon to be well-accepted theory of diathesis (Boyer 1818-1826).

The possible contagiousness of the disease remained a hotly debated point around the turn of the century. The question was broached in 1802 by the London Society for Investigating the Nature and Cure of Cancer, and research instigated over the next couple of decades settled the point fairly conclusively. Guillaum Dupytrech (1778 - 1834), for example, after feeding his dogs with cancerous meat noted no ill effect upon their health and, very courageously, Jean-Louis Alibert (1766 - 1837) and Laurent-Theodore Biett (1781 - 1840) actually innoculated themselves with the matter discharged from ulcerating tumours without subsequently contracting the disease (quoted by Boyle and Cayol 1812-1822).

1.1.11. THE LATTER PART OF THE NINETEENTH CENTURY AND THE BEGINNING OF THE MODERN ERA OF BREAST SURGERY.

During the late 1800's changes occurred with the introduction of anaesthesia by Morton in 1846, the development of antiseptic surgical technique by Lister in 1867 - a cause largely championed by James Syme in Glasgow - and a growing understanding of lymphatic spread of the disease, culminating in what may be regarded as a revolution in surgical treatment.

Charles Hewitt Moore (1821 - 1870) surgeon to St Luke's and the Middlesex Hospital in 1867 encouraged more extensive surgery for cancer of the breast. He believed that local recurrences are due to continuation of the growth of fragments of the primary tumour and thus may often be seen in the breast tissue residual after surgery, usually emanating from the area immediately adjoining the wound and extending centrifugally outwards. When operating, the surgeon should therefore excise the tumour thoroughly along with any tissues likely to be diseased - including skin, fat, muscle, and lymphatic tissue. His work laid the foundation for the development of more radical surgical techniques adopted by many eminent surgeons around that time, and culminating in Halsted's classical radical mastectomy (taken from Power 1934).
1.1.1.1 Halsted's classical radical mastectomy

In 1882, William Stewart Halsted, surgeon to the John Hopkins Hospital in Baltimore, began to practice an operation designed to maximise the prevention of local recurrences, and thus prolong life. His results were first reported briefly in 1890-1, and then more fully in a brilliant paper of 1894.

Halsted believed, according to the accepted doctrine of the time that "cancer in the breast in spreading centrifugally preserves in the main continuity with the original growth and before involving the viscera may become widely diffused along surface planes" (Halsted 1907).

He began to practice a technique by which breast, axillary nodes, intervening lymphatics and pectoralis major muscle were all removed together in one continuous block dissection, an operation which lasted four hours. The principles of this procedure were splendidly conceived - the obvious and logical solution to the problem - and entirely in keeping with the accepted theories of cancer surgery at that time.

1.1.1.2 The results of Halsted's radical mastectomy vs. earlier "incomplete" operations.

Many authors considered Halsted's radical mastectomy to be the end of the story of breast cancer surgery. Cooper (1941) summarised the results of the gradually more extensive operative techniques employed from the mid-nineteenth century up to and including the Halsted radical operation. Even allowing for the many possible inaccuracies in the data and the vagueness of many early authors regarding the outcome of their treatment, the historical analysis shows a clear-cut trend of progressively higher survival rates coinciding with the gradual emergence of the radical mastectomy.

1.1.1.3 The "super-radical" mastectomy

In Europe and the United States, Halsted's radical mastectomy was soon well-established as the operation in cases of primary carcinoma of the breast. The accepted underlying principle behind the method - that of a predominantly lymphatic centrifugal dissemination of the tumour from its primary location within the breast - was soon taken to its logical conclusion with surgery being extended in "super-radical" operations in every possible direction - proximally to include neck dissection (Dahl-Iverson 1951); to include the lymph nodes of the internal mammary chain (Handley et al 1956); with removal of the whole plastron of the chest and dissection of the mediastinal lymph nodes (Wangensteen and Lewis 1960). In advanced cases, a whole arm was sometimes lost.
It soon became apparent that these terrifically mutilating operations did little to improve the patient's survival, and detracted considerably from her quality of life, and happily, super-radical techniques soon fell into obscurity and the standard Halsted radical mastectomy reigned unchallenged until the 1970's - probably the longest period in the history of this disease when any consensus of opinion existed regarding the choice of treatment!

1.1.12. THE TREND BACK TOWARDS MORE CONSERVATIVE FORMS OF BREAST SURGERY.

The Halsted radical mastectomy was aimed at providing maximum local control at a time when most patients presented with fairly gross locally extensive disease. Handley (1975), for example, describes Halsted's original case histories as "a remarkable collection of rather advanced disease by present day standards" (see figure 6). He gives the example of an 8 x 7cm tumour being described as "a small infiltrating scirrous with metastases in the axilla".

**FIGURE 6:** One of Halsted's patients demonstrating the advanced stage of the disease often seen at that time.
In addition to patients presenting at ever earlier stages of the disease, our ideas regarding distant dissemination of the cancer have changed dramatically. The old idea of a centrifugal, predominantly lymphatic spread of the disease from a primary tumour to local lymph nodes, and only then on to distant sites has been superseded by increasingly convincing evidence of distant blood-borne metastatic spread at an extremely early stage in the disease - even before the primary tumour is large enough to be palpable.

These facts, in parallel with the advent of radiology, have now led, almost a century after the development of the radical mastectomy, to an almost 180-degree turnaround - the trend today seems to be increasingly in the direction of more conservative surgical operations. As with the earlier parts of the breast cancer story, things have happened very gradually and amid considerable controversy.

1.1.13. THE CURRENT "STATE OF THE ART"

On 1st - 3rd October 1986, the King Edward's Hospital Fund for London held a "consensus development conference" on the question of "treatment of primary breast cancer". The statement issued as a result of the conference represents a summary of how breast cancer might be treated today in view of current understanding of the disease. Some extremely interesting points were raised, and the panel's main recommendations included the following points -

"Once breast cancer is confirmed, factors which may influence the choice of treatment include the patient's age, menopausal state, tumour size and local extension, spread to regional lymph nodes, and presence of distant metastases."

"The effects of all (surgical, radiotherapy) procedures on survival rates, and quality of life...require careful evaluation."

"There is no evidence that mastectomy or more extensive surgery, as opposed to local removal of the tumour leads to longer survival".

"The risk of local recurrence is greater with breast conservation. However, this risk can be reduced substantially by radiotherapy, although there is no evidence that this prolongs life".

"For tumours which are multifocal or affect a large proportion of the breast, mastectomy will often be the best surgical treatment".

"Gross spread to the axilla is normally treated by surgical axillary clearance, and radiotherapy is reserved for recurrence. However, these patients will usually have large
primary tumours, so that modified radical mastectomy may be preferable. Women with
locally advanced cancer affecting the skin or underlying muscle, and those... with
metastatic disease will generally benefit from radiotherapy, endocrine therapy, and/or
chemotherapy (Consensus Development Conference 1986).

1.1.14. THE PROBLEMS OF BREAST CANCER

1.1.14.1. Incidence

Breast cancer is the commonest cancer of women in the Western World, accounting for
20% of new cancers of females in Britain (OPCS Cancer Statistics 1988). At some stage
during her life, between 1 in 10 to 1 in 12 of women in Britain is likely to develop
breast cancer (overall rate of 83.7 / 100,000 women / year in 1984, equivalent to 1 woman
in 11.9; OPCS 1988). The incidence has risen by over 1% per annum during the last 50
years (it rose by 45% between 1935-1969: Feinleib & Garrison 1969) and at present
increases by 2% per annum (Chamberlain 1989). The present estimated lifelong probability
of developing breast cancer amongst girls born in 1987 was calculated as 1 girl in 10
(Spratt et al. 1988).

1.1.14.2. Mortality

The mortality from breast cancer in Britain has been the highest in the world for several
years and continues to increase (Boyle et al. 1989), the standardised mortality rate varying
from 29.44 to 37.50 / 100,000 women / year in different parts of Britain (mean 33.8). This
unenviable position has been noted since 1973 (WHO 1973-76, 1977, 1982-84) and
compares with mortality rates of 27 / 100,000 / year across Europe and the USA, down to
5 for Japan, 0.73 for Thailand and to 0.07 for Nicaragua (Segi Institute 1982).

In Britain this means over 15,000 deaths a year, or the deaths of 300
women each week from breast cancer.

1.1.14.3. The Clinical Problem

Perhaps the major clinical problem of breast cancer is metastasis. Unlike benign breast
tumours, most breast cancers will spread to grow in distant parts of the body.
If a breast cancer is detected and surgically removed, whether by lumpectomy or
mastectomy, before it has spread or metastasized, then the patient might be 'cured'
(Haybittle 1983). If the cancer has already metastasized, the patient is likely to die from the
growth of these metastases and the management required is different. It is therefore
important to try and detect breast cancer before it has metastasized and to have some way of knowing this event - *a marker indicative of metastasis*.

The initial purpose of this project has been to seek a marker predictive of metastasis, as determined by long term follow-up, survival and cancer recurrence.

1.14.4 The Patient’s Problem - ‘The Sword of Damocles’.

If a tumour is benign, the patient can be discharged from follow-up after the stitches / dressing are removed. However, where the tumour is malignant, the patient’s life follows a totally different course whatever the stage of disease. If metastasis is detected around the time of diagnosis (eg. by axillary lymph node involvement), then chemotherapy or hormone therapy may start immediately. Even in the absence of evidence of metastasis, the patient may start on a trial of early adjuvant therapy.

Thus, because the disease cannot be accurately staged nor the likely outcome predicted for an individual, follow-up usually continues for the rest of the patient’s life.

No patient can be reassured that she is ‘cured’, at most she can be given a statistical probability of surviving. Undoubtedly there are some women who live for 20-30 years following diagnosis and die of causes other than breast cancer, *but how can we identify them?* Of all women who develop breast cancer, 80-85% die as a direct result of the disease (Langlands et al 1979); thus 15-20% might be considered ‘cured’.

Can such groups be identified at a stage which might be of practical use for the patient, even if only to save those statistically 'cured' from unnecessary adjuvant therapy?
1.2.1. T.N.M. STAGING.
1.2.1.1. Tumour size, attachment to overlying skin and underlying chest wall.
1.2.1.2. Lymph node involvement.
1.2.1.3. Distant metastases.

1.2.2. HISTOPATHOLOGICAL GRADING.

1.2.3. TUMOUR TYPE.
1.2.3.1. Lymphocytic infiltration.
1.2.3.2. Elastosis.
1.2.3.3. Mucoid change.
1.2.3.4. Vascular invasion.
1.2.3.5. Others.
1.2.3.6. Uncommon histological types.

1.2.4. FLOW CYTOMETRY.
1.2.4.1. Prognostic significance of tumour cell DNA content.
1.2.4.2. Correlation of DNA content with other prognostic features.
1.2.4.3. S-phase fraction.

1.2.5. HOST FACTORS.
1.2.5.1. Immune response.
1.2.5.2. Age.
1.2.5.3. Endocrine receptors.
1.2.5.4. Early detection.

1.2.6. BIOCHEMICAL TUMOUR MARKERS.
1.2.6.1. Carcinoembryonic antigen (CEA).
1.2.6.2. Human chorionic gonadotrophin (HCG).
1.2.6.3. Fucose.
1.2.6.4. Pregnancy associated macroglobulin.
1.2.6.5. Breast cyst fluid glycoprotein.
1.2.6.6. Urinary polyamines and nucleotides.
1.2.6.7. Placental alkaline phosphatase (PLAP).
1.2.6.8. Gamma glutamyl transpeptidase.
1.2.6.9. Others.
1.2.6.10. Conclusion.
1.2.7. GROWTH FACTORS, THEIR RECEPTORS, AND ONCOGENES.
1.2.7.1. Epidermal growth factor and its receptor.
1.2.7.2. Oncogenes.

1.2.8. MONOCLONAL ANTIBODIES.
1.2.8.1. Application to monitoring tumour burden.
1.2.8.2. Use in targetting toxins.

1.2.9. PROGNOSTIC CRITERIA IN ADVANCED DISEASE.
1.2.9.1. Original clinical staging.
1.2.9.2. Hormone receptor status.
1.2.9.3. Site and extent of metastatic disease.
1.2.9.4. Disease free interval.
1.2.9.5. Response to therapy.
1.2. INDICATORS OF PATIENT PROGNOSIS IN BREAST CANCER.

Very many diverse factors have been described as being of prognostic significance in breast cancer. Some of the more important, most widely accepted, or more intensively studied are described in this section.

1.2.1. T.N.M. STAGING

The extent of the disease at presentation is usually assessed by the clinical T.N.M. ("Tumour / Nodes / Metastasis") staging system which was originally developed more than 35 years ago. The T.N.M. sytem is fully described in the U.I.C.C. (Union Internationale Contre le Cancer) handbook, but, briefly, it is a simple index of:

1. T: primary tumour size and attachment to overlying skin or underlying chest wall.
2. N: the presence or absence of axillary lymph node involvement.

The index is believed to reflect the biological age of the tumour, or the extent of its progression along the course of its natural history; but may also be, to some extent, indicative of the aggressiveness or metastatic potential of the cancer (Fisher 1980; 1984) and the nature of the interactions between tumour and patient (Devitt 1983). The purpose of such a staging system is to aid in the selection of the most appropriate treatment regime by distinguishing between patients whose disease is localised - and whose prognosis is good under standard treatment - from those who will require a more aggressive approach to control disseminated, advanced disease.

1.2.1.1. Tumour size, attachment to overlying skin and underlying chest wall.

Tumour size may be estimated by clinical examination, or determined by mammography and other imaging techniques. It is thought to directly reflect prognosis - a smaller tumour either being younger or more slow growing than a larger one, and thus less likely to have reached the critical volume required for metastatic cells to begin to separate and colonise distant sites (Cutler and Myers 1967; Duncan and Kerr 1976; Fisher et al 1969). Fisher et al (1969), for example, in a study of two thousand breast cancers demonstrated a significant correlation between tumour size, the likelihood of axillary node involvement, and five year recurrence and survival rates. In lymph node positive patients (with between 1 - 3 nodes involved), for example, the five year recurrence rate was 44% in patients with
small 1 - 2cm tumours, but as high as 63% in those with lesions greater than 6cm.

Predictions of prognosis based upon tumour size data alone should be interpreted with caution, however, as the situation is not quite as straightforward as it might seem. Fisher et al (1969) found, for example, that a number of patients with large (>6cm) tumours were shown histologically to be lymph node negative, and had a very much better prognosis than might otherwise have been expected from consideration of tumour size alone. Foster and Neville (1981) in looking at a series of 437 primary infiltrating ductal breast carcinomas were unable to detect a significant difference in lymph node involvement and subsequent development of distant metastases when comparing tumours of greater versus less than 2cm diameter.

Other well established indicators of prognosis include local skin involvement (oedema, ulceration or fixation) (Cutler and Myers 1967; Haagensen 1971); generalised inflammation of the breast (Lucas and Perez-Mesa 1978); and tumour fixation to underlying chest wall (Cutler and Myers 1967; Haagensen 1971).

1.2.1.2. Lymph node involvement

Lymph node involvement has long been recognised as one of the most important pointers to prognosis in breast cancer (eg see Handley 1904). Gross involvement of the axillary lymph nodes may be determined by clinical examination, but simple clinical assessment of the axilla alone correctly predicts tumour invasion of the nodes in only about one third of patients (Haagensen 1971; Wallace and Champion 1972), and surgical resection with detailed histology is required to gain an accurate picture of lymph node status. The greatest clinical significance is attached to the absolute number of lymph nodes involved with tumour, regardless of the total number examined, their position, or tumour burden (Fisher and Slack 1970; Huvos et al 1970; Lacour et al 1976). Prognosis worsens with each additional node found to be involved, but as a general rule, patients with greater than four nodes involved are taken to have a considerably poorer outlook than those with between nought and three positive nodes (Fisher et al 1969).

Other factors heralding a poorer prognosis include fixation of the axillary nodes (Cutler and Myers 1967; Haagensen 1971), and involvement of supraclavicular nodes (Cutler and Myers 1967) and those of the internal mammary chain (Lacour et al 1976; Donegan 1977).

1.2.1.3. Distant metastases.

Alvord (1975) calculated that metastatic cells are first shed from a tumour at around the time of the twenty-first cell division, and the process continues steadily thereafter. By the time
the primary tumour is palpable, therefore, it will in most cases have already disseminated in micrometastases around the body. Depending upon the rate at which such deposits grow, they may already be manifest at the time of primary diagnosis or may remain occult for very many years. In a majority of cases, such disseminated disease will ultimately result in the death of the patient, either months, years or even decades after first presentation (Haagensen 1971; Fisher et al 1975).

The studies of Brinkley and Haybittle (1975; 1984) suggest that only an extremely small proportion of women with breast cancer have anything approaching normal life expectancy, no matter how thorough the primary therapy; perhaps as many as 35% of patients with "early" disease (clinical stage I; no lymph node involvement) will die within ten years of presentation as a result of widespread metastatic disease (Fisher et al 1975).

At the time of primary diagnosis, it is only possible to pinpoint those unfortunate patients whose prognosis is particularly poor due to already clinically advanced overt disease. It has been estimated, for example, that in the case of bony metastases, as much as half of the bone can be destroyed before the lesion becomes apparent on X-ray (Edelstyn et al 1967); similarly, grossly involved liver may remain impalpable, and only extremely large (>2cm) discrete deposits can be effectively visualised by liver scintigraphy (Friedell et al 1957). Even more modern techniques, for example ultrasound - which is generally quoted as being sensitive enough to detect liver deposits of less than 1cm diameter - are unable to detect disease until a relatively late stage. It is unfortunate that instead of being in a position to pronounce patients that are free of metastatic disease "cured", the technology presently available only allows the doctor to identify disease at a very late stage where little can be done to combat it effectively.

1.2.2. HISTOPATHOLOGICAL GRADING.

The biological aggressiveness of a tumour is often reflected by its histology - histopathological grading on the basis of such features as the mitotic activity, degree of differentiation of tumour cells, and nuclear pleomorphism provide a guide to the extent of proliferative activity; whilst evidence of tumour invasion of blood vessels and lymphatics may indicate a high metastatic potential (Tickle et al 1978; Bettelheim et al 1984). As early as 1902, for example, Van Hansemann demonstrated the existence of a direct relationship between the degree of loss of glandular organisation and abnormal mitoses and the degree of malignancy.

A number of grading systems have been devised which attribute varying degrees of prognostic emphasis to a variety of histopathological and cytopathological parameters, the resulting grade being taken to reflect the aggressiveness of the tumour and to correlate with
probable patient prognosis, at least in the short term. Haagensen (1933), for example, devised an enormously complex grading system based on considerations of no less than fifteen separate parameters of cell morphology, stromal reaction, and tumour cell growth characteristics. Tumours were divided into three basic grades, which in a study of 150 primary tumours correlated well with prognosis. His system was, however, far too complex to be of any great practical value for routine usage.

Hartveit (1971) based his grading system on only four basic cytological criteria, including definition of cell borders, degree of nuclear crowding, degree of nuclear lobulation, and nuclear diameter. Considerable expertise is required to use such a system effectively, and it is subject to considerable observer error.

A much simpler and widely adopted grading system is that established by Bloom and Richardson (1957); by their method, tumours are scored according to degree of tubule formation, nuclear pleomorphism, and the frequency of hyperchromatic and mitotic nuclei. They are graded I, II, or III according to their cumulative totals. Although somewhat subject to observer error, in experienced hands it appears to be a very useful system - the grading corresponding roughly to good (grade I), intermediate (grade II), and poor (grade III) prognosis. A very similar system, and that adopted for the grading of breast cancers in this study, is that described by Scarff and Torloni (1968) and adopted by the World Health Organisation. It is more fully detailed in section 2.1.2.5.

1.2.3. TUMOUR TYPE

Histological tumour type, as well as grade, is believed by many workers to influence prognosis - since the original observations of Dennis (1891) and von Hansemann (1893), many attempts have been made to identify adequate prognostic indices for the various histological tumour types, and to define their typical behaviour, spread, and level of aggression. Much "evidence" is anecdotal, however, and actual statistical proof appears elusive.

Cancers of the breast are believed to arise commonly from within the ducts, and less frequently from intralobular ductules and their related alveoli (only around 4-6% of breast carcinomas fall into this latter category). If detected early enough in its natural history, the tumour may be restricted to one of these sites and is described as "carcinoma in situ" if it shows no evidence of invasion or extension. At a slightly later stage, tumours of both lobular and duct origin may be observed extending along the ductal system but without evidence of penetrating basement membrane and infiltrating adjacent non-epithelial tissues. In situ carcinomas are rarely detectable by clinical examination, most usually presenting as an accidental finding when breast tissue has been taken for other reasons. They are by definition pre-invasive and if completely excised should, theoretically, herald an excellent
prognosis for the patient.

Most breast cancers are not detected until a much later stage in their natural history by which time they will have almost certainly become invasive. Infiltrating ductal carcinoma is by far the most common type observed in any routine histopathology department, and although it may manifest in a whole spectrum of morphological guises, none of the observed histopathological features presents any reliable prognostic indicator of clinical consequence.

A broad spectrum of microscopical growth patterns may often be seen within a single tumour. The epithelial component, for example, may exhibit degrees of differentiation ranging from easily recognisable tubules to complete anaplasia, and in some examples quite excessive mucin production gives rise to the "colloidal" type of carcinoma. Linked to these characteristics stromal components such as fibrosis, elastosis, lymphocytic infiltration, and tumour infiltration of blood vessels are sometimes seen. Their possible prognostic significance is discussed below.

1.2.3.1 Lymphocytic infiltration

Lymphocytic infiltration of the tumour has been considered a favourable prognostic feature by many workers (Black et al 1975), although perhaps not of such importance as other features. Hamblin (1968), for example, using a multivariate analysis technique, ranked it fifth in importance as a prognostic indicator.

1.2.3.2. Elastosis

There is a close correlation between the onset of infiltration of a carcinoma and the appearance of an abnormal increase in elastic tissue in the breast (Foster and Neville 1981). Extensive elastosis is, however, viewed by many to be a very favourable prognostic sign. A direct relationship, for example, has been demonstrated between degree of elastosis and disease-free interval and survival following surgery (Shivas and Douglas 1972; Masters et al 1976). This may be due to a strong correlation between elastosis and the presence of oestrogen receptor activity - an extremely important prognostic feature - as many as 95% of those tumours showing extensive elastosis are oestrogen receptor positive (Masters et al 1976).
1.2.3.3. Mucoid change

May vary from the production of a small quantity of mucopolysaccharide by isolated tumour cells, which is probably of very little prognostic significance, to the copious sea of abundant mucin seen in the pure colloidal-type of breast carcinoma - a histological type associated with a particularly good prognosis (Foster and Neville 1981).

Typically, colloidal carcinomas are seen in older patients, and are associated with a long history of a large breast mass (Silverberg et al 1971). They are very slow growing, infiltrating skin, connective tissue and lymph nodes much later than is seen in other tumour types and are associated with long disease free interval and favourable survival prospects (Foster and Neville 1981). This appears to be due not to the presence of the mucin itself, but rather to the slightly less aggressive nature of the tumour cells associated with it (Cheatle and Cutler 1930).

1.2.3.4. Vascular invasion

As metastatic spread to distant sites is generally believed to be via blood borne metastatic cells, frank invasion of blood vessels is believed by some workers to be an unfavourable prognostic sign (Sampat et al 1977).

1.2.3.5. others

Other histological features of less certain prognostic value include tumour invasion of perineural space (Taylor and Norris 1970), fibrosis (Alderson et al 1971), and tumour necrosis (Fisher et al 1978).

1.2.3.6. Uncommon histological types

Although a number of systems have been proposed for the classification of breast cancers according to histology, that adopted by W.H.O. (1981) is probably the most commonly used.

It is illustrated in Table 1, overleaf.
TABLE 1: W.H.O. Histological classification of breast tumours:

<table>
<thead>
<tr>
<th>I. Epithelial Tumours</th>
<th>II. Mixed Connective</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Benign</td>
<td>Tissue and Epithelial Tumours</td>
</tr>
<tr>
<td>1. Intraductal papilloma</td>
<td>A. Fibroadenoma</td>
</tr>
<tr>
<td>2. Adenoma of the nipple</td>
<td>B. Phyllodes tumour (cytosarcoma phyllodes)</td>
</tr>
<tr>
<td>3. Adenoma</td>
<td>C. Carcinosarcoma</td>
</tr>
<tr>
<td>a. Tubular</td>
<td></td>
</tr>
<tr>
<td>b. Lactating</td>
<td></td>
</tr>
</tbody>
</table>

| B. Malignant                |                              |
| 1. Non-invasive             |                              |
| a. Intraductal carcinoma    |                              |
| b. Lobular carcinoma in situ|                              |

| 2. Invasive                 |                              |
| a. Invasive ductal carcinoma|                              |
| b. Invasive ductal carcinoma with predominant intraductal component | |
| c. Invasive lobular carcinoma|                              |
| d. Mucinous carcinoma       |                              |
| e. Medullary carcinoma      |                              |
| f. Papillary carcinoma      |                              |
| g. Tubular carcinoma        |                              |
| h. Adenoid cystic carcinoma |                              |
| i. Secretory (juvenile)     |                              |
| carcinoma                   |                              |
| j. Apocrine carcinoma       |                              |
| k. Carcinoma with metaplasia|                              |
| i. Squamous type            |                              |
| ii. Spindle-cell type       |                              |
| iii. Cartilaginous and osseous type | |
| iv. Mixed type              |                              |
| l. Others                   |                              |

| 3. Paget's disease of the nipple | |

By far the most frequently seen types are the invasive ductal and invasive lobular carcinomas - together comprising some 70% of all breast cancers. These carry by far the poorest prognosis. A number of exceedingly rare variants including adenoid cystic carcinoma, secretory carcinoma, papillary and tubular carcinoma have been reported to form small but prognostically favourable sub-groups (McDivitt and Steward 1966; Caranzo and Taylor 1969; Murad et al 1981; Deos and Norris 1981), as have medullary, papillary and colloidal carcinomas (Rilke et al 1978; 1984), as illustrated in Table 2, overleaf.
TABLE 2: Survival Figures of Breast Cancer Histological Sub-Types

<table>
<thead>
<tr>
<th>Incidence</th>
<th>Patients Surviving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5yrs</td>
</tr>
<tr>
<td>Invasive ductal</td>
<td>63 %</td>
</tr>
<tr>
<td>Invasive lobular</td>
<td>8.5 %</td>
</tr>
<tr>
<td>Medullary</td>
<td>5 %</td>
</tr>
<tr>
<td>Papillary</td>
<td>2 %</td>
</tr>
<tr>
<td>Colloidal</td>
<td>2 %</td>
</tr>
</tbody>
</table>


1.2.4. DNA ANALYSIS BY FLOW CYTOMETRY

In recent years, there has been considerable interest in the possible clinical significance of abnormalities in the cellular DNA content of human tumours (Barlogie et al 1983). Earlier studies using single-cell microdensitometry of Feulgen-stained tissue sections established that aneuploidy (abnormal DNA content) is common in breast cancers, and showed some correlations with prognosis (Atkin 1972; Atkin et al 1979; Auer et al 1984), but the techniques involved were time-consuming, labour-intensive, and had limited resolving power. In addition, clinical information such as oestrogen receptor status and lymph node involvement was incomplete for many patients studied, thus rendering evaluation of prognostic significance of the technique in comparison to other prognostic variables extremely difficult.

The introduction of rapid and accurate mechanised flow cytometric techniques has revolutionised DNA ploidy analysis and opened up the way to intensive research.

Initially, studies were required to be carried out on fresh, unfixed tissues, and although providing excellent data carried the drawback that archival material could not be analysed, resulting in the necessity for long-term prospective follow-up before any prognostic significance of results obtained could be assessed.

The development of a flow cytometric method suitable for use with fixed, paraffin-embedded material by Hedley et al (1983; 1985) has allowed the analysis of archival material and ready evaluation of ploidy data in relation to the known prognosis of patients whose clinical outcome is already documented.
1.2.4.1. Prognostic significance of tumour cell DNA content

The work of Bohm and Sandritter (1975), using a single-cell method, first clearly demonstrated a strong association between increased DNA content/aneuploidy and malignancy. Benign tumours were shown to express a basically diploid profile, with some increase in the tetraploid region, indicative of increased proliferative activity. The majority of malignant tumours, however, possessed an obviously aneuploid, abnormal, sub-population of cells. (Examples of diploid and aneuploid DNA histograms are given, with a more complete explanation, in section 2.2.4. of Materials and Methods).

Auer and colleagues (1980) examined a retrospective series of 112 archival fine needle biopsies from patients with primary carcinoma of the breast. They classified the resulting DNA histograms into four distinct types, ranging from the normal diploid profile (which they called type I) to bizarre and distinctly aneuploid pattern (which they called type IV). Significant differences in patient prognosis were demonstrated between the groups. Most (81%) of patients with survival times of less than 2 years - patients with obviously highly malignant, biologically aggressive tumours - exhibited bizarre, aneuploid DNA profiles (classified as type III or IV), significantly deviant from those of normal breast epithelium. The vast majority (80%) of patients surviving in excess of 10 years - those with biologically less aggressive cancers - showed basically diploid DNA profiles (of type I or II), within the limits of normal proliferating breast epithelium.

Later work by Auer et al (1984), aimed at further investigating the prognostic significance of DNA analysis of neoplastic cells, compared the DNA histograms of a cohort of 36 patients who had survived for more than 15 years post-operatively and of 42 individuals who had died within 2 years of diagnosis. Similar results were obtained as in their original study, strongly suggesting that cellular DNA content in mammary carcinomas may be closely correlated with patient prognosis.

Other studies have also demonstrated a significant difference in the survival of patients with diploid compared to those with aneuploid tumours (Atkin 1972; Atkin and Kay 1979). Dowle et al (1987), however, in analysing a series of 354 patients with primary operable breast cancer, although demonstrating superior short-term survival in patients with diploid tumours, were unable to detect a difference in overall survival or in disease free interval after longer-term follow-up (of median duration 7 years). The study of Owainti et al (1987) appears to be in agreement with this study, again demonstrating early short-term advantage to patients with diploid tumours - during the first thirty months following surgery, DNA aneuploidy was associated with a higher rate of recurrence and shorter survival - but overall, failing to demonstrate a significant survival advantage to either group.
The data of Hedley et al (1984), in analysis of 165 patients entered in a multicentre adjuvant chemotherapy trial suggests that although relapse-free survival curves for patients with aneuploid and diploid tumours are significantly different, tumour cellular DNA content does not appear to influence patient survival following relapse. Aneuploid tumours may have a different natural history in comparison with diploid tumours, or may simply be more responsive to adjuvant chemotherapy.

1.2.4.2. Correlation of DNA content with other prognostic features

Many attempts have been made to correlate cellular DNA content of breast carcinomas with other recognised prognostic criteria, such as oestrogen receptor status, lymph node involvement, tumour grade, stage, and so on:

**Oestrogen receptor content**

The relationship between steroid hormone receptor status and cellular DNA content is weak. Most authors have suggested a trend towards diploid tumours being more likely to express oestrogen receptor than their aneuploid counterparts, although data has not always succeeded in achieving statistical significance (Olszewski et al 1981; Raber et al 1982; Owainti et al 1987; Taylor et al 1983; Masters et al 1987). Other groups have failed to demonstrate any clear association (Thorud et al 1986; Dowle et al 1987; Hedley et al 1984).

Stuart-Harris and colleagues (1985) have presented the results of a fairly small study aimed at assessment of the relationship between tumour cell ploidy and patient response to endocrine therapy at relapse. Patients with diploid tumours showed either increased response to hormonal manipulation, or improved survival, compared to those with aneuploid tumours.

**Menopausal Status**

A number of studies have attempted to correlate tumour ploidy with patient menopausal status. Some results suggest a higher proportion of aneuploid tumours in post-menopausal patients (Thorud et al 1986; Dowle et al 1987), others, however have failed to demonstrate a difference between pre- and post-menopausal groups (Hedley et al 1984; Raber et al 1982).

**Extent of Disease**

Many studies point to a strong positive correlation between extent of disease and cellular DNA content - the more advanced the stage of the disease, the more likely the tumour appears to be to express abnormal DNA ploidy (Ewers et al 1984; Olszewski et al 1981; Hedley et al 1985). Similarly, aneuploidy appears to correlate with large tumour size and
advanced histological grade (Dowle et al 1987), lymph node involvement (Hedley et al 1984; Ewers et al 1984) and a poor degree of differentiation (Raber et al 1982; Masters et al 1987).

It remains unclear as to whether tumour cellular DNA content should be regarded as an independent prognostic factor in breast cancer. Hedley et al (1984) and Dowle et al (1987), for example, each having performed complex multivariate analysis have failed to confirm independent prognostic significance.

Although there is little clear evidence to suggest that ploidy analysis will be a major prognostic factor comparable to, for example, degree of lymph node involvement, tumour endocrine receptor expression or histological grading, most studies do indicate that it does carry at least some degree of prognostic weight. At present a research tool in this country, flow cytometrical DNA analysis requires further exploration before its worth may be wholly understood.

1.2.4.3 S-phase fraction

A second parameter to be calculated from the DNA histogram is the S-phase fraction (SPF), which provides a crude estimate of the percentage of cells undergoing active cell division (in the S-phase of the cell cycle) and thus of the proliferative activity of the tumour. I am aware of only two studies which have directly examined the prognostic significance of SPF in breast cancer, and both have demonstrated a high S-phase fraction (therefore high proliferative rate) to predict poor patient prognosis in breast cancer (Hedley et al 1987; O'Reilly et al in press).

In centres where histological tumour grading is performed by one expert pathologist, the prognostic value of SPF appears not to show independent prognostic significance (Hedley et al 1987). Other studies disagree, however - for example, O'Reilly et al (in press) in a very recent paper, did demonstrate SPF to show a strong, but not quite statistically significant, trend towards independent prognostic significance. Many authors have observed that the grading of tumours is highly subjective and prone to extreme disagreement between pathologists (Delides et al 1982), and the objective technique of DNA flow cytometry to determine SPF may overcome these problems by providing a mechanised and reproducible alternative to histological grade.
1.2.5. HOST FACTORS:

1.2.5.1 Immune response

Alterations in immune function are known to occur in relation to human cancer, such disturbances becoming increasingly marked as disease progresses (Harris and Copeland 1974).

Most studies of immunological activity in patients with breast cancer have focussed upon non-specific indices of immune function such as delayed hypersensitivity skin tests (Nemoto et al 1974; Pinsky et al 1974), lymphocyte transformation (Wanebo et al 1976; Whittaker and Clark 1971), and quantitative studies of peripheral lymphocyte (Papates et al 1976; Keller et al 1976), and suppressor cell counts (Fujimoto et al 1976a and b). The results obtained indicate that specific recognition of tumour antigens probably does occur, and that an immune response is mounted against them; this response, however, becomes increasingly weakened as the disease process advances. It appears that generally immune reactivity - as assessed by these, perhaps inappropriate, means - has little prognostic significance except in that biological events in the natural history of the disease dictate subsequent variations in antigen expression and immunogenicity. Patients with early disease and healthy controls will thus show significantly stronger immune response than patients with advanced systemic disease.

1.2.5.2. Age

Survival in young vs. old patients.

Some prognostic indices include age amongst the variables to be considered (Bauer et al 1983; Fisher et al 1980) yet numerous reports conflict as to the precise significance of age as a guide to prognosis in breast cancer. Some studies, for example, have reported a poorer prognosis for young patients (Stoll 1976; Noyes et al 1982; Fisher et al 1980; Ribeiro and Swindell 1981), others that younger women fare better (Langlands and Kerr 1979; Mueller et al 1978), and others have failed to demonstrate any difference at all (Redding et al 1979; Herbsman et al 1981; Cutler et al 1969). Such conflicting results make the subject extremely confusing, and there still appears to be little agreement as to the precise prognostic significance of patient age at diagnosis.

Such confusion can to some extent be explained by differences in the selection criteria of patients in different studies, the fairly small numbers of subjects sometimes considered, but perhaps most significantly, differences in the age grouping used in analysis, since in many studies patients have been simply lumped together as older or younger than fifty years. Such a broad dividing line may obscure many of the differences in the data. Nemoto et al (1980), for example, reported the poorest five year survival in the youngest
patients (aged < 35 years) and this finding has been confirmed by other workers (Noyes et al 1982; Ribeiro and Swindell 1981). Such aggressive disease in young women is perhaps easily explained - by the time a breast cancer reaches such a size as to become clinically apparent it may have been present for anything between two and twenty years, depending, of course, on the rate at which the cells are proloiferating (Spratt 1977). In a very young woman, then, it is extremely unlikely that the cancer will have been present for twenty years or more (a woman, for example, in her late twenties or early thirties is simply not old enough); very young patients are thus more likely than their older counterparts to present with aggressive, rapidly proliferating cancers (Stoll 1986).

Another sub-set of young patients (if we take "young" to mean less than fifty years old), those approaching the menopause, appear to show a surprisingly good prognosis - it has been known for many years that the advent of a natural menopause appears to prolong recurrence free survival (A.M.A. Committee on Research 1960; Clemmensen 1948), as well as survival following first recurrence (Cutler et al 1969; Delarue 1955). More recent long-term studies confirm these findings, suggesting that the longest survival rates of all breast cancer patients are those seen in women presenting in the few years prior to their menopause (Langlands and Kerr 1979; Herman 1972). Attempting to assess the prognosis of women with breast cancer under the age of fifty years may include any combination of these heterogeneous groups as a single category of "young" patients - this may explain to some extent the discrepancies seen in the conflicting data of different studies.

The prognostic significance of age in elderly breast cancer patients is also far from straightforward. Many sources report that older patients more frequently present with long-standing, locally advanced disease (Mueller et al 1978; Stoll 1976; Stoll and Ackland 1970) which would suggest, perhaps, that very slowly proliferating, less biologically aggressive tumours are more commonly seen in these women (Stoll 1986) - a favourable prognostic sign. Several sources, however, have reported that the rate of death from breast cancer increases rather than decreases as patients become older (eg. Mueller et al 1978), perhaps as a result of their generally more advanced disease, or perhaps it is that while optimal efforts are made in the treatment, however unpleasant it may be, of young women, less intensive therapy is often offered to the aged. Death from causes other than breast cancer will also clearly be more frequent in the elderly rather than young patients, and it does appear likely that cause of death, in many cases, is wrongly ascribed to the malignancy (Brinkley et al 1984; Silman 1979).

Histopathology
The literature suggests the presence of consistent differences with regard to classical histopathological grading of tumours seen in younger versus older women, reflecting, perhaps, different growth characteristics and metastatic potential between these groups.
Many reports note a higher proportion of poorly differentiated tumours (Brightmore et al 1970; Barnet and Eisenberg 1964), and less evidence of elastosis and fibrous stroma (Fisher et al 1975; Wallgren et al 1977) in the tumours of younger women; and there also seem to be differences in the distribution of different tumour types: infiltrating duct and lobular carcinomas as well as the medullary type being found most commonly in the youngest age group (20 - 44 years), tubular carcinoma in patients aged 45 - 54 years, and mucinous carcinoma in older women (> 55 years) (Fisher et al 1975).

**Tumour Behaviour**

It appears that tumour behaviour may at least in part be affected by the age group of the patient. In keeping with the idea that tumours in very young patients are likely to exhibit unusually aggressive biological behaviour, Treves and Holleb (1958) reported women under the age of thirty five with positive lymph nodes to have a significantly shorter disease free survival than older patients. Other workers disagree, however, reporting earlier reactivation in patients of, variously, less than 84 years of age (Fisher et al 1975), 65 years of age (Papadrianos et al 1965), or failing to detect any difference at all in pre-versus post-menopausal women (Valagussa et al 1978). By way of explanation for these discrepancies, similar arguments apply as described previously: By grouping patients together in fairly broad age-bands, subtle differences are swamped; similarly, the factors influencing local, regional and distant recurrence to different organs may be quite disimilar and the grouping of heterogeneous data on often fairly short-term follow-up is bound to result in misleading conclusions (Stoll 1986).

In examining recurrence events in slightly more detail, it seems that patient age really is extremely important. Little difference is seen between age groups when considering the proliferative rate of local operation-site recurrences (Stoll 1986; Devitt 1971; Karabali-Dalamagas et al 1978), however, such growth does not necessarily mirror the inherent capacity of the tumour for invasion - a much truer index of prognosis (Tubiana et al 1981) and significant differences are seen in the invasive potential of tumours in young versus old patients.

It has been noted, for example, that elderly patients much more commonly present with large, locally advanced tumours (Langlands and Kerr 1979; Mueller et al 1978; Stoll and Ackland 1970), but that first recurrence is often significantly delayed in comparison to younger patients with the same stage disease (Stoll 1986). In addition, the first evidence of recurrence is frequently of loco-regional, rather than distant, origin (DiPietro et al 1981; Vianda et al 1973). Younger women, in comparison, are much more likely to recur first in bone or viscera - a much more life-threatening situation - and often experience a rapid succession of such blood-borne metastases within a very short time (frequently within 12 to 18 months ). The likelihood of such a "shower" of metastases
decreases with increasing patient age (Stoll 1976).

1.2.5.3. Endocrine receptors

It is generally accepted that patients whose tumours are positive for the expression of endocrine receptors show prolonged survival in comparison to those who are receptor negative (Blamey et al 1980; Crowe et al 1982; Howell et al 1984). The reason for this remains unclear, but it is most probably due, at least in part to the very strong correlation between the presence of such receptors and the patient's positive response to endocrine therapy in advanced disease delaying its natural course in many cases by months or even years (Howell et al 1984; Powles 1981; McGuire 1978).

Some workers have, however, shown the presence of such receptors to be associated with a more favourable prognosis in the absence of any hormonal manipulation. The time to first recurrence, for example, has been reported by some authors to be significantly extended in oestrogen receptor positive patients (Crowe et al 1982; Cooke et al 1979; Knight et al 1977). Others, however, have demonstrated only a transient difference (Aamdal et al 1984), perhaps limited to certain sub-groups of women (Blamey et al 1980; Saez et al 1983).

Studies involving progesterone receptors are equally contradictory; again, some reports suggest longer recurrence-free interval in receptor positive patients (Saez et al 1983; Mason et al 1983), where others have failed to demonstrate any difference between progesterone positive and negative cases (Howell et al 1984; Allegra et al 1979).

The reasons for such discrepancies remain unclear, but may simply be due to variations in methodology and so forth, making direct comparisons of different studies invalid. It is unfortunate that at present the prognostic significance of the progesterone and oestrogen receptor remains uncertain. Endocrine receptors are, however, of tremendous value in assessing the individual patient's likelihood of responding to endocrine manipulation therapy once metastatic disease has become apparent.

Interestingly, steroid-receptor status appears to be positively associated with possible morphological expressions of tumour differentiation and proliferative rate in breast cancer. Very many workers have, for example, demonstrated a strong correlation between both oestrogen receptor (eg. most recently Rasmusson et al 1981; Thoreson et al 1981; 1982; Howat et al 1983) and progesterone receptor status (Thoresen et al 1982; Howat et al 1983) and histological tumour grade; as well as between steroid receptor status and degree of elastosis of the tumour (Rasmusson et al 1981; Howat et al 1983), and with degree of lymphocytic infiltration (an inverse relationship) (Rasmusson et al
1981; Howat et al 1983; Fisher et al 1981). No real relationship has yet been demonstrated between oestrogen / progesterone receptor status and axillary lymph node involvement (Gentili et al 1981), arguably our finest predictor of patient prognosis, and it seems, therefore, that the value of steroid receptor status assay as a prognostic indicator is at present limited, appearing to function rather as an index of the tumour's sensitivity to endocrine manipulation.

1.2.5.4. Early detection

Most breast cancer patients will ultimately die as a result of the progression of widespread metastastic disease which would have been present, but remained undetectable, at the time of primary diagnosis. If diagnosis could be made at a much earlier stage than at present, before dissemination occurs, or when metastases were tiny enough to be eradicated by presently available adjuvant treatment, it seems likely that fewer patients would die of their disease. It is with this ideal in mind that numerous screening programmes have been established in recent years, testing asymptomatic women for early lesions - in most cases by a combination of mammography and skilled clinical examination.

The first randomised study of the effect of screening on breast cancer mortality was that of the New York Health Insurance Plan (H.I.P.), begun in 1963 and involving 62,000 women aged between 40 and 64 years at entry, and randomly assigned to a screening and control (routine clinical care) groups (Shapiro 1977; 1982). The screening procedure involved interview, clinical examination and mammography at three yearly intervals with 65% of the screening group undergoing at least one such regime.

A total of 584 breast cancers were detected amongst 62,000 women within the first five years of the study; 133 of these in the screened group. 33% of tumours detected during screening were picked up by mammography alone, being still too tiny to be localised by clinical examination - that these cancers were diagnosed at an earlier stage than those of the control group is reflected in the degree of axillary lymph node involvement: 70% node negative (screening) vs. 46% (controls).

Earlier cancer detection in the screened group is also reflected in the comparative mortality figures; very encouraging at the ten year mark with a 30% reduction in mortality in the screened vs. control group. However, the advantage is only seen in older patients (aged >50 years at entry), and disappears after fifteen years of the study.

Similarly, case survival rates for the screened and control groups were markedly different at four and eight years, but the advantage had disappeared by the ten year mark.
Although the results of such a trial are extremely interesting, they have yet to
demonstrate that screening for early breast cancer will ultimately decrease the number of
deaths from the disease as was originally hoped, and they should be interpreted with
some degree of caution owing to biases present in the data (Zelen 1976). Perhaps the
most important of these is "lead time" bias which exists as a result of early detection
simply alerting patient and physician to the presence of the disease at an earlier stage in
its natural history - the patient will thus be aware of the disease for a longer period of
time, and her survival will apparently be extended, even if the progression of her
disease is actually unaffected (Feinlieb and Zelen 1969; Shapiro et al 1974).

Another factor which must be taken into account is "length bias" sampling which may
occur owing to the wide variation in rates at which different individual breast cancers
grow - slow growing tumours are most likely to be detected by screening at a
comparatively early stage in their natural history; rapidly proliferating tumours, on the
other hand, are more likely to become apparent at a later stage and in the interval between
successive screenings. Patients selected by screening are thus much more likely to be
those with slow-growing, limited- potential disease; a prognostically favourable group.
Length bias is extremely difficult to correct for (Gershon-Cohen et al 1963).

Finally, the women themselves may not be truly representative of the population as a
whole (Shapiro 1977) - for example, women volunteering for inclusion in such a
screening programme are self-selected on the basis that they themselves are more than
averagely concerned by their risk of breast cancer. Once included in the study, those
allocated to the control (non-screened) group, may, having been alerted to the possibility
of their being at risk, seek mammography etc. elsewhere. Clearly randomisation of
patients in such a study simply into screening and non-screening groups will not
overcome the bias inherent in such a project.

The H.I.P. study, and others like it (eg Verbeek et al 1984; Collette et al 1984) leave
vital questions as to whether screening simply appears to extend survival by selecting in
favour of inherently slow-growing, prognostically favourable tumours - or actually
reduces mortality by detecting cancers before they have metastasized - largely
unanswered. The real potential of early detection in decreasing breast cancer deaths
remains unproven. It seems that at least a short-term reduction in mortality can be
achieved as a result of screening, but this benefit may subsequently disappear, or be
merely artefact. Results at present are largely inconclusive, and it is clear that many more
randomised studies with very long-term follow-up will be required before it is clear
whether breast cancer prognosis is significantly altered by such early detection
programmes, or not.
Perhaps the finest example of the practical value of a selective and sensitive biochemical tumour marker is that of the secretion of human chorionic gonadotrophin (H.C.G.) by choriocarcinomas: monitoring patient H.C.G. serum levels allows accurate staging of disease progression, and the sensitivity of the test is such that a tumour bulk of as little as $10^4$-$10^5$ malignant trophoblastic cells can be detected (Bagshawe 1973). The sensitivity of the test in conjunction with effective therapy has led to highly successful management of these tumours.

The potential value of a comparable biochemical tumour marker substance in breast cancer is only too apparent. Indeed, perhaps the most pressing need in breast cancer therapy today, is for a sensitive assay for the detection of residual disease once the primary tumour has been removed. All currently available detection methods for metastatic disease (bone scan, liver scan, ultrasound, X-rays etc.) are so insensitive that, unfortunately, tumour deposits generally remain occult until of such a bulk that successful eradication is impossible; furthermore, adjuvant treatment would make considerably more sense if clinicians had the means to select patients with metastatic disease most likely to benefit from it. Such a tumour marker could be of great value in clinical assessment of patient response to treatment, allowing unsuccessful, potentially harmful regimes to be discontinued at an early stage.

In common with other tissues of the female reproductive system, breast cancers may express oestrogen, progesterone and peptide hormone receptors; they may synthesize milk proteins in the same manner as active normal breast; and may, as with many other malignant tissues, and, indeed, normal breast, possess the ability to produce a variety of other substances, such as carcinoembryonic antigen (C.E.A.), enzymes, ferritin and so on (Coombes 1981).

Very many investigations in recent years have been aimed at evaluation of various biochemical markers expressed by breast cancer cells. The best known, and most promising, are probably carcinoembryonic antigen (C.E.A.), human chorionic gonadotrophin (H.C.G.), fucose, pregnancy associated alpha macroglobulin, breast cyst glycoprotein, urinary polyamines and nucleosides, placental alkaline phosphatase (PL.A.P.) and gamma glutamyl transpeptidase.

1.2.6.1. Carcinoembryonic antigen (C.E.A.)

Perhaps the most promising of potential "biomarkers" - and the most intensively studied is carcinoembryonic antigen (C.E.A.), a glycoprotein first detected in the serum and
tumour tissue of patients with colonic carcinoma (Gold and Freedman 1965), and subsequently in individuals with other malignant disease, such as tumours of the lung, ovary and breast (Hansen et al 1974). Borthwick et al (1977) reported that more than half of their series of patients with primary carcinoma of the breast and 70% of patients with metastatic disease had elevated C.E.A. levels, in comparison with only approximately 10% of normal controls. Most other studies report very comparable results with elevated serum levels in around 70-80% of patients with disseminated disease (Chu and Nemoto 1973; Tormey et al 1975; Myers et al 1978; Laurence et al 1972). Interestingly, fluctuations in serum C.E.A. levels have been reported in patients mirroring their clinical response to therapy (Borthwick et al 1977).

Cove et al (1979) demonstrated C.E.A. in the cytosols of 68% of primary breast carcinomas, and demonstrated that serum concentration of the antigen reflected initial tumour regression and subsequent progression after primary treatment. Quayle (1982) noted, however, that C.E.A. levels may not necessarily be valuable in monitoring disease progression, as observed fluctuations in serum levels may be representative of the interaction between such variables as synthesis, excretion and catabolism.

1.2.6.2. Human chorionic gonadotrophin (H.C.G.)

The study of Turmey et al (1977) suggests that human chorionic gonadotrophin (H.C.G.) may be detectable in the serum of as many as half of all breast cancer patients. There is evidence that the level of H.C.G. reflects tumour mass, and may mirror response to therapy. In addition, patients with lower levels of H.C.G. are likely to show a greater response rate to cytotoxic drugs.

1.2.6.3 Fucose

Several studies have reported elevated serum levels of the carbohydrate fucose in breast cancer patients compared with normal controls and individuals with benign breast disease. Again, reports indicate a high incidence (around 85-90%) of raised serum levels in patients with metastatic disease, a rise in level preceding obvious clinical recurrence, and a decline during response to therapy (Evans et al 1974; Waalkes et al 1978; Tatsumura et al 1977).

1.2.6.4. Pregnancy associated alpha macroglobulin

Anderson et al (1976) reported an elevation in pregnancy associated alpha macroglobulin level between 1 and 20 months prior to the appearance of clinically confirmed recurrent disease in a series of thirty breast cancer patients studied.
1.2.6.5. Breast cyst fluid glycoprotein

Haagensen and colleagues (1977) have evaluated the potential of a glycoprotein present in breast cyst fluid as a potential biomarker. Their results suggest the presence of elevated levels in most patients with obvious metastatic disease, and to a slightly lesser degree in patients with localised malignancy and with gross cystic disease.

1.2.6.6. Urinary polyamines and nucleosides

The levels of urinary polyamines and nucleosides have been reported to be raised in patients with malignant disease (Cohen 1977; Waalkes et al 1975).

1.2.6.7. Placental alkaline phosphatase (PL.A.P.)

Placental alkaline phosphatase (PL.A.P.) has also been forwarded as a potentially valuable marker in breast cancer (Nathanson and Fishman 1971; Wada et al 1979). Although further assessment is required (Rasmusson et al 1987), this enzyme does appear promising in the monitoring of patient disease status (Coombes 1981).

1.2.6.8. Gamma glutamyl transpeptidase

Gamma glutamyl transpeptidase is well known for its association with neoplastic and preneoplastic liver disease in animal models (Richards 1983). Chu and Douglas (1986) have demonstrated its association with hepatic metastases in human cancer.

1.2.6.9. Others

A wide variety of potential biochemical markers in breast cancer have been tested by Buckman (1984); they are listed below -

**Milk proteins**
- Casein
- Lactalbumin

**Enzymes**
- Sialyltransferase
- 5'-Nucleotidase

**Antigens**
- Response to T antigen
  - (in vivo/in vitro)
- Tissue polypeptide antigen

**Proteins**
- Immunoglobulin
- B2-Microglobulin
- G.C.D.F.P.

**Hormones**
- B-subunit of HCG
- Calcitonin

**Ferritin**
- Spleen type
- HeLa type

**Leukocyte**
- Adherence inhibition

**Miscellaneous**
- Immune complex, ASP, anti-MMTV, Sialic acid, Cu/Zn ratio,
Others include C-reactive protein (CRP); human tissue polypeptide antigen (TPA); and alpha-acid glycoprotein (alpha-AGP) (Williams and Buchanan 1987). All have given very disappointing results and none is specific for breast cancer.

1.2.6.10. Conclusion

Coombes et al (1981) in an extensive survey aimed at assessing the value of a large number of potential biochemical markers in breast cancer concluded that although several markers were found to be elevated in the majority of patients with advanced disease, only three - C.E.A., PL.A.P., and gamma glutamyl transpeptidase - in combination provided satisfactory monitoring of patient disease status. They reported a "lead interval" of approximately three months in around half of the patients studied before metastatic disease became clinically apparent.

Rasmusson et al (1987) in evaluating the value of C.E.A., PL.A.P., and others concluded that C.E.A. exhibited the greatest potential as a tool for early detection of relapse in breast cancer and for prognostic evaluation. PL.A.P. was described as being of limited value.

In conclusion, although a variety of biochemical markers have been described in relation to breast cancer disease status and prognosis, none is specific or unique to the disease. General markers such as C.E.A., H.C.G., or PL.A.P. may be of some limited value in monitoring patient recurrence status or response to therapy, and attempts have been made to use a series of markers to predict more accurately prognosis or relapse (eg Coombes 1981) At present, however, it is perhaps fair to say that biochemical tumour markers, although of great potential, are of rather limited clinical use.

1.2.7. GROWTH FACTORS. THEIR RECEPTORS. AND ONCOGENES

1.2.7.1. Epidermal growth factor and its receptor

Epidermal growth factor is a 55 amino acid, 6kDa molecule containing 3 disulphide linkages (Cohen 1983). It is found in many tissues, including breast, and is secreted in milk where its concentration is approximately 20-90ng/ml (Harris and Neal 1987). The physiological functions of epidermal growth factor remain unclear, but experimentally it has been shown to induce premature eye opening and tooth eruption in mice (Cohen 1962), maturation of foetal lung (Goldin and Opperman 1980), and may play a role in

The action of the epidermal growth factor is mediated through a specific membrane-bound receptor which has been identified at the cell surface in many different tissues including normal breast and breast cancers (Sainsbury et al 1985a; Fitzpatrick et al 1984). It is a 160-180kDa molecule, in two parts, with a 621 amino acid external domain (responsible for the binding of ligands including epidermal growth factor and transforming growth factor alpha) and a cytoplasmic 52 amino acid portion, linked by a short transmembrane section (Harris and Neal 1987; Downward et al 1984; Ullrich et al 1984).

The structure of the epidermal growth factor receptor has been shown to be similar to that of the c-erb-B-2 oncogene product, the oncogene product apparently representing a truncated form of the growth factor receptor (Downward et al 1984).

Binding of several growth factors, including epidermal growth factor, to their membrane-bound receptors has been shown to result in the activation of tyrosine-specific protein kinase. Phosphorylation of tyrosine residues is an unusual event in the normal cell, but has been commonly observed in malignant and transformed cells (Cooper et al 1982).

Epidermal growth factor receptor expression has been demonstrated to be associated with histologically poorly differentiated tumours (Sainsbury et al 1985c) and there also appears to be a strong inverse relationship between expression of the growth factor receptor and oestrogen receptor status (Sainsbury et al 1985a; Fitzpatrick et al 1984; Harris and Neal 1987). Correlations have also been demonstrated between growth factor receptor expression and increasing tumour size, and with lymph node involvement (Harris and Neal 1987).

Sainsbury et al (1985a) assayed a series of 104 primary breast cancers and 14 lymph node metastases for the presence of receptors for epidermal growth factor. 32% of the primaries and 71% of the metastases showed appreciable levels of E.G.F.-receptor. These results suggest that the presence of E.G.F.-receptor is associated with increased metastatic potential, and that the growth of some poor-prognosis oestrogen-receptor negative tumours may be regulated by growth factors binding EGF-receptor. Further studies (Sainsbury et al 1985b) indicate that human breast cancer fibroblast cell lines express E.G.F.-receptors and also synthesize an E.G.F.-like molecule - it has been proposed that E.G.F.-receptor positive breast cancers may be stimulated by E.G.F.-like substance derived from surrounding fibroblasts (Sainsbury et al 1985b).
1.2.7.2 Oncogenes

DNA sequences homologous to the transforming genes of certain RNA tumour viruses have been identified in normal, untransformed cells (Stehelin et al 1976; Spector et al 1981; Chance 1981), suggesting a possible association between these "oncogenes" and transforming potential (Slamon et al 1984).

The functions of oncogenes are not well understood, but they are conserved with great fidelity amongst vertebrates, suggesting a role in critical physiological functions (Bishop 1983; Shilo and Weinberg 1982). Some have been shown to code for proteins involved in control of cellular proliferation, such as protein kinases, DNA-binding proteins (Cline 1989) or for known growth factors or growth factor receptors, such as the c-erb-B gene and the epidermal growth factor receptor (EGFR) (Downward et al 1984).

Over-expression of certain oncogenes as a result of chromosomal translocation, genetic mutation or gene amplification may be implicated in transformation to malignancy as a result in over-production of normal or aberrant gene products leading to proliferative advantage or altered cell growth characteristics (Slamon et al 1984).

Alterations in the expression of several oncogenes by human breast cancers is reported in the literature. A number of these, including those of the ras gene family (Agnantis et al 1986; Ohuchi et al 1986; Theillet et al), c-scr (Rosen et al 1986) and c-myc (Tavassoli et al 1989; Escot et al 1986) appear to be commonly over-expressed in breast cancers, and this may have prognostic significance (see Gelmann and Lipmann 1987 for review).

Perhaps the most intensively studied oncogene with regard to breast cancer, and the one which appears to be of greatest importance prognostically, is c-erb-B 2.

The *c-erb-B* 2 oncogene

*c-erb-B* 2 encodes a protein which is thought to act as a transmembrane growth factor receptor of glandular epithelium (Yokota et al 1986).

Its expression is amplified in 10-40% of primary breast cancers (van de Vijver et al 1988; Slamon et al 1987; Barnes 1989) and a higher proportion of their metastases (Cline 1989).

A possible association between amplification of *c-erb-B* 2 gene and poor patient prognosis remains controversial, although consensus of opinion seems to suggest that it is real (Barnes 1989).

Slamon and colleagues (Slamon et al 1987;1989 Slamon and Clark 1988) report that
amplification is a significant and independent predictor of disease free interval and overall survival time in patients with breast cancer - a conclusion supported by Wright et al (1989) and the recent study of Lovekin et al (1989), but disputed in a number of other papers (eg. Berger et al 1988; Gusterson et al 1988; van de Vijver 1988).


Correlations with large tumour size (van de Vijver et al 1988; Borg et al 1989) poor histological grade (van de Vijver et al 1988; Barnes et al 1988; Wright et al 1989), high proliferative rate and steroid receptor negativity (Borg et al 1989; Wright et al 1989) have also been suggested.

It is interesting that over-expression of c-erb-B 2 has been reported to be particularly prominent in comedo type breast cancers, a histological tumour type associated with low steroid receptor content, high percentage of cells in S-phase, high mitotic index, pleomorphic nuclei with prominent nucleoli, severe necrosis, and particularly poor prognosis (van de Vijver et al 1988; Gusterson et al 1988; Borg et al 1989).

Perhaps it is not so surprising that oncogenes - genes coding for proteins which in many cases are believed to play critical roles in the growth and proliferation of normal cells - should be over-expressed in malignant tumours, and, furthermore, should be associated with cells of particularly aggressive malignancy.

The importance of c-erb-B 2, and other oncogenes, as potential prognostic indicators in breast cancer have yet to be fully evaluated; their relationship to other, established, markers such as lymph node status, grade, and S-phase fraction remains unclear.

**1.2.8. MONOCLONAL ANTIBODIES**

Numerous monoclonal antibodies have been raised against breast cancer-associated antigens and assessed for their specificity, and for correlation with established markers of breast cancer prognosis, such as histological grade, and the presence of lymph node and distant metastases. The subject is too diverse to be adequately reviewed here, but a number of the more important or more intensively studied monoclonal antibodies are listed below.
B72.3
Colcher et al (1981) and Nuti et al (1982) described a monoclonal IgG, called B72.3, raised against human metastatic breast cancer cells which, in an immunoperoxidase staining method, recognised around half of the primary breast cancers and two thirds of the metastases screened; it also bound 80% of colon cancer samples, and also other adenocarcinomas but did not cross-react with normal breast, other normal tissues or sarcoma (Johnson et al 1986; Johnston et al 1985). Reactivity appeared independent of tumour differentiation, but correlated with patient age (Sawtell et al 1984). The reactive antigen, "tumour associated glycoprotein 72", or "TAG-72", is a high molecular weight (>10^6 daltons) mucin-like molecule, it was detectable on the cancer cells of 22/23 malignant effusions taken from 21 patients with metastatic breast cancer (Johnson et al 1985).

Ki67
A monoclonal antibody Ki67 recognises a nuclear antigen present in proliferating cells (Gerdes et al 1983). It can be readily applied to frozen sections of breast cancer, and binding shows significant correlation with poor histological grade and high S-phase content (Walker and Campljohn 1988).

anti-M.F.G.M.
A number of antibodies have been raised against human milk fat globule membrane (M.F.G.M.) (a description of M.F.G.M. is given in section 4.4.1.2.of the Discussion) they show variable specificity, binding normal resting and lactating breast; primary breast cancers; and metastases (Arklie et al 1981; Foster et al 1982; Taylor-Papadimitriou et al 1981) as well as breast cells in culture (Edwards et al 1980).

Others
Antibodies have also been raised against many other antigens. Edwards (1980), for example, has described a monoclonal antibody which was raised against human breast fibroblasts - it was found to bind to most differentiating tissues, including breast. Colcher et al (1981) described an interesting antibody, initially raised against primary breast cancer metastasis to liver, which reacted with most primary breast cancers and also with their metastases.

1.2.8.1. Application to monitoring tumour burden:

No monoclonal antibody has yet been reported that can be used to detect tumour in asymptomatic individuals as an early diagnostic test, however, several have been reported that may be used to monitor tumour burden in breast cancer patients. For example, a monoclonal antibody, called DF3, (Kufe et al 1984) has been employed in radioimmunoassay to monitor the appearance of a 300,000 dalton antigen in patients.
Results indicate that 33/43 (76%) patients with metastatic breast carcinoma had elevated levels of serum antigen, in comparison with 3/36 controls (Hayes et al. 1985). The binding of the monoclonal antibody to tissue sections of breast cancer has been demonstrated to correlate with histological tumour grade and oestrogen receptor content of the tumours, and appears to be an independent marker of tumour differentiation. It also binds strongly to lactating mammary epithelium, and may thus be recognising a novel differentiation antigen (Lundy et al. 1985).

Rainsbury and colleagues (1983) produced a monoclonal anti-MFGM antibody, called LICR-LON-M8 which, labelled with radioactive indium 111, appeared valuable in visualising skeletal metastases in breast cancer patients.

Antibodies have also been raised against a high molecular weight glycoprotein (>500,000d) present in normal breast and breast cancers, its distribution being markedly altered in the latter. Elevated levels of the glycoprotein have been reported in the serum of patients suffering from metastatic breast cancer (Harvey 1984).

At the third Hamburg Symposium on Tumour Markers in December 1985, much emphasis was placed on the potential of new monoclonal antibodies, especially directed against "cancer antigen 15-3" (CA15-3), as tumour markers in breast cancer. CA15-3 is claimed to be both a sensitive and specific marker of breast cancer.

Investigations suggest that serum levels are elevated in patients with breast cancer; but, inconveniently, also in women with gynaecological disorders such as cancer of the ovary, endometrium (Paulick et al. 1986), uterus and cervix; some benign gynaecological disease; as well as nursing and pregnant women and normal healthy individuals (Kreienberg and Mobus 1986). Serum levels do, however, appear to mirror pre-operative tumour mass and correlate with lymph node status and the presence of distant metastases to some extent - post-operatively, patients showing tumour progression have high serum levels of CA15-3, those clinically free from the disease low levels (Schrock et al 1986). Assay seems to be of limited practical value as an early diagnostic screening test in breast cancer (Paulick et al. 1986; Crombach et al. 1986) but does carry potential as a means of detection of early metastatic disease or for monitoring the success of palliative treatment (Paulick et al. 1986; Van Dalen et al. 1986; Souchon et al. 1986; Crombach et al. 1986).

A number of workers have compared CA15-3 with other tumour markers, most notably CEA. Overall, CA15-3 appears to be a more sensitive marker than CEA (Van Dalen et al. 1986; Souchon et al. 1986; Jager et al. 1986); CA-15-3 and CEA employed in combination giving a more sensitive assay for detection of metastatic disease than either marker alone (Hoffman et al. 1986; Kreienberg and Mobus; 1986 Jager et al. 1986;
Schmid et al 1986). Between 80-90% of patients with metastatic disease show elevated serum levels of either CEA, CA15-3, or both (Kreienberg and Mobus 1986; Jager et al 1986).

1.2.8.2. Use in targetting toxins:

Specific monoclonal antibodies directed against tumour-associated antigens also carry the exciting possibility of use in specific cytotoxic therapy.

Although a minority of monoclonal antibodies actually possess the intrinsic ability to kill target cells expressing their antigen (eg see Houghton 1986; Hersey et al 1986), most potential immunotherapeutic approaches are likely to rely upon conjugation of specific antibody to a cytotoxic drug, poisonous lectin, or radionucleotide.

**Toxin-conjugated monoclonal antibodies**

The toxic lectin ricin has been investigated extensively as cytotoxic agent when conjugated to specific monoclonal antibodies. It has been shown to be active in culture and in animal models. Frankel et al (1984), for example, screened 95 hybridomas, the result of 52 fusions, for useful monoclonals raised against membrane extracts of human breast cancer cell lines. Twelve antibodies recognised most primary breast cancers and metastases. Ten of these were conjugated to the intensely toxic A chain of the lectin ricin; six of these showed specific killing of breast cancer cells in vitro.

One great disadvantage of using ricin as a poison is that it is intensely toxic to normal as well as malignant cells, and **specific delivery to target cells only** is essential in this type of approach (Wright and Cox 1987).

**Drug-conjugated monoclonal antibodies**

Cytotoxic drugs have also been linked to monoclonal antibodies for potential use in cancer therapy (eg. see Rowland et al 1985; Deguchi et al 1986). Many problems attend this approach - not least being the non-specific uptake by normal tissues, the high levels of antigen binding required to have any effect, tumour antigen heterogeneity leading to emergence of drug-resistant clones, and lack of drug internalisation by cells (Wright and Cox 1987).

**Radionucleotide-conjugated monoclonal antibodies**

Radionucleotide-conjugated monoclonal antibodies appear to carry the greatest potential at present - one tremendous advantage being that radionucleotides produce their cytotoxic effects over several cell diameters, thus negating the requirement for cell internalisation or even for direct binding. Ready localisation of radioactive material means that tracing of delivery and treatment efficacy is relatively straightforward.
Small clinical trials using radionucleotide-conjugated monoclonal antibodies have already been initiated (eg. see Carasquillo et al 1984; Pectasides et al 1986).

1.2.9. PROGNOSTIC CRITERIA IN ADVANCED DISEASE

The vast majority of breast cancer patients will ultimately die as a result of disseminated metastatic disease. For such individuals, the interval between diagnosis of the primary tumour and the appearance of secondary disease may vary from anything between a few months to several decades; however, once metastases become manifest, survival does not vary greatly among patients (Devitt 1976). Once this phase is reached, a quite different set of prognostic criteria apply.

1.2.9.1. Original clinical staging

Axillary node status of the patient at the time of primary treatment appears to be unrelated to survival after first recurrence, whether it be loco-regional (Patanaphaneet al 1984), or at a distant site (Brunet et al 1984). Similarly, the original clinical staging of the disease at diagnosis has little bearing upon prognosis once metastatic disease is apparent (Stoll 1986).

1.2.9.2. Hormone receptor status

Post-relapse survival appears to be significantly extended in endocrine receptor (oestrogen receptor and progesterone receptor) positive patients, most probably due, in the main, to the fact that it is these patients who are most likely to respond favourably to endocrine manipulation - sometimes delaying the natural course of the disease by many months or even years (Howell et al 1984; Powles 1981; McGuire 1978).

1.2.9.3. Site and extent of metastatic disease

Individual patient survival diminishes with increasing numbers of sites involved with metastatic disease, and also with the size of the deposits themselves.

Visceral metastases of brain, spinal cord, liver, peritoneum and lymphangitis carcinomatosa of lung carry a particularly poor prognosis - perhaps because of the gross extent of organ involvement often seen by the stage that detection becomes possible - mean duration of survival being in the order of only around six months. Discrete deposits in lung, and involvement of pleura herald an average life expectancy of approximately twenty months (Cutler et al 1969). Patients with osseous metastases fare
slightly better, having a significantly more favourable prognosis than those with either visceral or soft tissue involvement. Sclerotic lesions in particular herald favourable survival prospects (Stoll 1986).

1.2.9.4 Disease free interval

Many authors have reported that patients with extended disease free interval following primary treatment are likely to enjoy prolonged survival following relapse (Cutler et al 1969; Myers et al 1973). However, although there is considerable evidence that this may be true as far as loco-regional disease is concerned, it is not generally true of visceral involvement (Bedwinek et al 1981; Toonkel et al 1983; Stoll 1976).

In recent years, our concepts regarding the growth of breast cancers have changed dramatically. Instead of an idea of all tumours proliferating steadily, with cell doubling times remaining constant at anything between days, months or even years, their progression only interrupted by odd periods of unexplained dormancy (Spratt et al 1963), we now believe tumour growth to undergo periods of accelerated or decelerated proliferation during different stages of the disease course. In addition, there is increasing evidence that cell populations within the tumour may change considerably over time, the trend being ever towards de-differentiation and the selection of more aggressive, more metastatic, and treatment resistant clones.

It is this phenotypic instability which makes those prognostic variables highly relevant at an early stage in the disease, obsolete at a later point in tumour progression.

1.2.9.5 Response to therapy

Perhaps the factor most powerfully influencing post-recurrence prognosis, is patient response to systemic therapy.

It is well established that patients with endocrine receptor positive tumours are more likely to respond to endocrine manipulation. In addition, Stoll (1977) has related the likelihood of response to patient age and menopausal status. Such a relationship does not appear to apply to response to cytotoxic drugs.

Many other complex variables may influence the patient's response to treatment. Complete tumour regression may be associated with longer survival than partial regression: the extent of regression being generally dependent upon the proportion of proliferating cells within the tumour mass - the most complete remissions thus being associated with tumours with a high proliferative cell content. Complete, although often temporary, regression following combination chemotherapy is thus most common in
patients with aggressive disease and short (<5 years) disease free interval (Decker et al 1979).

It is clear that the prognosis of the patient post-relapse is largely mirrored by the duration of tumour regression following therapy. This in turn will be largely dependent upon (1) the extent to which the tumour load was diminished by treatment and (2) the rate of subsequent re-growth. Overall, longer remissions are generally seen in patients who generally showed extended disease free interval following primary treatment (Swenerton et al 1979).
1.3.1. DEFINITION.

1.3.2. EARLY HISTORY.
1.3.2.1. Toxic plant extracts.
1.3.2.2. Lectins from diverse sources.

1.3.3. INHIBITION OF LECTIN INDUCED CELL AGGLUTINATION.

1.3.4. LECTINS IN BLOOD GROUPING.

1.3.5. LECTIN BINDING SUGAR SPECIFICITY.

1.3.6. SELECTIVE LECTIN BINDING TO MALIGNANTLY TRANSFORMED CELLS.

1.3.7. THE LECTINS OF *HELIX POMATIA* - THE EDIBLE OR ROMAN SNAIL.
1.3.7.1. Discovery.
1.3.7.2. Blood group specificity.
1.3.7.3. Carbohydrate specificity.
1.3.7.4. Isolation.
1.3.7.5. Three dimensional structure.
1.3. LECTINS - A BRIEF HISTORICAL INTRODUCTION

1.3.1. DEFINITION

The term "lectin" was first coined by Boyd and Shapleigh in 1954. It is derived from the Latin verb "legere" which means to pick out, to select or to choose. It was originally applied to a group of proteins, isolated predominantly from plants, which exhibited remarkable sugar-binding specificity and which agglutinated cells.

Subsequently, many similar proteins have been described from a diversity of biological sources as wide ranging as mammals, slime moulds, fungi, invertebrates, and bacteria; they can be either soluble in biological fluids, or membrane-bound. Consequently, considerable controversy has arisen over the definition of this very heterogeneous group.

Today the generally accepted definition of the term "lectin" is that outlined by Goldstein et al (1980) and adopted by the Nomenclature Committee of the International Union of Biochemistry: A lectin is "a carbohydrate-binding protein of non-immune origin that agglutinates cells and/or precipitates polysaccharides or glycoconjugates".

This definition implies that lectins are:

1) Multivalent - two or more sugar-binding sites are necessary for the cross-linking of animal or plant cells in agglutination, or of glycoconjugates/polysaccharides in precipitation.

2) Of non-immune origin - to distinguish lectins from antibodies directed against carbohydrates, which may also act as agglutinins. Many lectins have been described from plants, fungi, bacteria, and other organisms which do not synthesize immunoglobulins; and in contrast to immunoglobulins which are structurally homologous, lectins are structurally diverse, varying considerably in molecular weight, amino-acid composition, metal requirement, and three-dimensional structure - in this respect, and in their sugar specificity, they much more closely resemble enzymes.

3) Not enzymes - the definition excludes most sugar-binding enzymes (glycosidases, glycosyltransferases, glycosylkinases etc.) as it specifies more than one binding site. Under some circumstances, sugar specific enzymes with multiple combining sites agglutinate cells and/or precipitate glycoconjugates, and so may act as a lectin.

4) In spite of similarities to true lectins from the same source, toxins which bear only one
sugar binding site - such as the toxic B chain of ricin - should not be called lectins since they do not agglutinate cells or precipitate glycoconjugates.

The actual functions of lectins within the organisms that produce them remain for the most part unknown. In plants, it has been proposed that lectins may play some crucial role in the transport or storage of carbohydrates in seeds (Ensfgraber 1958; Kauss and Glaser 1974); binding of nitrogen fixing bacteria in root hairs (Hamblin and Kent 1973) inhibition of fungal growth (Albersheim and Anderson 1971) and the feeding of insects (Janzen et al 1976).

1.3.2. EARLY HISTORY

1.3.2.1. Toxic plant extracts

Lectins were first described during early investigations into the toxic principles of plant extracts used at that time in medicine, but have subsequently been shown to be present within a diverse range of plants, animals, fungi and bacteria. They first aroused interest during the late 1800's and the early years of this century.

During this period, "haemagglutinins" were shown to be proteins; they played an important part in establishing some of the fundamental principles of immunology; their ability to selectively agglutinate cells and precipitate glycoproteins and polysaccharides was established - as was the inhibition of these reactions by certain carbohydrate-rich mixtures.

The lectin story really begins with Stillmark, who during work for his doctoral thesis in 1887 - 1888, at the University of Dorpat in Estonia, one of the oldest universities in Czarist Russia, investigated extracts of seeds of the Euphorbeaceae. A toxin had been described in an aqueous extract of castor oil seeds, Ricinus communis, (figure 7, overleaf) by Dixon in (1887). Stillmark (1888; 1889) isolated and semi-purified a protein, which he called "ricin" from the same source and tested its effects upon erythrocytes, liver cells, leukocytes and epithelial cells, noting an agglutination reaction "like in clotting". Erythrocytes of different animal species reacted in different ways.
Bruylants and Venemann (1884) in Belgium and Warden and Waddel (1884) working under Koch in India, had already published studies on the toxin of *Abrus precatorius*, the jequirty bean (Figure 8, overleaf). Warden and Waddel named the substance, which they assumed to be a protein, "phytoalbuminose". Hellin (1891) went on to investigate the properties of this toxin, which he called "abrin", and showed that it would agglutinate erythrocytes and precipitate serum.
Power and Cambier (1890) isolated a toxin from the bark of the black locust (*Robinia pseudoacacia*) (figure 9, overleaf), which they named "robin", and later Mendel (1909) prepared a haemagglutinin from the seeds of the same plant. Previously, the haemagglutinating effect of plant extracts had been naively and incorrectly attributed to the toxin. For abrin and ricin it was relatively recently that the lectins responsible for toxicity were distinguished from those causing agglutination of cells (Olsnes and Pihl 1982).
Elfsrand (1897; 1898) demonstrated dramatic differences in the agglutinating effect of crotin, a lectin from Croton tiglium, on erythrocytes of a large number of different animals - ranging in intensity from no discernable effect or mild clumping to complete lysis; and Stillmark's original observations of the variable effects of ricin on the red blood cells of different animals were confirmed by several other workers.

1.3.2.2 Lectins from diverse sources

Landsteiner, an immunologist perhaps best remembered for his discovery of the ABO blood group system, along with his co-worker Raubitschek (1907) described a number of haemagglutinins from seeds of the Leguminosaeae and Viciaceae families. They described these substances as being proteins; soluble in water, but not in alcohol; thermolabile; non-dialysable; as giving positive buiret and xanthoprotein reactions; and as capable of
being salted out by electrolytes. Landsteiner also noted the variability of the agglutination effect upon human erythrocytes but, perhaps surprisingly, makes no mention of blood group specificity.

Over the next twenty years, a great many papers appeared describing dozens of new, non-toxic, haemagglutinins from a diversity of sources. Perhaps owing to ease of availability, most concentrated upon botanical material, and it became apparent that seeds of the *Leguminosae; Euphorbeaceae;* and *Solanaceae* were particularly rich sources. However, lectins were described in bacteria and viruses (Kraus and Ludwig 1902; Flexner 1912; Lansteiner 1906), fungi (Ford 1907), invertebrates (eg Noguchi 1922), and even the venom of snakes (Flexner and Noguchi 1902).

### 1.3.3. INHIBITION OF LECTIN INDUCED CELL AGGLUTINATION

Stillmark, in his thesis of 1888, noted that the presence of serum effectively inhibits the agglutination of erythrocytes by ricin. Kraus (1902) went on to investigate this phenomena, and later Landsteiner and Raubitschek (1909) showed that porcine gastric mucin could also reverse lectin-induced clumping of cells. The significance of these observations was at the time overlooked, but they were the first indication of the carbohydrate-binding properties of lectins.

Watkins and Morgan (1952), more than forty years later, were the first to show that simple sugars are capable of inhibiting lectin activity.

### 1.3.4. LECTINS IN BLOOD GROUPING

Landsteiner (1907) was perhaps the first to note that crude lectin extracts did not always agglutinate the red blood cells of all individuals to an equal extent; it was, however, not until thirty years later that the reason for this became apparent with the first reports of blood group specific lectins.

In 1935, Sugishita described two agglutinins from *Anguilla japonica*, the Japanese eel - one clumped human erythrocytes non-specifically, the second showed a particular affinity for those of blood group O individuals; and in 1944, Jonsson described an agglutinin from *Anguilla anguilla* which also showed affinity for blood group O cells. These substances were originally assumed to be antibodies and their non-immune origin was not realised until relatively recently.
In 1949, Boyd and Reguera reported that a crude saline extract of dried lima beans
(*Phaseolus lunatus* sp. *limensis*) would clump human blood group A erythrocytes, but had
no effect on group B or O cells.

![FIGURE 10: Haemagglutination. Blood group A erythrocytes clumped by a lectin from *Dolichos biflorus*, the horse gram, which binds N-acetyl galactosamine moieties.](image)

An enormous amount of interest in the search for blood group specific lectins followed (see
Makela 1957; Bird 1959; Boyd 1963 for reviews), notably several papers appeared
reporting results of the screening of many hundreds of plant extracts for possible use in
blood typing.

Perhaps owing to ease of availability and to the simple preparative procedures involved, it
was predominantly plant species which were screened in vast numbers for the possible
blood group specificity of their lectins. Between 1945 and 1964, more than one hundred
plant species were shown to possess blood group specific agglutinins. However, a number
of extracts from other, more bizarre sources were also tested. The sera of some fish was
demonstrated to possess intraspecies selectivity in agglutinating activity and some species
did appear to show blood group affinity (Cushing 1952; 1953; Sindermann 1958; 1961).
Agglutinins of varying specificity were described from the venom of *Vipera aspis* (Dujarric
de la Riviere et al 1954).

Blood group typing is now routinely performed by using anti-A or anti-B antibodies.
However, lectins do remain useful in typing as no natural anti-O antibody is available, and
are routinely used for detecting secretors - individuals who secrete blood group substances

in their saliva, urine, and other body fluids (Sharon 1977).

Morgan and Watkins (1959) were the first to demonstrate that blood group specificity of lectins was a direct result of the sugar-binding specificity of the agglutinin. They showed that agglutination by lima bean lectin, specific for blood group A, could be inhibited by N-acetyl-D-galactosamine; whilst agglutination by *Lotus tetragonolobus* lectin, specific for blood group O cells, was inhibited by α-methyl-L-fucose. This was one of the first pieces of evidence for the presence of carbohydrates on the cell surface.

1.3.5. LECTIN BINDING SUGAR SPECIFICITY

The specificity of a lectin is usually expressed in terms of the simple monosaccharide which inhibits its effect with the greatest molecular efficiency. For example, D-galactose for peanut lectin (*Arachis hypogaea*); N-acetyl-D-galactosamine for Horse gram (*Dolichos biflorus*) etc. However it is an important point that the actual receptor that a lectin molecule "sees" is generally larger and more complex than a simple monosaccharide: usually it contains several sugar molecules and sometimes amino acids as well. Two lectins with the same "sugar specificity" may thus actually recognise quite different structures, and may exert quite different effects upon living cells.

In looking at blood group specificity, the remarkable selectivity of lectins is reflected in their distinguishing between erythrocyte sub-groups; for example, *Dolichos biflorus* lectin reacts more strongly with red cells of blood group A1 than those of A2; *Vicia graminia* lectin will agglutinate group N cells, whilst *Iberia amava* lectin recognises group M cells. Another interesting example is that of Lima bean lectin and Soyabean lectin - both bind N-acetyl-galactosamine, but only Lima bean lectin is blood group A specific. This is because it is specific for the alpha-linked configuration of the sugar molecule; soyabean will bind both the alpha- and beta- linked type. Both configurations may be found on cell surfaces, but only the alpha-linked configuration is seen on group A erythrocytes (Sharon and Lis 1977).

1.3.6. SELECTIVE LECTIN BINDING TO MALIGNANTLY TRANSFORMED CELLS

Aub was one of the first scientists to investigate the idea that the difference between normal and malignantly transformed cells lay in alterations in their surface components. He incubated malignant and normal cells with a range of enzymes and noted that a crude preparation of lipase from wheatgerm agglutinated malignant cells but left their normal counterparts unaffected (Aub et al 1963). Further investigations revealed that it was not after all the lipase which was responsible for the selective clumping of the cancer cells, but a lectin, now known as wheatgerm agglutinin (Burger and Goldberg 1967).
The work was taken up by Burger of Princetown University, who purified the lectin and employed it in extensive studies of the surface changes which attend malignant transformation.

Subsequently, Sachs and Inbar of the Weizmann Institute discovered that Concanavalin A, at that time a much more readily available lectin, would also preferentially agglutinate malignant cells; and Sachs et al. demonstrated a similar property in soyabean agglutinin (Sachs and Inbar quoted by Sharon 1977).

In the relatively short period since these original observations, an enormous number of lectins have been tested and found to selectively agglutinate transformed, but not normal, cells from diverse sources (See Burger 1970; Rapin and Burger 1974; Nicolson 1974; 1976; Poste 1975; for review). Generally, lectins will agglutinate transformed cells at a very much lower concentration than that required to clump their normal counterparts. This selective activity is independent of the means by which the transformation was achieved. For example, normal rat, hamster, and mouse fibroblasts show no agglutination at 500μg/ml of Concanavalin A, but strong agglutination is seen after transformation with SV-40 virus, polyoma virus, X-rays, or chemical carcinogens at concentrations of the lectin as low as 1μg/ml (Inbar and Sachs 1969; Inbar et al 1972; Weber 1973).

1.3.7. THE LECTINS OF HELIX POMATIA - THE EDIBLE OR ROMAN SNAIL.

FIGURE 11: Helix pomatia, the edible or Roman snail.
1.3.7.1 Discovery

Lectins were first reported to be present in the haemolymph of invertebrates during the early years of the twentieth century. Cantacuzene (1915) tested the haemolymph of *Helix pomatia*, the edible or Roman snail, but failed to detect haemagglutinating activity. It was not until fifty years later that the lectin present in the albumin gland of the animal was discovered by Prokop et al (1965).

The work of Vretblad (1979), using the technique of isoelectric focussing on pooled or individual samples, has since demonstrated the presence of at least a dozen distinct *Helix pomatia* isolectins.

1.3.7.2 Blood group specificity

*Helix pomatia* lectin will selectively agglutinate human erythrocytes of blood group A; but not O or B (Prokop et al 1965a and b; Hammarstrom and Kabat 1969).

1.3.7.3 Carbohydrate specificity

GalNAc-α-1,3GalNAc > α-GalNAc > α-GlcNAc >> α-Gal

The carbohydrate-binding specificity of *Helix pomatia* lectin has been investigated using either displacement assays of, for example, its binding to Sephadex (Ishyama and Uhlenbruck 1972); or by inhibition of the precipitation of blood group A substance (Hammarstrom and Kabat 1969).

Hammarstrom and Kabat (1969) attempted to inhibit lectin-induced precipitation of blood group A substance by competitive inhibition with a number of simple sugars. Mannose, glucose, galactose, glucosamine and galactosamine had no inhibitory effect; N-acetyl-glucosamine showed weak inhibitory activity; but by far the most efficient monosaccharide inhibitor was N-acetyl-galactosamine. The alpha form being preferred to the beta form of the molecule.

More recently, Baker et al (1983) demonstrated GalNAc-α-1,3GalNAc to be an even more effective inhibitor.

The lectin's carbohydrate specificity makes it a useful probe for the detection of terminal
non-reducing N-acetyl-alpha-galactosaminyl end groups on molecules or at the cell surface (Prokop et al 1965a and b; Hammarstrom 1973; Uhlenbruck and Prokop 1966).

1.3.7.4 Isolation

Isolation of *Helix pomatia* lectin may be performed by affinity chromatography using insolubilised human or hog blood group A substance (Hammarstrom and Kabat 1969; Hammarstrom 1973) - or N-Acetyl-galactosamine on Sepharose beads - followed by elution with N-Acetyl-galactosamine (Vretblad et al 1979). An alternative procedure is adsorption onto Sephadex G100 or G200 beads - although the lectin has only slight affinity for alpha-glucose, the large number of available binding sites provided by the beads appears to be sufficient for effective adsorption. The lectin may be eluted with either N-Acetyl-galactosamine; galactose; glucose; or mild acid (Ishiyama and Uhlenbruck 1972; Kuhnemund and Kohler 1969).

1.3.7.5 Three dimensional structure

Hammerstrom and his colleagues (see below) have analysed the three dimensional structure of the *Helix pomatia* lectin molecule in some detail.

It contains a high proportion of proline residues, and many acidic and hydroxylic amino acids (Hammarstrom and Kabat 1969; Hammarstrom 1973 Hammarstrom et al 1972). There are eighteen half-cystine residues (no free sulphhydryl groups) and ten methionine residues, and a very high content of galactose and mannose (Hammarstrom and Kabat 1969; Hammarstrom et al 1972). The lectin has an Mr of 79,000. It consists of six identical polypeptide chains each with an Mr of 13,000. Each chain has an intrachain disulphide bond and a single carbohydrate binding site. The chains are linked in dimers, each with an Mr of 26,000, by interchain disulphide bonds; and the dimers are then linked together by non-covalent interactions (Hammarstrom et al 1972; Hammarstrom and Kabat 1971).
1.4.1. CONCEPT.

1.4.2. TUMOUR LECTINS.

1.4.3. THE USE OF NEOGLYCOPROTEINS IN DETECTION OF TUMOUR LECTINS.

1.4.4. PURIFICATION AND CHARACTERISATION OF TUMOUR LECTINS.

1.4.5. ENDOGENOUS LECTINS OF MAMMARY TUMOURS.

1.4.6. DIFFERENCES IN EXPRESSION OF ENDOGENOUS TUMOUR LECTINS IN RELATION TO METASTATIC POTENTIAL.
1.4. LECTINS OF HIGHER VERTEBRATES AND THE CONCEPT OF HUMAN "ENDOGENOUS LECTINS".

1.4.1 CONCEPT

Over the past decade or so, a number of lectins have been described in higher vertebrate - including human - tissues; and there is much interest in the, as yet largely unidentified, role of these glycoconjugates.

The lectins of higher vertebrates have been described as either

(1) integrated into the cellular membrane - and thus, apparently, instrumental in the transport of glycoconjugates into cells (Ashwell and Hartford 1982), or

(2) freely soluble - and perhaps involved in the secretion / organisation of extracellular glycoconjugates (Barondes 1984).

A brief review of some of the better characterised lectins of higher vertebrates is given in Appendix I.

1.4.2. TUMOUR LECTINS

In recent years there has been a sudden upsurge of papers reporting endogenous lectin expression by malignant cells. The biochemical characterisation of these proteins, and the demonstration of differences in their distribution between tumours of differing biological behaviour raises evidence for the possibility that endogenous tumour lectins and their interaction with glycoconjugates may influence the pathogenesis of cancer.

Hans-J. Gabius and co-workers have published copiously on the subject of endogenous tumour lectins, their list of publications include at least two comprehensive overviews of the subject (Gabius 1988; Gabius et al 1986).

1.4.3 THE USE OF NEOGLYCOPROTEINS IN DETECTION OF TUMOUR LECTINS

A group of synthetic compounds termed "neoglycoproteins" have been widely employed in the detection of endogenous tumour lectins. They are synthesized by linking a specific sugar - or sometimes a complex oligosaccharide chain - to an inert, non-glycosylated protein, most usually bovine serum albumin (BSA). These novel moieties can be readily labelled, for example with biotin, facilitating their use as simple and direct histochemical probes for lectins in tissue sections.
Great success has been achieved using this approach, and the distribution of endogenous tumour lectins has been mapped in systems as diverse as human meningiomas (Bardosi et al 1988), murine Ehrlich's tumour (Kojima and Gabius 1988) and human normal placenta vs chorion-epithelioma (Gabius et al in press), as well as numerous others. Comparative studies using a selection of neoglycoproteins have so far shown that analyses of this type reveal differences between normal and tumour tissues, between different types of tumour, and within a single class of tumour (Gabius 1988); for example, in malignancies of breast (Gabius et al 1988), lung (Kayser and Gabius 1988), and brain (Bardosi et al 1988).

1.4.4. PURIFICATION AND CHARACTERISATION OF TUMOUR LECTINS

Lectins have been isolated from preparations of tumour cell lines, and from human and animal tumours. Most commonly the experimental approach has been conventional affinity chromatography followed by SDS-PAGE. Some lectins, purified in this manner, appear to be novel carbohydrate-binding proteins, never described before, for example, a mannan specific lectin, molecular weight 68kDa., isolated from human teratocarcinoma (Gabius et al 1985a) or a β-galactoside binding protein, apparent molecular weight 58kDa., in human Ewings sarcoma (Gabius et al 1986b); others, for example, in human epithelial tumour (Gabius et al 1985b) and rodent rhabdomyosarcoma (Gabius et al 1984) are normal constituents of embryonic or adult tissues.

1.4.5. ENDOGENOUS LECTINS OF MAMMARY TUMOURS

Differences have been described in the binding of biotinylated neoglycoproteins between normal, benign and malignant human breast tissues. (Schauer et al 1988; Gabius et al 1988). Benign and malignant breast lesions revealed increased binding to a mannosyl-receptor probe, in comparison to normal breast. Cytoplasmic receptors for lactose and N-acetyl-glucosamine could be detected only in certain types of malignancy, nuclear receptors for these neoglycoproteins were detected in nuclei of normal breast specimens. Staining patterns with galactosyl-receptor probes also appeared to differ between various types of breast cancer (Gabius et al 1988).

Gabius et al (1986a) using monoclonal antibodies against three β-galactoside specific lectins has demonstrated heterogeneity of the endogenous lectin patterns in breast cancers and differences between normal, benign and malignant breast. In normal breast, endogenous lectins were expressed by different cell types: lectins with apparent molecular weights of 14.5 kDa. and 18 kDa. were preferentially localised by fibroblasts, smooth muscle, endothelial and myoepithelial cells. A 29kDa. lectin was mostly localised in luminal cells and their secretory product. In breast carcinomas, the three lectins had different levels of expression as detected by antibodies, with only occasional cells staining with anti-14.5,
more numerous cells staining with anti-18, and almost all staining with anti-29. In benign lesions, the L-14.5 was absent, L-18 and L-29 were present.

1.4.6. DIFFERENCES IN EXPRESSION OF ENDOGENOUS TUMOUR LECTINS IN RELATION TO METASTATIC POTENTIAL

Raz et al (1984) raised a series of monoclonal antibodies against B16 melanoma galactoside-specific lectin. One of the antibodies bound several types of cultured human and murine cells, including melanoma, sarcoma and carcinoma. The antibody was used in a quantitative analysis of endogenous lectin on the surface of non-malignant, malignant and metastatic cells of rodent and human origin. It appeared to react strongly with the transformed cell variants, whereas normal cells presented negligible or relatively low densities of cell surface lectin (Raz et al 1986).

In a comparative study, monoclonal antibodies were raised against two lectins, molecular weight 14kDa. (L-14.5) and 34kDa. (L-34), purified from a murine fibrosarcoma cell line. Immunoprecipitation studies revealed the presence of only L-14.5 in normal rat embryonal fibroblasts, but in contrast, both L-14.5 and L-34 were found in oncogene-transfected, immortalised cell clones derived from normal rat cells, and untransformed clones selected for expression of transformed, malignant and metastatic phenotypes. In the latter system, a marked increase in the amount of cellular and cell surface lectin was observed upon progression to the metastatic phenotype. These results suggest that expression of endogenous tumour-cell surface lectins is associated with transformation and metastasis (Raz et al 1987a).

cDNA clones coding for the L-14.5 and L-34 have been isolated. RNA and DNA blot analyses revealed that the two lectins represent products of two different genes. Differential levels of specific mRNA's were found when low- and high-metastatic counterparts of the fibrosarcoma cell line were compared, and these genes were not expressed in detectable amounts in normal mouse liver (Raz et al 1987b).

Differences in lectin expression between rodent tumour cell lines with high and low metastatic potential have been demonstrated by Gabius and associates (Gabius et al 1986b; 1986c; 1987). For example, the metastatic variant of a tumour model cell line derived from virally transformed rat fibroblasts has been shown to differ qualitatively from the parental clone in that each express a number of novel carbohydrate-binding proteins (Gabius et al 1986c).

These studies, and others, are of great relevance in that they may go some way towards helping our understanding of some of the complex interactions perhaps involved in the transformation to malignancy and to metastatic competence.
1.5.1. STUDIES ON TUMOUR CELL LINES.
1.5.1.1. Terminal sialic acid and metastatic potential in wheatgerm agglutinin (WGA) resistant mutants.
1.5.1.2. Peanut agglutinin (PNA) as a marker of metastatic potential.
1.5.1.3. Other lectins.

1.5.2. STUDIES ON LECTIN BINDING TO BREAST CANCERS.
1.5.2.1. Con A and lentil (glucose/mannose-binding) lectins bind selectively to malignant but not to benign breast.
1.5.2.2. Wheatgerm (GlcNAc/sialic acid-binding) lectin as a possible marker of histological tumour differentiation.
1.5.2.3. L-PHA, a lectin from Phaseolus vulgaris, (binds complex oligosaccharide) binds more strongly to malignant than benign breast.
1.5.2.4. Ulex europaeus I (L-fucose binding) lectin - a proposed marker of tumour metastasis to local lymph node.
1.5.2.5. Peanut (Gal-GalNAc-binding) lectin and steroid receptors.

1.5.3. STUDIES ON HELIX POMATIA LECTIN BINDING TO BREAST CANCER.
1.5. LECTIN BINDING AND CANCER METASTASIS

The remarkable sugar-binding specificity of lectins makes them splendid tools for investigation of the carbohydrate populations present on the surfaces of cells. Numerous studies have catalogued the lectin binding characteristics of normal and malignant human and animal tissues, as well as cell lines in culture. Of particular interest are the investigations of the differences in carbohydrate expression between tumours of high- and low- metastatic potential.

1.5.1. STUDIES ON TUMOUR CELL LINES

1.5.1.1. Terminal sialic acid and metastatic potential in wheatgerm agglutinin (WGA) resistant mutants

A number of studies, a few of which are described below, have centred upon "lectin resistant" cells. In addition to their sugar binding properties, the cytotoxicity of many lectins makes them excellent probes for selection of cell variants with altered cell surface carbohydrates. Cells often retain their resistance to a particular lectin even when cultured in its absence for several months, indicating a stable mutation with low reversion rates. The phenotypes of such mutants have been characterised in many studies and the biochemical basis for their lectin resistance elucidated.

The development of wheatgerm lectin-resistance by tumour cells has been linked with loss of metastatic potential in a number of cell lines: Dennis and co-workers, for example, have intensively studied the oligosaccharides expressed by murine tumour cell lines with varying metastatic potential. Growth of a wheatgerm lectin-resistant spontaneous mutant line, MDW4, which is non-metastatic in syngeneic mice in harsh comparison to the highly metastatic wheatgerm lectin-binding parent line, MDAY-D2 (Dennis et al 1984). Dennis et al (1985) have also developed a second mutant line, resistant to Bandeiraea simplicifolia lectin II (B.S.II), from the MDW4 cells; the double-mutant B.S.II resistant cells showed a partial return to the original malignant parent phenotype. Detailed analysis of carbohydrate expression by these cell classes suggests that the MDW4 cells are deficient in sialic acid and galactose. Sialation of terminal chain carbohydrates may enhance metastatic potential (Altevogt et al 1983; Dennis et al 1986).

Several other studies have demonstrated a difference in metastatic potential between wheatgerm binding and non-binding cell lines. Irimura et al (1986) demonstrated enhanced WGA-binding to sialoglycoproteins in a highly metastatic lung colonising variant of a murine large-cell lymphoma/lymphosarcoma cell line; Benedetto et al (1989) and Elia et al
(1988) showed a marked decrease in metastatic ability in wheatgerm resistant tumour cell variants of the normally highly aggressive Friend leukemia cell line. The major difference in the metastatic and non-metastatic variants appears again to be the increased sialation of high molecular weight glycoproteins in wheatgerm binding cells. Ishikawa and colleagues (1988;1989) selected a spontaneous WGA-resistant variant from the human malignant melanoma MeWo line. The WGA-resistant cells had lost their ability to metastasise and were found by biochemical analysis to be phenotypically similar to the non-metastatic, WGA-resistant MWD4 cells of Dennis et al.

A poorly metastatic variant of the normally highly aggressive murine T-cell lymphoma ESb cell line differs from the parent strain in its binding of N-acetylgalactosamine lectins. In the parent line, the N-acetylgalactosamine residues are masked by sialic acid (Altevogt et al 1983; Fogel et al 1983). Again the authors conclude that masking of terminal carbohydrate residues by sialic acid is of crucial importance in the metastatic process. The presence of terminal sialic acid on the high molecular weight glycoproteins at the surface of cells of these cultured tumour lines appears to be strongly related to their metastatic competence.

1.5.1.2. Peanut agglutinin (PNA) as a marker of metastatic potential

A number of studies have strongly implicated peanut lectin as a marker of increased malignant potential in various tumour systems. Limas and Lange (1986), for example, in a very straightforward paper, looked at PNA binding to fresh and paraffin-processed sections normal and neoplastic urothelium. Peanut lectin did not bind to normal urothelium; but bound to 10% of non-invasive and 65% of invasive carcinomas.

Steck and Nicolson (1983;1984) in their work on the rat mammary adenocarcinoma cell line 13762NF (13762NF mimics human breast carcinoma in its pathology and pathogenesis; it spontaneously metastasises to a number of sites including lymph node and lung) examined the lectin-binding affinities of two clones, one of high- and one of low-metastatic potential. In this study, the cells were first treated with the enzyme neuraminidase to strip any sialic (neuraminic) acid present. Highly metastatic cells bound strongly to peanut lectin; wheatgerm lectin binding decreased with increasing metastatic potential; and the binding of Concanavalin A was the same in all cell clones. The intensity of PNA labelling of a de-sialated galactoprotein band, of molecular weight 580 kDa., detected by SDS-PAGE was seen to correlate with clone metastatic potential. In addition, a second band, of ~80kDa., corresponding to a sialoglycoprotein decreased in labelling intensity on clones of increasing metastatic potential. The results suggest quantitative changes in cell surface glycoproteins rather than major qualitative alterations are associated with differences in the metastatic behaviour of these tumour cell clones.

In a similar study (Badenoch-Jones 1987) on a highly metastatic variant, "clone 4", of the
otherwise poorly metastatic rat mammary adenocarcinoma line DMBA-8, the highly metastatic cells were demonstrated to bind peanut agglutinin and *Ulex europaeus* agglutinin I (UEAI). In SDS-PAGE four *Ulex* lectin-binding bands of subunit molecular weight greater than 100,000 were identified in the metastatic variant which were not present in the parent cell line. The bands were also identified in another highly metastatic rat mammary adenocarcinoma MAT 13762-B.

Barnett and Eccles (1984) examined the binding of a panel of fluorescein-labelled lectins to cell lines isolated from a mouse mammary adenocarcinoma which differ markedly in their morphological and metastatic properties. Galactose moieties, detected by PNA-binding, and N-acetylgalactosamine residues, detected by soyabean lectin, were expressed by all highly metastatic clones; but only to a limited extent by clones of limited metastatic potential. Pre-treatment by neuraminidase abolished these differences, increasing PNA- and SBA-binding to poorly metastatic lines, suggesting that galactose and N-acetylgalactosamine residues were present, but were masked by sialic acid. Further investigations, involving an ingenious technique in which tumour cells were incubated on cryostat sections of normal tissues, strongly suggest that the expression of galactosyl- or N-acetylated galactosaminyl- groups on highly metastatic cells facilitates their attachment to lectin-like receptors on liver cells and contributes to their capacity for growth and metastasis in this organ.

In a fascinating study by Yamura et al (1985) immunisation of mice with soluble tumour associated antigens isolated by affinity chromatography on a PNA-agarose column appeared to inhibit metastasis of Lewis lung carcinoma (3LL) to lung. Peanut binding glycoproteins ("receptors") were isolated from the 3LL cells and administered by sub-cutaneous injection shortly following surgical excision of the primary tumour. A decrease in the incidence and size of subsequent lung metastases was noted. This effect was abolished if immunisation was delayed, or if splenectomy was performed simultaneously with primary tumour excision; this latter observation perhaps implicating lymphoid involvement in the control of metastatic tumour growth.

1.5.1.3. Other lectins

*Ulex europaeus*

A number of studies have demonstrated differences in the binding of various other lectins to tumours of high- and low-metastatic potential. Kahn et al (1988), for example, studied the binding of a panel of lectins to primary tumours of liver and lung metastasizing variants of the murine Lewis lung carcinoma and their metastases; the results implicate a *Ulex europaeus* I lectin-binding glycoprotein in the metastatic process; and Irimura et al (1987) have isolated a high molecular weight surface glycoprotein from human adenocarcinomas
of distal colon and rectum which binds *Ulex europaeus* lectin I. Increased expression of UEAI-reactive molecule was related to transformation of colorectal epithelial cells; and decreased expression appeared to be associated with progression and metastatic potential.

**Concanavalin A**

Yamori and colleagues (1984) found a reduction in Concanavalin A binding sites in highly metastatic variants of the murine colonic adenocarcinoma 26 cell line; however, Stanford et al (1986), in looking at a series of rabbit hepatocarcinoma metastatic variants correlated the presence of Concanavalin A binding sites with successful metastasis to lung, although the binding sites appeared to be lost during subsequent tumour outgrowth into lung tissues.

**Soyabean**

Disagreement exists as to the involvement of soyabean lectin-binding sugars as markers of metastasis. Buckley and Carlsen (1988) found increased soyabean lectin binding in a metastasizing tumour cell-enriched line derived from the poorly metastatic R3230AC rat mammary adenocarcinoma. Lang and colleagues (1987), however, found soyabean lectin binding increased in poorly metastatic variants of the ESb murine lymphoma line - soyabean lectin binds to the original highly metastatic parent line if the cells are stripped of terminal sialic by neuraminidase.

**Griffonia simplicifolia** (also called *Bandeiraea simplicifolia*)

Varani et al (1983) and Grimstead et al (1984) both correlated the binding of *Griffonia simplicifolia* lectins to high metastatic potential. Varani and colleagues (1983) found that a murine cell line variant which did not bind to GS isoelectin B4, a lectin with strict specificity for terminal alpha-D-galactopyranosyl residues, had lost its ability to metastasise. Grimstead et al (1984) also report a greater number of cell surface terminal alpha-D-galactopyranosyl groups, as detected by soyabean agglutinin binding, in the highly metastatic cells versus the poorly metastatic cells derived from the same murine fibrosarcoma.

These interesting results do not altogether agree with those of Dennis et al (1985), previously described in section 1.5.1.1., who found the absence of *Griffonia simplicifolia* (*Bandeiraea simplicifolia*) lectin binding with an increase in metastatic potential.

**1.5.2. STUDIES OF LECTIN BINDING TO BREAST CANCERS**

A few studies have specifically addressed the question of lectin binding to primary breast cancers; some have demonstrated differences in the carbohydrates expressed by normal and hyperplastic breast tissue in comparison to malignant tissue, while some workers have attempted to identify a correlation between heterogeneous lectin binding patterns of tumours and degree of differentiation, or metastatic potential.
1.5.2.1. Con A and lentil (glucose/mannose-binding) lectins bind selectively to malignant but not to benign breast:

Louis et al (1983) looked at the binding of 9 commonly studied lectins [Arachis hypogaea (peanut); Bandeiraea simplicifolia (also called Griffonia simplicifolia), Dolichos biflorus (Horse gram); Maclura pomifera (Osage orange); Tetragonolobus purpureas (Lotus bean or winged pea); Triticum vulgaris (wheatgerm); Ulex europaeus I (gorse); Con A (from Canavalin ensiformis, jack bean); and Lens culinaris (lentil)], to a series of 95 non-malignant (including normal resting and lactating breast and a range of benign lesions) and 69 malignant (carcinoma in situ, infiltrating tumour and Paget's disease of the nipple) breast samples. The aim of the study was to attempt to distinguish differences in lectin binding between benign, normal, and malignant breast.

The results of the study were really quite startling: Con A and lentil lectin bound very strongly to all malignant (infiltrating and non-infiltrating) lesions, but did not bind to normal tissue or benign disease, suggesting abnormal glycoprotein expression by malignant cells; this selectivity in Con A binding has been confirmed by Dansey et al (1988). Both lectins bind glucose and mannose; which is interesting in the light of other studies which have identified abnormal glycoprotein with increased mannose residues on the surface of a variety of malignant cells (Chandrasekaran and Davidson 1979; Lis and Sharon 1973; Atkinson and Bramwell 1980;1981).

The authors suggest that in foci of epitheliosis, patches of staining may represent premalignant change. Furthermore, changes in staining pattern from predominantly cytoplasmic to membranous may indicate a progression to a "more malignant" type as abnormal glycoprotein, detected by Con A and lentil lectin, becomes concentrated at the cell surface and may eventually be shed into circulation (Chandrasekaran and Davidson 1979).

Dansey et al (1988), in a study of 86 breast cancers, reported an absence of Con A binding to normal breast, but positive staining in 29/84 cancers - the proportion of tumours staining increasing with progressively poorer histological grade. Con A binding appears to be a weakly significant marker of poor patient prognosis - patients with Con A negative tumours enjoying prolonged disease free survival in comparison to their Con A positive counterparts - however, independent prognostic significance disappears when tumour grade is included in the analysis.

1.5.2.2. Wheatgerm (GlcNAc/sialic acid-binding) lectin binding as a possible marker of histological tumour differentiation:

Rosemary Walker has published a series of papers describing lectin binding to sections of normal, hyperplastic and malignant breast (Walker 1984a; 1984b; 1984c; 1984d; Walker et
al 1985; Walker and Day 1986). She reports consistent binding of WGA to paraffin sections of normal and hyperplastic breast (Walker 1984a), but great heterogeneity in its binding to malignant cells (Walker 1984b), an observation confirmed by Franklin (1983). The N-acetyl-glucosamine/sialic acid binding wheatgerm lectin (WGA) has been demonstrated to agglutinate tumour cells to a greater degree than normal cells (Aub et al 1965), and, more recently, to distinguish in culture malignant cells of high and low metastatic potential (Dennis et al 1984; 1985; Irimura et al 1986; Benedetto et al 1989; Elia 1988; Ishikawa 1988; 1989). The extent to which tumour binds WGA appears, with a few exceptions, to directly mirror its degree of histological differentiation. A complex relationship between lectin binding and the presence of axillary lymph node involvement has also been reported - most tumours where many cells bind WGA were node negative; where a moderate degree of binding was noted, most were node positive; and where few cells bound the lectin no correlation was apparent, although the number of cases was small. As the cancers included in this paper were excised only 1-3 years prior to the study, long-term follow-up information regarding patient prognosis and distant metastatic involvement was not available (Walker 1984b).

Interestingly, inhibition tests using simple unlabelled sugar suggests that WGA binding to normal tissue is through N-acetyl-glucosamine residues (Walker 1984a), but to malignant tissues is through sialic acid (Walker 1984b) which is of note in the light of other studies on tumour cell lines implicating the masking of sialic acid residues as a crucial step in the progression to metastasis (eg Altevogt et al 1983; Fogel et al 1983; Dennis et al 1986).

1.5.2.3. L-PHA, a lectin from *Phaseolus vulgaris* (binds complex oligosaccharide) binds more strongly to malignant than benign breast:

Dennis and Laferte (1989a) looked at the binding of L-PHA ("leukoagglutinin"), a lectin from *Phaseolus vulgaris*, red kidney bean, which binds a complex N-acetylglucosamine-β-1-6 mannose-alpha-1-6-mannose-β-1-branched asparagine linked oligosaccharide, to a series of breast cancers and benign lesions. Around half of the malignant tumours bound strongly to the L-PHA, but benign breast lesions bound much more weakly. Expression of L-PHA receptor oligosaccharide is dependent on the production of the enzyme N-acetylglucosamine transferase. The authors suggest the need for further studies to determine whether the presence of these oligosaccharides is associated with metastatic disease and reduced patient survival time.

1.5.2.4. *Ulex europaeus* I (L-fucose binding) lectin - a proposed marker of tumour metastasis to local lymph node:

Walker (1984c) has also looked at the binding of the L-fucose specific lectins from *Lotus
tetragonalobus (LTA) and Ulex europaeus I (UEAI) to frozen sections of a series of 80 breast cancers. It is interesting that although both lectins theoretically bind the same sugar, their staining pattern on breast tissue was quite distinct; LTA consistently bound normal and hyperplastic epithelium, but binding to cancers was variable, and showed no correlation with differentiation or the presence of lymph node metastases, while the binding of UEAI to normal and hyperplastic breast was variable, but its binding to cancers did appear to be associated with the absence of lymph node involvement, an observation confirmed by later studies (Walker and Day 1986). Franklin (1983) reported consistent staining by UEAI of normal and benign breast tissue but reduced binding to cancers. He attributed loss of staining in malignancy to deletion of blood group H antigen, but did not investigate its potential link with metastatic potential.

It is interesting that Badenoch-Jones (1987) reported conflicting results in his study of a rat mammary adenocarcinoma cell line DMBA-8. Highly metastatic "clone 4" variants of the normally poorly metastatic parent line were shown to bind strongly to UEAI. In S.D.S.-P.A.G.E., 4 distinct UEAI-binding bands were identified in clone 4, the bands were also present in extracts from cells of a similar highly malignant cell line, MAT 13762-B, but not in the poorly metastatic DMBA-8 cells.

1.5.2.5. Peanut (Gal-GalNAc-binding) lectin and steroid receptors:

Newman et al (1979) examined binding of fluorescein-conjugated peanut lectin (PNA) to mature and immature rat mammary tissue; cultures of a rat mammary cell line, rama 25; and paraffin-sections of human normal and malignant breast. PNA-receptors were localised exclusively on the apical (luminal) surfaces of epithelial cells of normal breast. In malignant tissue, quite different staining patterns were observed, related to degree of morphological differentiation: in well-differentiated tumours, PNA bound predominantly to cellular periphery (plasma membrane) and material within lumina; in poorly- differentiated tumours, PNA binding was rarely observed, but occasional intense cytoplasmic staining was seen.

These results were mirrored by PNA-binding patterns in rat mammary tissue at different stages of development. Lectin binding could be inhibited by D-galactose and \( \beta \)-D-galactosyl-(1-3)-N-acetyl-galactosamine.

Unfortunately, studies comparing the relationship of PNA binding to breast cancers with degree of differentiation have yielded confusingly conflicting results, with some workers, like Newman, showing a clear correlation between PNA-binding and degree of differentiation (Walker 1985; Cooper 1984) and others finding no association (Calafat and
Equally equivocal results have arisen from work addressing a possible correlation between PNA-binding and steroid receptor status of breast cancers. Early work by Klein et al (1981; 1983) suggested that a distinction could be made between hormone-sensitive and -insensitive rat mammary tumours, and, furthermore, that those patients who would benefit from endocrine therapy could be predicted on the basis of PNA-lectin binding to tumour. An association between PNA-binding and oestrogen receptor status of breast cancer patients has been confirmed by Walker et al (1985) and in culture, only those mammary carcinoma cell lines known to be oestrogen sensitive have been demonstrated to bind the lectin (Daxenbichler et al 1986). However, Stanley et al 1986 have failed to detect any correlation between PNA-binding of breast cancers and hormone receptor status.

### 1.5.3. STUDIES ON HELIX POMATIA LECTIN BINDING TO BREAST CANCER

The remarkable carbohydrate-binding specificity of lectins makes them splendid tools for the investigation of changes in oligosaccharide expression by cells. Their potential value may lie in the detection of change with disease, particularly malignancy, and perhaps more vitally with the detection of carbohydrate moieties associated with the transformation of cancer cells to metastatic competence.

Changes in lectin binding to tissues with disease and with malignancy are well documented. In addition, many authors have noted variable lectin binding patterns among otherwise morphologically similar tumours. However, few attempts have been made to link such variable carbohydrate expression with differences in clinical behaviour; and it is here, perhaps, that lies the great potential of lectins as selective, specific, carbohydrate-binding probes.

Leathem and colleagues have described the binding of a number of lectins of different sugar specificities to normal human tissues (Leathem and Gardner 1981) and to normal and malignant breast (Leathem et al 1983; 1984; 1985; Leathem, unpublished data). Some results are summarised in Table 3:

**TABLE 3:** Lectin binding to primary breast cancers. (Leathem - previously unpublished data).

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Poke</th>
<th>Lotus</th>
<th>WGA</th>
<th>PNA</th>
<th>SBA</th>
<th>HPA</th>
<th>BSA</th>
<th>ConA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>12</td>
<td>12</td>
<td>15</td>
<td>12</td>
<td>8</td>
<td>14</td>
<td>7</td>
<td>11</td>
</tr>
<tr>
<td>(A) epithelial luminal surface</td>
<td>10</td>
<td>12</td>
<td>15</td>
<td>12</td>
<td>8</td>
<td>14</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>0%</td>
</tr>
</tbody>
</table>
A hitherto little-studied alpha-N-acetyl-galactosamine-binding lectin isolated from the albumin gland of the Roman or edible snail, *Helix pomatia*, was identified as binding intensely to the luminal surface of normal breast ducts and acini, and giving strong luminal surface and cytoplasmic localisation in most, but not all, of a small series of breast cancers. The lectin also bound strongly to some normal and malignant breast secretions. Of eight lectins studied (pokeweed; *Lotus*; wheatgerm; peanut; soyabean; *Helix*; *Bandeiraea*; and Con A), *Helix pomatia* lectin gave the strongest and most precise localisation in normal breast, and showed considerable variation in staining intensity and pattern in breast cancers (Leathem et al 1983; Leathem, unpublished data).

These results were partially confirmed by Beham et al (1985) who documented the binding patterns of five lectins (peanut; *Ulex europeas* I (gorse); *Helix pomatia*; soyabean; wheat germ; *Bauhinia purpurea*) to a series of 56 breast cancers and attempted to correlate the proportion of cases binding each lectin with tumour grade and histological type. *Helix pomatia* lectin binding was the most variable of all lectins studied. The results are summarised in Table 4:
TABLE 4: *Helix pomatia* lectin binding to a series of 56 breast cancers. (After Beham et al 1985)

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Grade</th>
<th>Total No. of Cases</th>
<th>Approximate Percentage of Cases Binding <em>Helix</em> Lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-invasive Intraduct</td>
<td>I</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>20</td>
<td>100%</td>
</tr>
<tr>
<td>Invasive Ductal</td>
<td>I</td>
<td>1</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4</td>
<td>0%</td>
</tr>
<tr>
<td>Invasive Lobular</td>
<td>I</td>
<td>1</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>Mucinous</td>
<td>II</td>
<td>4</td>
<td>100%</td>
</tr>
<tr>
<td>Medullary</td>
<td>II</td>
<td>3</td>
<td>33%</td>
</tr>
<tr>
<td>Adenoid Cystic</td>
<td>I</td>
<td>1</td>
<td>100%</td>
</tr>
</tbody>
</table>

None of the 10 lobular carcinomas in the study bound *Helix pomatia* lectin. Binding was extremely variable in invasive ductal tumours; but the apparent inverse trend between grade and lectin positivity should perhaps be interpreted with extreme caution owing to the number of cases involved (ie a single grade I tumour).

Analysis of *Helix pomatia* lectin binding in conjunction with clinical data by Leathem and colleagues revealed a highly significant association between metastases to local lymph nodes and binding of the lectin to primary cancer cells (p<0.003) (Leathem et al 1984; 1985; and Leathem unpublished data), as illustrated in Figure 12.


p < 0.003

Key: o = lymph node negative cases
+ = lymph node positive cases
The binding of this snail lectin appeared to parallel the biological behaviour of the primary cancer more closely than tumour size or histological grade, as shown in Table 5.

**TABLE 5: Helix pomatia lectin binding to primary breast cancers - association with histological grade, tumour size, and lymph node status. (Leathem - unpublished data 1984)**

<table>
<thead>
<tr>
<th>case number</th>
<th>histological grade</th>
<th>tumour size (cm)</th>
<th>lymph nodes involved</th>
<th>% cells of primary tumour binding to lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYMPH NODE NEGATIVE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>II</td>
<td>1.5</td>
<td>0/20</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>III</td>
<td>1</td>
<td>0/12</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>II</td>
<td>3</td>
<td>0/9</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>II</td>
<td>1</td>
<td>0/11</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>II</td>
<td>2</td>
<td>0/12</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>II</td>
<td>0.5</td>
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These findings, although based on a relatively small number of cases (n = 20) seemed sufficiently exciting to warrant further investigation; it is from such a basis that the present study was undertaken.
MATERIALS AND METHODS
2. MATERIALS AND METHODS

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2.2.1. SELECTION OF CASES FOR DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY.

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2.2.7. CELL CYCLE ANALYSIS: THE S-PHASE FRACTION (S.P.F.).

2.2.8. DNA INDEX ANALYSIS - D.I.
2.1. BREAST CANCER PATIENT "FOLLOW-UP": ESTABLISHMENT OF A BREAST CANCER PATIENT "LIBRARY"

For each breast cancer patient included in the cohort described in sections 2.2, 2.3 and 2.4 a detailed follow-up survey was carried out.

2.1.1. SOURCES OF FOLLOW-UP INFORMATION

The clinical progress of all patients from the time of diagnosis until December 1987 (total follow-up period 15-20 years) was plotted by means of follow-up information gathered from the following sources:

1) At the Middlesex Hospital: through the Cancer Registry and Cancer Death Registry; the Oncology and Radiotherapy Department records; patients' hospital notes; post-mortem
reports; histology reports; and blood bank records.

2) Through letters to the patients' general practitioner, and to the Family Practice Committee.

3) Through letters to Thames Cancer Registry, Clifton Avenue, Belmont, Sutton, Surrey, SM2 5PY.

4) Through letters to NHS Cancer Registry, Smedley Hydro, Southport, Merseyside, PR8 2HH.


2.1.2. FOLLOW-UP INFORMATION GATHERED

Comprehensive follow-up information was recorded for each patient on a three-page form (specimen form included as Appendix II).

The principle clinical criteria included:

2.1.2.1. Patient age / menopausal status.

Menopausal status at presentation was initially recorded from patient notes, but, as the information was lacking for a majority of patients, it was necessary to make assumptions on the basis of patient age. Women aged 50 years or less were considered to be pre-menopausal; those older than 50 years, post-menopausal.

2.1.2.2. Lymph node status

290/373 patients (77%) had undergone, as part of their surgical treatment, some form of axillary clearance or sampling. For these individuals it was possible to record the total number of axillary lymph nodes histologically involved with tumour as a fraction of the total number examined. From this, the percentage of lymph nodes involved was calculated.

2.1.2.3. Blood group

Blood group was, unfortunately, seldom stated in patient notes. Although all patients at the time of surgery would have been cross-matched by the hospital blood bank, records are kept for a maximum of seven years only. For most patients, therefore, blood group remained unknown.
2.1.2.4. Tumour size

Both a (pre-operative) clinical estimate, and the actual measured cut surface of the tumour were recorded in notes / histology reports for all patients. Tumour cut surface measurements were used in preference to clinical estimates in subsequent analyses, on the basis that, clearly, they provide a more accurate indication of tumour size. Tumour size was recorded to the nearest whole centimetre.

2.1.2.5. Histological grade

Histological grade was generally not given in Middlesex Hospital histology reports.

Haematoxylin and eosin preparations (see section 2.3.3.I.) for each patient were graded according to the WHO criteria (Scarff and Torloni 1968). Briefly, this system requires that each slide is scored for (1) tubule formation; (2) hyperchromatism and mitosis; (3) irregularity of size, shape and staining of nuclei.

(1) Tubule formation
Well-marked tubule formation or acinar arrangement, with cells grouped more or less regularly around a central space, is characteristic of a high degree of differentiation and indicates a favourable prognosis.

One point is awarded if the section shows well-marked tubule formation; two points if tubule formation is moderate; and three points if there is little or no differentiation, with cells growing in sheets or strands.

(2) Hyperchromatism and mitosis
The greater the number of hyperchromatic or mitotic nuclei, the worse the prognosis.

One point is awarded if only an occasional hyperchromatic or mitotic figure is seen per low-power field; two points if there are two or three such figures in most fields examined; and three points if the number is higher.

(3) Irregularity of size, shape and staining of nuclei
The greater irregularity of size, shape and staining of nuclei, the worse the prognosis.

One point is awarded if the nuclei are fairly uniform in size, shape and staining; two points if there is moderate variation; and three points if pleomorphism is marked.

Totals
The points allocated for each of the three criteria are added together.
3-5 points is considered indicative of low malignancy (grade I)
6-7 points, intermediate malignancy (grade II)
8-9 points, high malignancy (grade III)

Examples of tumours scored as grades I, II, and III are given in Figure 2 in the "Results" section.

All cases were examined by Dr A. Leathern and myself independently in batches of approximately 50 slides. Periodically, slides previously graded were re-assessed as "quality control". With experience, (almost) complete reproducibility was possible.

2.1.2.6. Interval to first recurrence

The interval to first recurrence was taken to be the time in months between primary diagnosis and the noting of the first symptom of a confirmed disease recurrence. For example, if the first symptom of recurrence was bone pain and the presence of bony metastases was later confirmed by bone scan, x-ray, biopsy or post mortem examination, the time of that first symptom was recorded. If there was no subsequent confirmation, that symptom was disregarded.

Recurrences were classified as - local (operation site, chest wall or glandular); regional (including pleural effusion, skin, other breast); distant visceral (liver, lung, brain etc); and distant bony.

2.1.2.7. Time to death

Time to death was recorded as the number of months between date of diagnosis and date of death.

2.1.2.8. Cause of death

Cause of death was recorded as being due to carcinoma of the breast if the patient obviously died as a result of metastatic disease; or if the patient had suffered a confirmed local, regional, distant visceral or bony recurrence.

Death was recorded as being due to a cause other than carcinoma of the breast if the patient died without evidence of metastatic disease and an alternative cause of death was recorded on the death certificate.
Patients who did not fit into either of the above categories were listed as having died of unknown cause.

2.2. ANALYSIS OF NUCLEAR PLOIDY AND CALCULATION OF S-PHASE FRACTION BY FLOW CYTOMETRY

For all tumours for which it was possible, analysis of nuclear ploidy and calculation of S-phase fraction were performed.

In recent years, there has been considerable interest in the possible clinical significance of abnormalities in the cellular DNA content of human tumours (Barlogie et al 1983). Earlier studies using single cell microdensitometry of Feulgen stained tissue sections established that aneuploidy (abnormal DNA content of cells) is common in breast cancers, and showed some correlations with prognosis (Atkin 1972, Auer et al 1984, Atkin and Kay 1979) but the techniques involved were time consuming, labour intensive, and had limited resolving power. In addition, clinical information such as steroid receptor status and degree of lymph node involvement was incomplete for many patients studied, thus rendering evaluation of prognostic significance extremely difficult.

The introduction of rapid and accurate mechanised flow cytometric techniques has revolutionised DNA ploidy analysis and has opened up the way to intensive research.

Initially, it was necessary to use fresh, unfixed tissues, and although providing excellent data, carried the drawback that archival material could not be analysed, resulting in the necessity for long-term prospective follow-up before any prognostic significance of results could be assessed.

The development of a flow cytometric method suitable for use with fixed, paraffin-embedded material by Hedley et al (1983) has allowed the analysis of archival material and ready evaluation of ploidy data in relation to the known prognosis of patients whose clinical outcome is already documented.

2.2.1. SELECTION OF CASES FOR DNA PLOIDY ANALYSIS BY FLOW CYTOMETRY

Ideally, all 373 breast carcinomas previously assessed for Helix pomatia lectin binding would also be analysed by flow cytometry for DNA ploidy. However, the relatively large amount of tissue required for this procedure (2 x 50 μm sections of formalin fixed, paraffin-embedded material, in which at least 80% of the cells should be malignant) was
not available in every case. Only the 358 patients for which sufficient archival tissue was available were included in the study.

2.2.2. PREPARATION OF CELL SUSPENSIONS FROM PARAFFIN-EMBEDDED MATERIAL

Flow cytometry was performed according to the method described by Hedley et al (1983) with slight modification by Camplejohn and MaCartney (1985).

In summary, 50µm sections of formalin-fixed, paraffin-embedded tissue were cut, dewaxed in xylene and rehydrated through graded alcohols (100%; 95%; 70%; 50%) to distilled water. They were digested in a 5mg/ml solution of pepsin (Sigma), pH 1.5, at 37°C for 30 minutes. The resulting suspension was passed through a 25 gauge needle and filtered through a 35µm pore nylon gauze to remove any residual cell clumping. It was then spun down for 3 minutes at 200 r.p.m., and the sediment resuspended in 5ml of a 1mg/ml solution of a fluorochrome, DAPI (4'-6-diamidino-2-phenylindol- dihydrochloride) (Boehringer Mannheim, West Germany) in Isoton (Coulter Electronics, Luton, England).

2.2.3. FLUORESCENCE FLOW CYTOMETRY

Samples were analysed using a Beckton-Dickinson (California, U.S.A.) F.A.C.S. (Fluorescence Activated Cell Sorter) analyser powered by a mercury arc lamp. The fluorochrome was excited by ultraviolet emissions of a peak wavelength of 360nm; and fluorescence was detected in the blue region of the spectrum, peak wavelength 490nm.

Between 10,000 and 15,000 cells were scanned in each sample.

2.2.4. THE DNA HISTOGRAM

Results of DNA analysis by flow cytometry are most commonly expressed as a simple histogram on which quantity of DNA is plotted on the abscissa versus cell number on the ordinate, as illustrated in Figure 13 (overleaf).

In most normal cell populations, the majority of cells are in G0/G1 phase of their cycle, and have a normal diploid or 2c DNA content. Smaller numbers of cells will be in G2 or M phases of mitosis and will thus contain a tetraploid or 4c content, or will be in S phase of active DNA synthesis where DNA values lying between 2c and 4c may be expected. An example of such a profile is seen in Figure 13a.

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2.2.5. PLOIDY

In looking at malignant tissues, the most vital information to be derived from analysis of DNA content is the presence or absence of cell populations possessing abnormal aneuploid DNA content. Such aberrant cells may be identified as a discrete peak on the DNA histogram lying outside the normally expected $2c$ or $4c$ regions. An example of such a profile is illustrated in Figure 13b.

FIGURE 13: Examples of DNA histograms obtained by flow cytometry. (a) Diploid profile - most cells have a normal $2c$ DNA content, while a small percentage, those in the G2 or M phase of the cell cycle, fall into the $4c$ or tetraploid region of the histogram. (b) Aneuploid profile - in addition to the $2c$ and $4c$ peaks, a third, distinct aneuploid peak is apparent.
2.2.6. C.V. - THE "COEFFICIENT OF VARIATION"

The reliability of the information represented by the DNA histogram is reflected in a value called the coefficient of variation or C.V. of the G0/G1 peak. A low C.V. is indicative of a high degree of accuracy. The coefficient of variation achieved with any one sample will depend upon (1) optimal adjustment of the flow cytometric apparatus, and (2) the quality of the material used (a higher C.V. is generally obtained with fixed, paraffin-embedded, rather than fresh tissue samples).

The C.V. is a numerical term which actually describes the variability within the sample. Mathematically, it is an expression of the standard deviation as a percentage of the mean.

It is calculated as follows:

If \( x' \) represents the DNA content of each of the cells examined within the sample; \( x^* \) is the mean DNA content; and \( n \) is the number of cells counted,

\[
\text{variance within the sample} = \frac{\sum (x' - x^*)^2}{n - 1}
\]

standard deviation, \( s \), is calculated by taking the square root of this value;

coefficient of variation is calculated by dividing standard deviation by the mean of the sample, and then expressing this total as a percentage, thus;

\[
\text{C.V.} = \frac{s \times 100}{x^*}
\]

The C.V. is a useful descriptive tool in situations in which a change in the conditions under which measurements are made alters standard deviation in the same proportion as it alters the mean. The C.V. then remains unchanged and is a useful measure of variability. Thus, the higher the C.V., the greater the variability within the sample, and thus, in this case, the less reliable the results.

As an arbitrary cut-off, samples with a C.V. greater than 10 were excluded from the analysis as being insufficiently accurate for inclusion.
2.2.7. CELL CYCLE ANALYSIS: THE S-PHASE FRACTION (S.P.F.)

Flow-cytometry allows estimation of the proportion of cells in a given sample which are in S or G2/M phase - a value reflecting the proliferative activity of cells present.

S-phase fraction values were calculated according to the method of Baisch et al (1975). In aneuploid tumours, the S-phase fraction of aneuploid cells only was calculated according to the simple formula:

\[
\frac{\text{number of aneuploid cells in S-phase}}{\text{total number of aneuploid cells}} \times 100
\]

It is not possible to calculate S-phase fraction under certain circumstances. These are:

1. In cases where more than one aneuploid peak is present, as the mathematics involved are too complicated.

2. If the aneuploid peak is less than 10% of the size of any diploid peak, as too few aneuploid cells will be in S-phase and results will be inaccurate.

3. If diploid and aneuploid peaks lie too close together (ie if DNA index <1.3), as the two peaks are not sufficiently distinct to allow accurate calculations to be made.

2.2.8. DNA INDEX ANALYSIS - D.I.

The DNA index or DI is defined as the ratio of tumour cell DNA content: normal cell DNA content (values expressed in both cases as peak channel number (Hiddeman et al 1984).

It is an expression of DNA content aberration. DNA index is calculated by the ratio of the mode (or mean) of the relative DNA content of the G0/G1 cells of the sample divided by the mode (or mean) of the relative DNA measurement of the diploid G0/G1 reference cells.

A DNA index of 1.0 indicates the presence of normal diploid cells only. The diagnosis of "abnormal DNA stemline" or "DNA aneuploidy" is reported when at least two separate G0/G1 peaks are reported.

DNA index, S-phase fraction value, the percentage of cells with aneuploid DNA; and coefficient of variance were all calculated using program and software supplied by Beckton-Dickinson.
2.3. HELIX BINDING TO PRIMARY BREAST CANCERS: RETROSPECTIVE STUDY.

2.3.1. SELECTION OF PATIENTS.

2.3.2. TISSUES.

2.3.3. HISTOLOGY.
2.3.3.1. Haematoxylin and eosin stain.

2.3.4. HISTOCHEMICAL STAINING METHOD - *HELIX POMATIA* LECTIN BINDING.
2.3.4.1. Controls
2.3.4.2. Scoring of stained sections.

2.3.5. PHOTOMICROGRAPHY.

2.4. CORRELATION OF LECTIN STAINING WITH CLINICAL FOLLOW-UP DATA / STATISTICAL ANALYSIS.

---

**PLAN OF INVESTIGATION**

- Identification of all breast cancers diagnosed at Middlesbrough Hospital 1967-1972
- Collection of formalin-fixed paraffin-embedded tissues
- Sections cut
- Determination of staining methodology
- Sections stained for binding of *Helix pomatia* lectin
- Staining evaluated
- Computer storage of data
- Correlation of staining with clinical follow-up / statistical analysis
- Collection of fresh tissues
- Isolation and characterisation of breast cancer metastasis-associated Helix-binding material
  - H.P.L.C.
  - Lectin affinity chromatography
    - S.D.S.-P.A.G.E. and Western style electroblotting
    - Dot-blotting
    - Ion exchange chromatography
2.3. HELIX POMATIA LECTIN BINDING TO PRIMARY BREAST CANCERS: RETROSPECTIVE STUDY

Formalin fixed, paraffin embedded sections of primary breast cancers were stained for the binding of Helix pomatia lectin.

Presence or absence of lectin binding was correlated with patient prognosis over a 15-20 year follow-up period.

2.3.1. SELECTION OF PATIENTS

500 consecutive cases of histologically confirmed primary carcinoma of the breast were selected from the histology reports of the Middlesex Hospital of January 1967 - June 1972. All patients had been treated surgically by mastectomy (Patey radical mastectomy, simple mastectomy, or partial mastectomy) or by simple or wide excision lumpectomy, and had no previous history of carcinoma of the breast, or of any other malignancy.

2.3.2. TISSUES

Blocks of formalin-fixed paraffin-embedded tissue taken from the original surgical specimen were retrieved from the archives of the Middlesex Hospital.

Material was available for 492 of the 500 patients; the remaining 8 patients, for whom blocks were missing from the archives, were omitted from the study.

2.3.3. HISTOLOGY

6 x 5 μm serial sections were cut by microtome from the tissue blocks. The first and last section from each block was stained with haematoxylin and eosin by the following method:

2.3.3.1. Haematoxylin and eosin stain

1) Sections dewaxed in xylene for 15 minutes, rehydrated through graded alcohols (100%; 95%; 60%) and brought to water.

2) Immersed in Harris's haematoxylin for 5 minutes.

3) Immersed in running tap water until nuclei, on microscopic examination, appeared deep blue.
blue.

4) Differentiated in 1% acid alcohol (approximately 10 seconds).

5) Rinsed in running tap water for 5-10 minutes, until nuclei, upon microscopic examination appeared deep blue and cytoplasm clear and colourless.

6) Immersed in eosin for 4 minutes.

7) Rinsed briefly in tap water to remove excess eosin.

8) Dehydrated through graded alcohols (60%; 95%; 100%) and immersed in xylene.

9) Mounted in Depex resinous mountant.

[Xylene and alcohol (99% methanol) from was obtained from BDH; Harris's haematoxylin, eosin and Depex resinous mountant from Raymond A. Lamb. A list of suppliers' addresses and telephone numbers is given in Appendix VII.]

[The formulae for 1% acid alcohol, Harris's haematoxylin, and eosin are listed in Appendix III.]

Sections were examined through a microscope to confirm the presence of malignant cells.

Of 492 cases examined, tumour tissue was present in only 373. For 119 patients, omitted from the study, only small samples of tumour had originally been processed, and the blocks had been cut through when the original pathological diagnosis was made leaving only normal breast tissue, evidence of benign breast disease, fibrous tissue or fat.

2.3.4. HISTOCHEMICAL STAINING METHOD - *HELIX POMATIA* LECTIN BINDING

Formalin-fixed, paraffin-embedded sections for each of the remaining 373 patients were stained for *Helix pomatia* lectin binding by an avidin-biotin sandwich technique. The reason for choice of this particular staining method is detailed in Appendix V.

The principles of this technique are illustrated in Figure 14, overleaf.
**Method:**

1) Sections dewaxed in xylene and rehydrated through graded alcohols (100%; 95%; 60%) to water.

2) Endogenous peroxidases blocked by incubation in a 3% solution of hydrogen peroxide in methanol for 20 minutes.

3) Sections treated with trypsin [1mg trypsin :1mg calcium chloride : 1ml Tris buffered saline] for 20 minutes at 37°C.

4) Incubated with *Helix pomatia* lectin 10µg/ml in TBS for 60 minutes at room temperature.

5) Incubated with polyclonal rabbit antiserum raised against *Helix pomatia* lectin at a
dilution of 1/200 in TBS for 60 minutes.

6) Incubated with biotinylated antisera raised in swine against rabbit immunoglobulins at a dilution of 1/400 in TBS for 60 minutes.

7) Incubated with avidin peroxidase at a concentration of 5µg/ml in TBS for 30 minutes.

8) Incubated with diaminobenzidine, a chromogenic substrate for peroxidase, at a concentration of 100µg/ml in TBS with 6µl of hydrogen peroxide added per 1ml of solution. Incubation time 10 minutes.

9) Counterstained in Mayers haematoxylin for 2 minutes and blued in running tap water.

10) Mounted in Depex resinous mountant.

[The recipe for Tris buffered saline (TBS) is given in Appendix III.]

[Hydrogen peroxide and methanol were obtained from BDH; trypsin (porcine, type II, crude), Helix pomatia lectin, avidin peroxidase and diaminobenzidine from Sigma; Rabbit anti-Helix was raised in our laboratory according to the protocol set out in Appendix IV; Biotinylated swine anti-rabbit was supplied by Dako; and Mayer's haematoxylin by Raymond A. Lamb.]

2.3.4.1. Controls

*Positive control:*
A case known to be strongly positive for Helix pomatia lectin binding was included with each batch of slides as a positive control.

*Negative controls:*
As negative controls (1) the lectin was simply omitted, and (2) the lectin was incubated with the section in the presence of 0.1M N-acetyl-galactosamine (Sigma).

2.3.4.2. Scoring of stained sections

Staining was recorded initially as a combination of both approximate percentage of cancer cells staining positively, plus an estimate of the intensity of the staining reaction, scored as either negative, or as +, ++ to ++++ (for example, 80% +++; 100% +; 25% +- etc).

Ultimately, however, cases were classified simply as "stainers" or "non-stainers".
2.4 Statistical analysis

Arbitrarily, the dividing line between stainers and none-stainers was laid as follows: "Stainers" were cases where 5% or more of cancer cells were clearly staining positively (with an intensity of at least +), or 50% or more of the cancer cells were weakly positive (with intensity +). "Non-stainers" were cases where less than 5% of the cancer cells were scored +, or where less than 50% of cancer cells stained +-. In practice, most cases were either intensely positive or completely negative, as seen in Figure 24 in the "Results" section.

The cases included in the study were all obvious cases of carcinoma, previously diagnosed by an experienced pathologist - cancer cells were easily identified. Although I am not trained as a pathologist, I was able to score slides relatively easily. Dr. Leathem and myself examined all cases independently of each other, and then compared our readings. With experience, a high degree of reproducibility was possible. As "quality control" we included in each batch of slides to be read a small number of cases that had already been scored on a previous occasion to be re-assessed blindly. Again, with experience, a high degree of reproducibility was possible.

2.3.5. PHOTOMICROGRAPHY

Colour photomicrographs were prepared with the aid of an Olympus BH2 microscope fitted with an Olympus OM-4T camera. Colorama 135mm (ASA 100/21 DIN) colour print film was used throughout.

2.4. CORRELATION OF LECTIN STAINING WITH CLINICAL FOLLOW-UP DATA / STATISTICAL ANALYSIS

Log-rank analysis, life-tables (prepared by the Kaplan-Meier method), Cox's proportional hazards regression analysis, and other statistics were calculated using a statistical analysis package produced by B.M.D.P. Software Incorporated (University of California Programs), and supplied by Statistical Software Inc. All calculations were performed under the supervision of Teresa Young (Department of Radiotherapy, Middlesex Hospital) using an IBM personal computer.
2.5. HELIX POMATIA LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER.

2.5.1. CHOICE OF CASES.
2.5.2. TISSUES.
2.5.3. HISTOLOGY.
2.5.4. HELIX POMATIA LECTIN BINDING.
2.5.5. SCORING OF SLIDES.

PLAN OF INVESTIGATION

- Identification of all breast cancers diagnosed at Middlesex Hospital 1967-1972
- Collection of patient follow-up data
- Identification of breast cancer metastases
- Collection of formalin-fixed paraffin-embedded tissues
- Sections cut
- Determination of staining methodology
- Sections stained for binding of Helix pomatia lectin
- Staining evaluated
- Computer storage of data
- WHAT IS THE HELIX BINDING MATERIAL?
- Collection of fresh tissues
- Isolation and characterisation of breast cancer metastasis-associated Helix-binding material
- H.P.L.C.
- Lectin affinity chromatography
- S.D.S.-P.A.G.E. and Western style electroblotting
- Dot-blotting
- Ion exchange chromatography
- Correlation of staining with clinical follow-up/statistical analysis
2.5. HELIX POMATIA LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER

Formalin-fixed, paraffin-embedded sections of metastases arising from primary breast cancers were stained for the binding of Helix pomatia lectin.

2.5.1. CHOICE OF CASES

46 consecutive cases were chosen from the post mortem reports of the Middlesex Hospital from January 1982 to December 1985 of individuals who at autopsy had shown evidence of widespread metastatic disease originating from a primary carcinoma of the breast.

2.5.2. TISSUES

Blocks of formalin-fixed, paraffin-embedded tissue taken at autopsy from sites where the post-mortem report indicated the presence of metastatic tumour deposits were retrieved from the hospital archives for all cases selected. In total, 97 blocks were chosen. The distribution of metastases in the 46 patients examined was as listed in Table 6.

---

**TABLE 6: Metastases to be stained with Helix pomatia lectin**

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>bone/bone marrow</td>
<td>19</td>
</tr>
<tr>
<td>liver</td>
<td>19</td>
</tr>
<tr>
<td>lung</td>
<td>15</td>
</tr>
<tr>
<td>lymph node</td>
<td>10</td>
</tr>
<tr>
<td>adrenal</td>
<td>6</td>
</tr>
<tr>
<td>skin</td>
<td>5</td>
</tr>
<tr>
<td>pleura</td>
<td>4</td>
</tr>
<tr>
<td>pituitary</td>
<td>4</td>
</tr>
<tr>
<td>brain</td>
<td>2</td>
</tr>
<tr>
<td>other</td>
<td>13 (including: pleura; contralateral breast; thyroid; uterus; spleen; pericardium; peritoneum; kidney; gall bladder; meninges)</td>
</tr>
<tr>
<td>total</td>
<td>97</td>
</tr>
</tbody>
</table>

---
In 7 cases, the patient had previously received surgery for her primary breast carcinoma at the Middlesex Hospital (the remaining 39 cases had been treated elsewhere). In these 7 cases, a block of the original primary tumour was retrieved from the hospital archives and stained in conjunction with blocks of subsequent metastases.

### 2.5.3. HISTOLOGY

6 x 5 μm sections were cut from each tissue block, and the first and last section stained with haematoxylin and eosin, as described in section 2.3.3.1. Sections were examined through the microscope and tissues present in each block identified.

### 2.5.4. *HELIX POMATIA* LECTIN BINDING

Sections were stained in duplicate for *Helix pomatia* lectin binding, using the avidin-biotin sandwich technique listed previously (section 2.3.4.).

### 2.5.5. SCORING OF SLIDES

As before, staining was recorded as a combination of approximate percentage of cells staining positively, plus an estimate of the intensity of the staining reaction, scored as either negative, or as +, + to ++++. 
2.6. ION EXCHANGE CHROMATOGRAPHY.

2.6.1. PRINCIPLES.

2.6.2. PREPARATION OF SAMPLES.
2.6.2.1. Primary breast cancers.
2.6.2.2. Breast cancer metastasis to liver.
2.6.2.3. Normal liver.
2.6.2.4. Blood group A, B, and O sera.

2.6.3. PREPARATION OF THE ION EXCHANGE COLUMN.

2.6.4. MONITORING OF MATERIAL ELUTED FROM THE ION EXCHANGE COLUMN.

2.6.5. ION EXCHANGE METHOD.

2.6.6. ANALYSIS OF COLLECTED FRACTIONS.
2.6.6.1. Dot-blotting.
2.6.6.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (S.D.S.-P.A.G.E.) of ion exchange fractions.

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PLAN OF INVESTIGATION

Identification of all breast cancers diagnosed at Middlesex Hospital 1971-1977

Collection of formalin-fixed paraffin-embedded tissues

Collection of patient follow-up data

Sections cut

Identification of breast cancer metastases

Determination of staining methodology

Sections stained for binding of Helix pomatia lectin

Staining evaluated

Computer storage of data

WHAT IS THE HELIX BINDING MATERIAL?

Correlation of staining with clinical follow-up/statistical analysis

Collection of fresh tissues:

Isolation and characterisation of breast cancer metastasis-associated Helix binding material

H.P.L.C.

Lectin affinity chromatography

S.D.S.-P.A.G.E. and Western style electrophoresing

Dot-blotting

Ion exchange chromatography
2.6 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography was performed in an attempt to separate the material in breast cancers which binds to the *Helix pomatia* lectin from other components.

2.6.1. PRINCIPLES

Ion exchange chromatography is one of the principal methods for fractionation of closely similar biological molecules from a highly complex mixture. The principle of ion exchange is that separation of molecules is achieved on the basis of their electrostatic charge. It is a widely used technique - as most biological molecules are polar and thus capable of being charged - it is applicable to numerous separation problems - and carries great resolving power.

The ion exchange matrix may be based on an inorganic compound, synthetic resin, or more commonly a polysaccharide, (cross-linked dextran, agarose, or cellulose) to which the functional groups are covalently linked - its properties, such as mechanical strength, flow characteristics, capacity and so on differ accordingly. The first ion exchangers designed for separation of biological molecules were based on cellulose (Peterson and Sober 1956) but suffered from disadvantages of low capacity and poor flow properties. These problems have been largely overcome in the wide range of modern ion exchangers currently available.

The ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. The charged groups are associated with mobile counter-ions, which readily exchange with other ions carrying the same charge. Both positively and negatively charged ion exchangers are available; positively charged ion exchangers carry negatively charged counter-ions (anions) and are thus termed "anion exchangers", similarly, a negatively charged ion exchanger is associated with positively charged counter-ions (cations) and is therefore called a "cation exchanger".

The separation in ion exchange is obtained by reversible adsorption of molecules onto the column. The sample is first applied to the column - molecules carrying the same charge as the counter-ions of the column are adsorbed, and counter ions, in exchange, are released. Unbound substances - molecules bearing an opposite charge to that of the columns counter-ions - can then be washed out from the exchanger bed unhindered. This mechanism is illustrated in Figure 15, overleaf.
Bound substances will have different affinities for the ion exchanger owing to differences in the charge they carry. These affinities can be controlled in two ways - by varying either the pH, or the ionic strength, of the buffer bathing the column. At low ionic strengths, competition for charged groups on the ionic exchanger is at a minimum and substances are bound strongly. Increasing ionic strength of the buffer bathing the column increases competition and reduces interaction between ion exchanger and the sample substances, resulting in their elution.

Alternatively, as the net charge on a molecule is dependent on pH, altering the pH towards the isoelectric point of a substance causes it to lose its net charge, desorb, and elute from the ion exchanger. Bound substances can thus be separated by sequential elution as a gradient of either changing pH or changing ionic strength is applied to the column.

In practice, continuous pH gradients are difficult to produce at constant ionic strength, since simultaneous changes in ionic strength, although small, do occur. Linear pH gradients cannot be obtained by mixing buffers of different pH in linear volume ratios since the buffering capacities of the systems produced are pH dependent. In addition, the buffer has to titrate the buffering action of the ion exchanger.
Continuous ionic strength gradients are easy to prepare and are highly reproducible - two buffers of differing ionic strength are simply mixed together, and if the volume ratio is changed linearly, the ionic strength changes linearly. The simplest linear ionic strength gradient - and the one employed in this set of experiments - is produced in a two chamber gradient-making apparatus, the starting buffer being of very low ionic strength, in this case distilled water, its molarity gradually increasing to equilibrium with a strong - eg 0.25M or 0.5M - sodium chloride solution.

2.6.2. PREPARATION OF SAMPLES

Samples for ion exchange were prepared as follows:

2.6.2.1. Primary breast cancers

It is a relatively rare occurrence for us successfully to obtain fresh breast cancer specimens from mastectomy and lumpectomy operations, as these are routinely fixed in formalin in the operating theatre. Biopsy for frozen section is also becoming an increasingly unusual procedure, being rapidly superceded by fine needle aspiration or needle biopsy. Over a period of three years from January 1985 - December 1988 we have obtained only seven. Primary breast carcinoma is very occasionally present at post mortem - unusually as a chance finding; sometimes as residual or recurrent disease in patients who have been receiving treatment, or in individuals who have refused surgical intervention. We have managed to collect only five fresh primary breast cancers from post mortem specimens. All fresh breast cancers have been kept stored frozen at -20°C.

1g of fresh tumour tissue was cut from each of the dozen primary breast cancers in our fresh tissue bank. The tumour tissue was pooled and homogenised using a "polytron" RT 20 OD tissue homogeniser (supplied by the Northern Media Supply Company Ltd.) with a minimum volume of distilled water, at high speed, until completely liquidised.

The resulting slurry was centrifuged using an "Eppendorf" centrifuge (supplied by Anderman), and separated into three distinct layers - an upper lipid layer; a pellet of solid cell debris; and a clear, middle, aqueous layer. It was this aqueous fraction that was used in ion exchange chromatography. A 2.5ml sample was used in each ion exchange separation.

2.6.2.2. Breast cancer metastasis to liver

Fresh breast cancer metastasis to liver was obtained from 4 patients at post mortem. The tumour tissue was dissected away from surrounding uninvolved tissue. 12g of tumour, 3g
from each case, was pooled, homogenised with a minimum volume of distilled water, spun down, and the aqueous fraction separated by the same method as employed for primary tumour. A 2.5ml sample was used in each ion exchange separation.

2.6.2.3. Normal liver

Fresh normal liver samples were obtained from four post mortem examinations. (Cause of death non-malignant disease). 12g of liver, 3g from each case, was pooled, homogenised, spun down, and the aqueous fraction separated as above. A 2.5ml sample was used in each ion exchange separation.

2.6.2.4. Blood group A,B and O sera

Serum was obtained from two main sources: (1) Whole blood which had been used for cross-matching hospitalised patients was obtained from the blood bank of The Middlesex Hospital; and (2) blood was also taken from healthy volunteers amongst my work colleagues.

In either case, it was allowed to clot at room temperature, and serum then separated from the contracted clot.

0.25ml sample was used in each ion exchange separation.

2.6.3. PREPARATION OF THE ION EXCHANGE COLUMN

10g of "fast flow" Q-sepharose anion exchange resin (Pharmacia) was washed repeatedly in excess distilled water. Beads were allowed to settle gently between washes and finings discarded.

A chromatography column (1cm diameter x 16cm height) was packed with 50cm$^3$ of the beads. The column was fed from a Pharmacia "P1" peristaltic pump connected to a perspex gradient maker (home-made) and drained to a Pharmacia "Frac 100" automatic fraction collector.
2.6.4. MONITORING OF MATERIAL ELUTED FROM THE ION EXCHANGE COLUMN

The protein content of the material eluted from the ion exchange column was monitored by measuring its optical density at a wavelength of 280nm using a Shimadzu UV-110-02 spectrophotometer.

2.6.5. ION EXCHANGE METHOD

1) Sample applied to column via pump, pump speed set at a flow rate of 1ml/minute.

2) Column washed with distilled water fed from one reservoir of the gradient maker until the optical density of material eluted from the column falls to zero.

3) Column subjected to a sodium chloride gradient rising from distilled water to either 0.25 molar or 0.5 molar sodium chloride solution over a total volume of 1 litre of solution.

Fractions of 5ml volume were collected from the column.

An ion exchange profile was constructed for each sample by plotting fraction number on the horizontal axis versus optical density reading at a wavelength 280nm on the vertical axis.

2.6.6. ANALYSIS OF COLLECTED FRACTIONS

The fractions collected from the ion exchange column were analysed in two ways: Initially, each fraction was tested for the presence or absence of *Helix pomatia* lectin binding material after "dot-blotting" onto nitrocellulose membranes. Then, fractions corresponding to the tips of "peaks" in the ion exchange profile were further analysed by polyacrylamide gel electrophoresis under denaturing conditions in the presence of sodium dodecyl sulphate and Western style blotting:

2.6.6.1 Dot-blotting

"Dot-blotting" is a simple technique for conveniently analysing a large number of dilute samples, such as the fractions collected from an ion exchange chromatography column, for the binding of a ligand such as an antibody, enzyme or lectin. The technique utilises the remarkable binding properties of nitrocellulose membrane for protein. A tiny aliquot of
each sample is applied as a dot to the nitrocellulose membrane where protein binds tightly. Unused binding sites of the membrane are then saturated by incubation with some simple blocking protein which will not interfere with subsequent staining procedure - bovine serum albumin, milk, or haemoglobin are perhaps most frequently used. The membrane can then be probed by basically the same methods which are used in the histochemical staining of tissue sections to give a coloured, fluorescent, or radio-active product and demonstrate the presence or absence of a particular substance.

1) 5μl samples of each fraction collected from the column were applied using a 50μl micro-syringe to a sheet of 0.2μm pore nitrocellulose paper (Schleicher and Schuell, supplied by Anderman) and allowed to air-dry for 5 minutes.

2) The nitrocellulose sheet was then incubated in a 2% solution of bovine serum albumin (Sigma) and 0.05% Tween 20 (Sigma) in TBS, with constant agitation, for 30 minutes.

3) It was then transferred to a solution of peroxidase labelled Helix pomatia lectin (Sigma) at a concentration of 1μg/ml in 0.05% Tween 20 in TBS, with constant agitation, for 2.5 hours.

4) The membrane was then washed extensively in 3 x 5 minute changes of 0.05% Tween 20 in TBS; and incubated in DAB/H2O2 for 10 minutes. Any fraction which contained Helix pomatia lectin binding material showed as a brown coloured dot.

2.6.6.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (S.D.S.-P.A.G.E.) of ion exchange fractions

Preparation of samples:
The fractions corresponding to each "peak" of the ion exchange profile graphs were pooled; transferred to dialysis sacs (Sigma) and dialysed overnight against running tap water to remove excess sodium chloride; and then concentrated against "aquacide" (CalBiochem) until the optical density measured at a wavelength of 205nm or 280nm was greater than 1.

S.D.S.-P.A.G.E. and Western blotting:
This material was then analysed by polyacrylamide gel electrophoresis in the presence of S.D.S. followed by Western style blotting as described in Section 2.9. The blots were then probed for the presence of Helix pomatia binding material.
2.7. AFFINITY CHROMATOGRAPHY.

2.7.1. PRINCIPLES.

2.7.2. PREPARATION OF HELIX POMATIA LECTIN AFFINITY CHROMATOGRAPHY BEADS.

2.7.2.1. Theory.
2.7.2.2. Coupling of Helix pomatia lectin to cyanogen bromide-activated agarose beads.

2.7.3. PREPARATION OF SAMPLES.

2.7.4. COLUMN AFFINITY CHROMATOGRAPHY.

2.7.4.1. Apparatus.
2.7.4.2. Application of sample.
2.7.4.3. Elution of unbound material.
2.7.4.4. Affinity elution of bound material.

2.7.5. REGENERATION OF THE GEL.

2.7.6. ANALYSIS OF FRACTIONS.

2.7.6.1. Estimation of yield.
2.7.6.2. Dot-blotting.
2.7.6.3. S.D.S.-P.A.G.E. and Western blotting.
2.7.6.4. Gel filtration by H.P.L.C.

2.8. GEL FILTRATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (H.P.L.C.)

2.8.1. PRINCIPLES OF GEL FILTRATION.

2.8.2. H.P.L.C. - PRINCIPLES.

2.8.3. H.P.L.C. APPARATUS.

2.8.4. METHOD.

2.8.5. MOLECULAR WEIGHT MARKERS.
2.8 Gel filtration by HPLC

2.7. AFFINITY CHROMATOGRAPHY

*Helix pomatia* lectin affinity chromatography was employed in an attempt to purify lectin binding material from crude, heterogeneous samples.

2.7.1. PRINCIPLES

Affinity chromatography is a technique by which almost any biological molecule can be purified on the basis of its chemical structure or its biological function. The molecule to be purified is specifically and reversibly adsorbed onto a complementary binding ligand immobilised onto an insoluble support matrix. Rapid, simple and highly selective separations result from the natural specificities of reacting molecules.

The first application of affinity chromatography was the adsorption of amylase onto an insoluble starch matrix in 1910, but it was not until Axen et al (1967) reported that molecules containing a primary amino group could be coupled to an insoluble polysaccharide matrix by the action of cyanogen bromide that affinity chromatography started to become established as a routine separation technique. Today numerous specific affinity adsorbants are commercially available, in addition to cyanogen bromide activated agarose beads convenient for ready immobilisation of almost any ligand.
Affinity chromatography may be performed with the beads packed into a chromatography column, or as a batch procedure. In either case the affinity adsorbant is exposed to a complex sample but selectively binds only molecules which have a structure complementary for that of the immobilised ligand. Unbound material is then eluted with a simple buffer; and finally the bound material is released. Bound material can be freed by either a change in pH which will alter the degree of ionization of groups at the binding site; or by increasing the ionic strength of the buffer bathing the beads; or by affinity elution using a solution of a specific eluting agent which will compete for binding sites on either the immobilised ligand or on the adsorbed molecules.

In column affinity chromatography, for sharp elution curves and efficient recovery of adsorbed ligands as low a flow rate as is practicable coupled with minimal diffusion should be used. In sample application, a low flow rate will ensure maximum binding as sample is in contact with the affinity adsorbant for an extended period. In affinity elution, the bound substance is in equilibrium with a small amount of free substance, and the eluting agent is either in competition with it or with the immobilised ligand. Thus, in such a dynamic system, a rapid flow rate would prevent fully effective interaction of molecules and result in sub-optimal recovery of affinity bound molecules.

The principles of affinity chromatography are illustrated in Figure 16:

**Principles of Affinity Chromatography**

1) lectin immobilised on gel  
2) Sample substance adsorbed  
3) bound substance released

**FIGURE 16:** Principles of affinity chromatography
2.7.2. PREPARATION OF HELIX POMATIA LECTIN AFFINITY CHROMATOGRAPHY BEADS

2.7.2.1. Theory

Cyanogen bromide reacts with vicinol diols of agarose, dextran and cellulose to produce a reactive matrix that can be subsequently derivitised with either spacer molecules or ligands containing primary amines. Cyanogen bromide activated beads can be purchased commercially and linked to ligands of the experimenter's choice by a simple method described below (section 2.7.2.2). The chemical reaction involved is illustrated in Figure 17:

![Diagram of cyanogen bromide activation of polysaccharides](after Dean P.D.G., Johnson W.S. and Middle F.A. 1987.)

FIGURE 17: Cyanogen bromide activation of polysaccharides.

2.7.2.2. Coupling of Helix pomatia lectin to cyanogen bromide-activated agarose beads

Swelling and washing the gel -
(All solutions are pre-cooled to 4 °C before use.)

2g cyanogen bromide (CNBr) activated sepharose 4B beads (Pharmacia) allowed to swell for 15 minutes in 20ml 1mM HCl, with continuous gentle agitation on a rotary mixer.

They were then washed 10 times in 20ml aliquots of 1mM HCl; after each wash, the beads were allowed to settle, and supernatant and finings poured off.
1g dry gel powder theoretically yields approximately 3.5ml of swollen gel.

(The addition of HCl preserves the activity of the reactive groups which hydrolyse readily at high pH.)

**Preparation of ligand to be coupled**
For each 1ml of gel, 2ml of a solution of *Helix pomatia* lectin was prepared, at a concentration of 5-10mg/ml in 0.1M NaHCO₃, pH 8.3, with 0.5M NaCl.

**Coupling of ligand to beads**
The gel was briefly washed in coupling buffer (0.1M NaHCO₃, pH 8.3, with 0.5M NaCl), then immediately transferred to the lectin solution, the ratio of volumes being 1 part gel : 2 parts lectin solution. Gel and lectin were gently mixed together, on a rotary mixer, for 2 hours or overnight.

Coupling is most efficient at pH 8-10 (where amino groups of the lectin are in an un-protonated form), and in the presence of a high concentration of salt (to minimise protein-protein adsorption caused by the polyelectrolyte nature of proteins).

NB. Tris and buffers containing amino groups must not be used, as they will couple to the gel.

**Blocking excess reactive groups**
The beads were allowed to settle and the supernatant discarded.

Beads were then washed extensively, with gentle agitation on a rotary mixer, with Tris/HCl buffer, pH 7.6.

**Washing the product**
Beads washed in 5 alternate cycles of (1) coupling buffer and (2) sodium acetate buffer, pH 4.5, both incorporating 0.5M NaCl, to elute any uncoupled lectin.

This step ensures that no free lectin remains ionically bound to immobilised lectin. Protein desorption occurs only when pH is changed. pH changes do not cause loss of covalently bound protein.
2.7.3. PREPARATION OF SAMPLES

Samples prepared from homogenised fresh primary breast cancers and from metastases to liver were prepared as described for ion exchange chromatography (Section 2.6.2). The samples were dialysed against running tap water overnight and then centrifuged at 10,000G to remove any tiny solid particles.

Pooled, frozen human milk was obtained from the "milk bank" of Queen Charlotte's Hospital, Chelsea. Samples were de-fatted by chloroform-methanol extraction according to the method described by Wessel and Flugge (1984) and listed below:

*de-fattting:*
1) 400μl methanol (BDH) vortexed briefly with 100μl milk, then centrifuged at 9000g for 10 seconds.

2) 100μl chloroform (BP) added; mixture vortexed, then centrifuged at 9000g for 10 seconds.

3) For phase separation, 300μl distilled water added; samples vortexed vigorously, and centrifuged at 9000g for 1 minute.

4) The upper methanol/aqueous layer and lower chloroform/lipid layer carefully drawn off and discarded.

5) 300μl methanol added to the residual protein disc; the mixture vortexed, and centrifuged for 2 minutes at 9000g to pellet the protein.

6) Methanol drawn off; protein pellet air dried and stored frozen prior to use.

For affinity chromatography, de-fatted milk pellet reconstituted in the original volume of normal saline.

2.7.4. COLUMN AFFINITY CHROMATOGRAPHY

2.7.4.1. Apparatus

A small column (75mm x 10cm) (Pharmacia) was carefully packed with 5cm³ beads. The column was fed from a Pharmacia "P1" peristaltic pump and drained to a Pharmacia "Frac 100" fraction collector. 1ml samples were collected. The optical density of these were read at 280nm using a Shimadzu UV-110-02 spectrophotometer.
2.7.4.2 Application of sample

2.5 ml of sample was applied through the pump over a period of 1 hour.

2.7.4.3 Elution of un-bound material

Un-bound material was eluted in normal saline. Eluted fractions were monitored by spectrophotometer until the optical density of the eluted material at 280nm fell to a steady low level. For this step, the flow rate at the pump was increased to 10ml/hour.

2.7.4.4 Affinity elution of bound material

Bound material was then eluted by 5ml of a 0.1M solution of N-Acetyl-galactosamine in normal saline. For this step the flow rate was reduced to 2.5ml/hour.

2.7.5 REGENERATION OF THE GEL

After use the beads were regenerated according to the following procedure:

1. Beads washed in ten-times their volume of 0.1M Tris/HCl buffer containing 0.5M NaCl adjusted to pH 8.5 with concentrated HCl.

2. Washed with ten-times their volume of 0.1M sodium acetate buffer containing 0.5M NaCl adjusted to pH 4.5 with concentrated HCl.

3. Re-equilibrated with starting buffer.

2.7.6 ANALYSIS OF FRACTIONS

2.7.6.1 Estimation of yield

The yield of *Helix pomatia* lectin binding material isolated by this method was estimated by protein analysis according to the method described by Bradford (1976):

(1) Standard solutions of bovine serum albumin (Sigma) at concentrations of 2.5 / 5 / 10 /15 / 20 / and 25µg/ml were prepared in a solution of 0.15M NaCl in distilled water.

(2) 0.1ml aliquots of each standard, and a 0.1ml aliquot of *Helix pomatia* lectin binding
material, were mixed into a series of test tubes each containing 5μl of Bradford's reagent (Sigma).

(3) The absorbance of the resulting mixtures were measured at a wavelength of 595nm using a Shimadzu UV-110-02 spectrophotometer.

(4) The absorbance readings of the range of albumin standards were plotted against their known protein concentrations to give a standard curve.

(5) The approximate protein concentration of the solution containing the lectin binding material was then calculated from the curve.

2.7.6.2. Dot-blotting

All fractions collected from the affinity chromatography column; and the starting sample, samples of the washes, and the eluted material prepared by the batch methods, were tested for Helix pomatia lectin binding by dot-blotting as described under section 2.6.6.1.

2.7.6.3. S.D.S.-P.A.G.E. and Western blotting.

The original samples; washes; and material eluted from the column by 0.5M N-acetylgalactosamine were dialysed against running tap water overnight and concentrated by dialysis against polyethylene glycol flakes ("Aquacide") (CalBiochem) until their optical density read at 280nm was approximately 1.0. They were then analysed by S.D.S.-P.A.G.E., under denaturing conditions; the polyacrylamide gels stained for total protein by Coomassie blue, and duplicate gels blotted onto nitrocellulose and the blots probed for Helix pomatia lectin binding, by the methods described in Section 2.9.

2.7.6.4. Gel filtration by H.P.L.C.

Samples of bound material eluted from the beads by 0.1M and 0.5M N-acetyl galactosamine; by 0.5M sodium chloride; and by regeneration of the beads after use were dialysed over night against Tris/HCl buffer pH 7.6, and concentrated against Aquacide until their optical density measured at 280nm was approx 1.0. They were then subjected to gel filtration by the technique of H.P.L.C. (high performance liquid chromatography) as described in section 2.8.
2.8 GEL FILTRATION CHROMATOGRAPHY BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (H.P.L.C.)

The purity of the material extracted from tissue homogenates by lectin affinity chromatography was subsequently assessed by the technique of gel filtration, performed in a high performance liquid chromatography (H.P.L.C.) system.

2.8.1. PRINCIPLES OF GEL FILTRATION

Gel filtration separates molecules according to size. Separation depends upon the different abilities of the various sample molecules to enter the pores of the gel. Very large molecules which are unable to enter the stationary phase, move through the chromatographic bed unhindered, and thus emerge from the column first. Smaller molecules which can actually enter the gel pores, move more slowly through the column, since they spend a proportion of their time in the stationary phase. Molecules are thus eluted in order of decreasing size. This is illustrated in Figure 18.

![Figure 18](image)

**FIGURE 18:** Principles of gel filtration chromatography. Small molecules are able to enter the beads and are thus retarded by the column. Larger molecules cannot enter the beads and pass unhindered. Molecules are thus eluted in decreasing order of size.
2.8.2. H.P.L.C. - PRINCIPLES

Most forms of column chromatography (such as the ion exchange chromatography described in section 2.6, lectin affinity chromatography in section 2.7, and gel filtration, above) rely on gravity or low pressure pump systems for supply of eluent to the column. Flow rates are necessarily slow, with the consequence that any bands of separated material passing through the column become broadened from the effects of diffusion. It is not possible simply to speed up the flow rate, as this would cause compression and damage to the gel matrix - further impairing flow and consequently reducing, rather than enhancing, resolution.

In the last decade, dramatic developments in chromatography technology have made available a range of chromatography matrices (developed for affinity chromatography, ion exchange, gel filtration etc) of small particle size, specifically designed to withstand high pressures and high flow rates. The resulting technique of "high performance (originally called "high pressure-") liquid chromatography" is relatively fast compared to conventional column chromatography, and gives excellent resolution coupled with reliable, reproducible separation results.

2.8.2. H.P.L.C. APPARATUS

H.P.L.C. was performed using a basic module supplied by Dionex. This machine was linked to a model 2238 "Uvicord SII" ultraviolet detector from LKB and a model 2210 two-channel chart recorder, also from LKB. Fractions emerging from the column were collected by a model 2212 "Helirac" automatic fraction collector from the same supplier.

Gel filtration chromatography was achieved in this system by use of a "T.S.K. 3000 S.W." column from Amersham.

2.8.3. METHOD

flow rate = 1ml/min
2 minute fractions were collected

1) The system was first equilibrated with 0.025M Tris-HCl (pH 7.5).

2) A 50μl sample was applied to the gel filtration column via the sample loop of the H.P.L.C. module.
3) 0.025M Tris-HCl (pH 7.5) was washed through the column until the optical density of the eluted material (measured at a wavelength of 280nm) fell to a steady zero (approximate running time 40 minutes).

N.B. All reagents must be of H.P.L.C. grade. Trizma base ("reagent grade") was purchased from Sigma; sodium chloride ("HiPerSolv") from BDH; and purified water was drawn from an "Elgastat Spectrum RO" water purification system supplied by Elga Ltd.

2.8.4. MOLECULAR WEIGHT MARKERS

The gel filtration molecular weight markers listed in Table 7 were run in parallel with the sample. Molecular weight markers were purchased from Sigma.

**TABLE 7: Gel filtration molecular weight markers**

<table>
<thead>
<tr>
<th>marker protein</th>
<th>approx. molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytochrome c</td>
<td>12.4</td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>29</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>66</td>
</tr>
<tr>
<td>alcohol dehydrogenase</td>
<td>150</td>
</tr>
<tr>
<td>beta-amylase</td>
<td>200</td>
</tr>
<tr>
<td>blue dextran</td>
<td>2,000</td>
</tr>
</tbody>
</table>
2.9. SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (S.D.S.-P.A.G.E.) AND WESTERN-STYLE ELECTROBLOTTING.

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2.9. SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (S.D.S.-P.A.G.E., AND WESTERN-STYLE ELECTROBLOTTING)

2.9.1. PRINCIPLES

Polyacrylamide gel is formed by the polymerisation of the acrylamide monomer into long chains, and the cross-linking of these by bi-functional compounds such as N,N’-methylene bisacrylamide (usually shortened to bisacrylamide or simply bis) reacting with free functional groups at chain termini (Figure 19).

Polymerisation is initiated by the addition of, most commonly, ammonium persulphate, or sometimes riboflavin; plus the catalyst N,N,N’,N’-tetramethylethlenediamine (usually shortened to Temed). Temed catalyses the formation of free radicals from persulphate, which in turn initiate polymerisation. Ultraviolet light causes the photodecomposition of riboflavin to release the free radicals necessary for polymerisation of the gel even in the absence of Temed, but the process occurs more reliably in its presence.
FIGURE 19: The chemical structure of (1) acrylamide and (2) N,N'-methylene bisacrylamide monomers, and of (3) polyacrylamide gel.

(1) acrylamide

(2) N,N'-methylene bisacrylamide monomer

(3) polyacrylamide gel
Polyacrylamide gel electrophoresis is often performed under harsh conditions designed to dissociate sample proteins into their component sub-units. Samples are boiled briefly in the presence of the strong ionic detergent sodium dodecyl sulphate (SDS) and a thiol compound such as mercaptoethanol. Proteins are denatured and combine with a constant ratio of SDS. The overwhelming negative charge provided by the SDS negates any intrinsic charge carried by the molecule so that electrophoretic separation of such polypeptide-SDS complexes have essentially identical charge densities and their electrophoretic separation is almost entirely due to molecular sieving.

Polyacrylamide gel electrophoresis is most commonly performed under a multiphasic or "discontinuous" buffer system where the gel buffer is different from the buffer in the electrophoresis chamber. In the system first described by Ornstein (1964) and Davis (1964), a relatively high molarity Tris-HCl buffer of pH 8.8 is incorporated into the gel, and a low molarity Tris-glycine buffer, pH 8.3, bathes the electrodes.

Resolution of protein bands is significantly improved by the incorporation of a "stacking gel" - a low molarity gel containing a pH 6.8 Tris-HCl buffer - above the separating gel. A fairly large, dilute sample can be applied to the stacking gel, where it becomes concentrated into a narrow band before it enters the separating gel, giving fine resolution.

The way in which very narrow bands or "stacks" of protein are formed in the stacking gel is a complicated phenomenon to explain. Briefly, the sample and the stacking gel contain a Tris-HCl buffer, pH 6.8, while the electrophoretic chamber is flooded in a Tris-glycine buffer. At pH 6.8, glycine is only weakly dissociated and its effective mobility in the electric field is low, chlorine ions are highly mobile, and proteins have a mobility intermediate between the two. When voltage is applied, chloride ions race ahead, trailing a zone of reduced conductivity behind. Conductivity is inversely proportional to field strength, and so this region attains a higher voltage gradient in which the trailing glycine ions can speed up and draw level with the chloride ions. Thus an equilibrium is attained where chloride and glycine ions travel at the same speed with a sharp boundary between them: at this boundary the proteins are concentrated in a narrow stack, usually only a few microns thick.

As the protein stack hits the interface between the low molarity stacking gel (pH 6.8) and the higher molarity separating gel (pH 8.8) the glycine becomes more highly dissociated at the higher pH and races ahead of the proteins to travel ahead with the chloride ions. Proteins now travel in a zone of uniform voltage and are separated according to size by molecular sieving effects alone.
2.9.2. METHODOLOGY

One-dimensional vertical slab SDS-PAGE under a discontinuous buffer system was performed according to the method of Laemmli (1970).

A 3% acrylamide gel was used for the stacking layer, with a 10% separating gel. Good resolution of protein bands was achieved in the molecular weight range 5000 - 100,000 Da.

2.9.2.1. Stock solutions for S.D.S.-P.A.G.E.:

(1) Acrylamide Solution (concentration of acrylamide = 30%)

Acrylamide 29.2g (29.2%)
Methylene bis acrylamide 0.8g (0.8%)
Distilled water to 100ml

Solution filtered through Whatman number 1 filter paper, and stored at 4°C in a dark glass bottle (stable for several months).

(2) Lower (separating) gel buffer
pH 8.8

Tris 18.17g (1.5M)
10% SDS solution in distilled water 4.0ml (0.4%)
Distilled water to 100ml.

pH of the buffer adjusted to 8.8 with concentrated HCl. Filtered through Whatman number 1 filter paper. Buffer stored at room temperature.

(3) Upper (stacking) gel buffer
pH 6.8

Tris 6.06g (0.5M)
10% SDS solution in distilled water 4.0ml (0.4%)
Distilled water to 100ml

pH of the buffer adjusted to 6.8 with concentrated HCl. Filtered through Whatman number 1 filter paper. Buffer stored at room temperature.
(4) Sample buffer

10% SDS solution in distilled water 0.5% w/v
upper (stacking) gel buffer pH 6.8 9% w/v
glycerol or sucrose 30% w/v
bromophenol blue 0.1% w/v

Sample buffer was divided into 0.5ml aliquots and stored frozen at -20°C.

(5) Electrophoresis (running) buffer

Tris 3.03g 0.25M
glycine 14.4g 0.192M
SDS 1.0g 0.1%

Made freshly for each run

(6) Coomassie blue solution
(for protein staining of gels)

2.5 g Coomassie brilliant blue (Sigma)
20% v/v methanol
10% v/v glacial acetic acid
70% v/v distilled water

(7) Destaining solution for Coomassie blue

20% v/v methanol
10% v/v glacial acetic acid
70% v/v distilled water

2.9.2.2. Apparatus

S.D.S.-P.A.G.E. was performed using a Biometra "minigel" system (supplied by Orme Scientific) powered by a "Biorad" powerpack

2.9.2.3. Preparation of polyacrylamide gels

The electrophoresis chamber, blotting apparatus, glass plates, spacers, and all glass wear were washed thoroughly in hot water and detergent; rinsed well in distilled water
and then in 70% methanol; and allowed to dry prior to each use.

Glass plates were clamped together with 1mm spacers between them, according to the manufacturers instructions. The assembled gel mould was then positioned vertically on a flat surface with the open end uppermost.

The lower (separating) gel (a 10% acrylamide gel) was prepared according to the following protocol:

**preparation of 10% acrylamide lower (running) gel:**

- Stock (30%) acrylamide solution 10 ml
- Lower (separating) gel buffer, pH 8.8, 7.5 ml
- Distilled water 11 ml

were mixed well in a glass conical flask with a side-arm and tight fitting stopper. The mixture was de-gassed under vacuum for 5 minutes. 100µl of a freshly prepared ammonium persulphate solution (at a concentration of 100µg/ml distilled water) and 46µl Temed were quickly swirled into the flask, and the gel was then rapidly pipetted between the two glass plates of the casting mould to a depth of approximately 2.5 cm from the top. A saturated solution of butanol in distilled water or a 0.1% solution of S.D.S. was layered onto the gel, and the gel was left to polymerise for approximately one hour at room temperature.

**preparation of 3% acrylamide upper (stacking) gel:**

An upper 3% acrylamide (stacking) gel was prepared by mixing:

- Stock (30%) acrylamide solution 1 ml
- Upper (stacking) gel buffer, pH 6.8 2.5 ml
- Distilled water 6 ml

The mixture was de-gassed under vacuum for 5 minutes.

The butanol or S.D.S. solution layering the lower gel was discarded, and the surface of the gel washed x 2 with the above mixture.

A comb was inserted into the top of the gel casting mould, according to manufacturers instructions.

50µl of ammonium persulphate solution and 10ul of Temed were quickly swirled into
the stacking gel mixture. The gel was then carefully pipetted between the glass plates of
the gel casting mould until level with the upper edge of the comb.

The gel was left to set at room temperature for 1 hour.

2.9.2.4. Assembly of the electrophoresis chamber

When the gel had fully polymerised, the casting mould was disassembled by removing
the clips and carefully easing out the spacer. The gel, still sandwiched between glass
plates, was then transferred to the electrophoresis chamber and clamped vertically into
position. Upper and lower reservoirs were flooded with electrophoresis buffer and the
casting comb was gently removed, under buffer, to reveal sample wells.

2.9.2.5. Sample preparation

Equal volumes of sample (protein concentration approximately 1mg/ml) and sample
buffer were mixed well in Eppendorf tubes. The tubes were then sealed, their caps
pierced, and they were lowered into a boiling water bath for between 3 - 5 minutes.
Samples were allowed to cool before loading.

25μl aliquots were loaded into the sample wells of the polyacrylamide gel using a fine
microsyringe. For each gel, molecular weight standards were run in parallel with test
samples.

2.9.2.6. Molecular weight standards

The molecular weight of a given sample protein can be calculated by comparing its
electrophoretic mobility with those of known markers. An approximately linear
relationship is obtained if the logarithms of the molecular weights of standard marker
proteins are plotted against their electrophoretic mobilities.

Pre-stained molecular weight markers were obtained from Sigma chemical company.
The conjugation of a blue dye to the marker proteins facilitates the visual monitoring of
migration during electrophoresis, and carries the added advantage that upon blotting, the
ready-stained protein bands remain visible and are unaffected by any subsequent staining
procedure. The marker proteins included in the kit were as listed in Table 8, overleaf.
TABLE 8: Molecular weight of pre-stained markers for S.D.S.-P.A.G.E.

<table>
<thead>
<tr>
<th>protein</th>
<th>source</th>
<th>approx subunit molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-2-macroglobulin</td>
<td>human plasma</td>
<td>180 (kDa.)</td>
</tr>
<tr>
<td>beta-galactosidase</td>
<td>E. coli</td>
<td>116</td>
</tr>
<tr>
<td>fructose-6-phosphate kinase</td>
<td>rabbit muscle</td>
<td>84</td>
</tr>
<tr>
<td>pyruvate kinase</td>
<td>chicken muscle</td>
<td>58</td>
</tr>
<tr>
<td>fumarase</td>
<td>pig heart</td>
<td>48.5</td>
</tr>
<tr>
<td>lactic dehydrogenase</td>
<td>rabbit muscle</td>
<td>36.5</td>
</tr>
<tr>
<td>triosephosphate isomerase</td>
<td>rabbit muscle</td>
<td>26.6</td>
</tr>
</tbody>
</table>

Unlabelled molecular weight markers (again, supplied by Sigma) were also sometimes used.

These are listed in Table 9:

TABLE 9: Molecular weight of unlabelled markers for S.D.S.-P.A.G.E.

<table>
<thead>
<tr>
<th>protein</th>
<th>source</th>
<th>approx subunit molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>myosin</td>
<td>rabbit muscle</td>
<td>205 (kDa.)</td>
</tr>
<tr>
<td>B-galactosidase</td>
<td>Escherichia coli</td>
<td>116</td>
</tr>
<tr>
<td>phosphorylase B</td>
<td>rabbit muscle</td>
<td>97.4</td>
</tr>
<tr>
<td>albumin</td>
<td>bovine plasma</td>
<td>66</td>
</tr>
<tr>
<td>ovalbumin</td>
<td>chicken egg</td>
<td>45</td>
</tr>
<tr>
<td>pepsin</td>
<td>porcine stomach mucosa</td>
<td>34.7</td>
</tr>
<tr>
<td>carbonic anhydrase</td>
<td>bovine erythrocytes</td>
<td>29</td>
</tr>
<tr>
<td>trypsinogen</td>
<td>bovine pancreas</td>
<td>24</td>
</tr>
<tr>
<td>B-lactalbumin</td>
<td>bovine milk</td>
<td>18.4</td>
</tr>
<tr>
<td>lysozyme</td>
<td>egg white</td>
<td>14.3</td>
</tr>
</tbody>
</table>

2.9.2.7. Electrophoresis

A voltage of 130 volts was applied across the gel until the sample had fully entered the stacking gel; the voltage was then increased to 190 volts until electrophoresis was complete (approximately 1 hour).

2.9.2.8. Coomassie blue total protein stain

For staining of total protein, gels were transferred to Coomassie blue stain for 1-2 hours. Destaining was then carried out in several changes of destaining solution until protein bands were revealed deep blue against a transparent background. The sensitivity of this protein stain varies between proteins but usually 0.5 µg/band is detectable.
2.9.3. WESTERN STYLE ELECTROBLOTTING

Western-style electroblotting is a simple technique by which protein bands, previously separated by S.D.S.-P.A.G.E., are transferred from the gel to a nitrocellulose membrane. The membrane can then be probed to reveal the identity of specific bands - for example, those which bind to a particular antibody, or in this case a specific lectin.

2.9.3.1. Recipes

1) anode buffer 1:
18.48g Tris dissolved in 1 litre 20% methanol in distilled water.

2) anode buffer 2:
3.025g Tris dissolved in 1 litre 20% methanol in distilled water.

3) cathode buffer:
3.025g Tris
5.24g amino caproic acid dissolved in 1 litre 20% methanol in distilled water.

2.9.3.2. Apparatus

Blotting was performed using an Ancos (Denmark) type "A" semi-dry electroblotter (supplied through Dakopatts) connected to the same "Biorad" powerpack as used for the electrophoresis.

2.9.3.3. Blotting method

The graphite plates of the semi-dry electroblotter were cleaned thoroughly with distilled water and wiped dry with soft tissue.

The anode plate was dampened with anode buffer I and the following "sandwich" was layered onto it:

a) 6 sheets Whatman number 1 filter paper trimmed to the approximate size of the gel and soaked in anode buffer I until saturated.

b) 3 sheets Whatman number 1 filter paper soaked in anode buffer II.

c) 1 sheet Schleicher and Schuell nitrocellulose membrane thoroughly pre-soaked in distilled water.
d) The polyacrylamide gel to be blotted.

e) 9 sheets Whatman number 1 filter paper soaked in cathode buffer.

All air bubbles were very carefully expelled from the sandwich by smoothing with the flat edge of a clean spatula, or with a glass rod, or ruler.

The cathode plate was lowered into position and 0.26 Amps/gel were applied across the sandwich for 1 hour.

This arrangement is illustrated in Figure 20:

![Diagram of semi-dry electroblotting arrangement](image)

**FIGURE 20:** Semi-dry electroblotting - arrangement of polyacrylamide gel, nitrocellulose membrane, filter paper, and electrodes.

### 2.9.3.4. Staining the blotted membrane

The membrane was first stained with Ponceau red total protein stain to check the efficiency of transfer, and then - after destaining - probed for the binding of *Helix pomatia* lectin.
1) **Ponceau red total protein stain**

After blotting, the nitrocellulose membrane was carefully removed from the blotter and transferred to a dish of a 1% solution of Ponceau red (Sigma) in distilled water, for 1 minute. It was then washed in several changes of distilled water until protein bands showed pink-red against a white background.

The membrane can then either be allowed to dry as a permanent record of total protein transferred from the polyacrylamide gel, or can be completely destained in distilled water and then further probed for *Helix pomatia* lectin binding.

2) **Helix pomatia lectin binding:**

   a) **Optimisation of staining methods**

   A single sample, liver metastasis A, was run in all 10 tracks of the gel slab. After blotting, the nitrocellulose sheet was cut into 10 strips. Each strip could then be stained separately, in order to determine the optimum conditions for staining Western blots for *Helix pomatia* lectin binding.

   The criteria by which the methods were judged were:

   i) sensitivity  
   ii) minimum non-specific background staining  
   iii) reliability / reproducability  
   iv) economy of reagents (especially of peroxidase labelled *Helix pomatia* lectin which is quite expensive!)

**Basic Method:**

1) Blot incubated with a 3% solution of bovine serum albumin (Sigma) in TBS for 30 minutes.

2) Incubated with peroxidase labelled *Helix pomatia* lectin

3) Washed extensively x 3 in TBS

4) Developed in DAB/H₂O₂
Variations of the basic method:
i) All solutions, washes etc prepared in TBS alone; or in TBS in the presence of 0.05% 0.1%; 0.25%; 0.5%; 1% Tween 20 detergent (Sigma)

ii) All incubations, washes etc performed either simply on the bench; or with continual agitation on a rotary mixer.

iii) Peroxidase labelled Helix pomatia lectin tested at concentrations of 1µg/ml; 2µg/ml; 4µg/ml; 8µg/ml /10µg/ml with incubation times of 30 minutes; 1 hour; 1.5 hours; 2 hours; 2.5 hours; 3 hours; 6 hours; 12 hours.

b) Final Standard Staining Method:
After optimisation of methodology, the following standard method was applied to the staining of all blots:

1) The nitrocellulose membrane, either directly from the blotter or after complete destaining following Ponceau red treatment was incubated in a 3% solution of bovine serum albumin in 0.05% Tween 20 (Sigma) in TBS for a 30 minutes, with constant agitation.

2) It was then transferred to a solution of peroxidase labelled Helix pomatia lectin (Sigma) at a concentration of 1µg/ml in TBS for 2.5 hours, with constant agitation.

3) The membrane is then washed extensively in several changes of 0.05% Tween 20 in TBS, and Helix lectin binding revealed by application of a chromogenic substrate for peroxidase, DAB-H2O2 for 20 minutes. The blot was then rinsed thoroughly in distilled water and allowed to dry.

2.9.4. SAMPLES FOR S.D.S./P.A.G.E. AND WESTERN STYLE BLOTTING

The following samples were analysed by S.D.S.-P.A.G.E./Western blotting in order to identify and characterise any Helix lectin binding bands present:

2.9.4.1. Fresh primary carcinoma of the breast

Samples of fresh primary breast carcinoma was taken from surgical specimens and autopsy material, as described in section 2.6.2.

1g of fresh tumour tissue was cut from each of the dozen primary breast cancers in our
fresh tissue bank. The tumour samples were homogenised at high speed until completely
liquidised.

The resulting slurry was centrifuged, and separated into three distinct layers - an upper lipid
layer; a pellet of solid cell debris; and a clear, middle, aqueous layer. The aqueous layer
was further de-lipidised and concentrated by chloroform-methanol extraction after the
method described by Wessel and Flugge (1984) as described in section 2.7.3.

The pellet of concentrated protein was re-dissolved overnight in a 0.1% solution of SDS at
an approximate concentration of 1mg/ml.

Reduction of the albumin component:
In some samples, a dominant albumin fraction caused some disturbance of the
electrophoretic run and made interpretation of gels and blots difficult (see Figures 56 and
59 in "Results" section). It was thus necessary to reduce the albumin component of some
samples. This was done very simply by mixing the sample with "affi-gel blue" (Biorad), an
ion exchange resin which according to the manufacturer's claims, selectively adsorbs
albumin.

1ml of "affi-gel blue" beads were mixed gently with 250μl of sample for 30 minutes. The
beads were then spun down, and the sample drawn off and run on S.D.S.-P.A.G.E.

If excess albumin still remained after treatment, the procedure was repeated as many times
as necessary, using fresh beads in each case.

2.9.4.2 Fresh breast cancer metastasis to liver
2.9.4.3 Fresh breast cancer metastasis to bone
2.9.4.4 Fresh breast cancer metastasis to lymph node
2.9.4.5 Fresh breast cancer metastasis to lung

Samples of breast cancer metastases to different sites were taken at autopsy of patients who
had died as a result of widespread metastatic breast cancer. Tissues were stored at -20°C
until required.

For each site, 1g tissue samples from a minimum of five separate cases were taken and
homogenised to a slurry. The liquidised tissue was then centrifuged to pellet any solid
particles, and then treated in exactly the same way as described in section 2.9.4.1.
2.9.4.6. Fresh normal human liver
2.9.4.7. Fresh normal human bone
2.8.4.8. Fresh normal human lung

Samples of normal tissue were taken from post mortem examinations of patients who had
died as a result of non-malignant disease. For each site, 1g tissue samples from a minimum
of five separate cases were taken and homogenised to a slurry. The tissue was then treated
as described in section 2.9.4.1.

2.9.4.10. Human breast milk

Pooled, frozen human milk was obtained from the "milk bank" of Queen Charlotte's
Hospital, Chelsea.

It was de-fatted by the method of Wessel and Flugge (1984) as described in section 2.7.3.
prior to use.

2.9.4.11. Human milk fat globule membrane

Milk fat globule membrane was prepared from milk by a simple phase partitioning method
which utilises the unique properties of the ionic detergent Triton X-114 (Bordier 1981).
Triton X-114 is soluble in aqueous buffers at low temperatures; but above 20°C separates
out as a distinct detergent phase - in general, hydrophilic, peripheral proteins are then found
in the aqueous phase, and the hydrophobic species in the detergent phase.

1) Sucrose (Sigma) was added to a 25ml sample of fresh milk to give a 4% solution.

2) An equal volume of distilled water was overlayed over the milk/sucrose in a 50ml
centrifuge tube, and the mixture spun at 3000 r.p.m./ 1500g for 15 minutes - the fat forms
a thick upper layer.

3) The upper layer was removed; and the aqueous fractions spun again to give a second
yield of cream.

4) Lipid was separated from protein in the cream layer by chloroform- methanol extraction
according to the method described by Wessel and Flugge (1984), and listed in 2.7.3.

5) The protein layer was gently agitated in a 2% solution of Triton-X114 in 10mM
Tris/HCl buffer, pH 7.4, at an approximate concentration of 1-3mg/ml, at 0-4°C, for 1
hour to solublise the milk fat globule membrane.
6) The mixture was centrifuged at 100,000g for 1 hour to pellet any insoluble material.

7) The soluble fraction was removed and layered on a cushion of 6% sucrose in Tris/HCl buffer (pH 7.4) in a clean centrifuge tube.

8) It was warmed to 25-30°C; then centrifuged at 100g for 5 minutes above 20°C. The detergent appears as an oily layer in the bottom of the centrifuge tube.

9) Solubilised membrane in the aqueous layer was then concentrated by chloroform / methanol extraction by the method of Wessel and Flugge described in 2.7.3., and re-dissolved at a concentration of 1mg/ml in 0.1% SDS solution.

[Triton X-114, which may be purchased from the Swiss company Fluka, was a gift from Dr. Udo Schumacher, Department of Anatomy, University of Munich.]

2.9.4.12. Normal human serum

Normal human serum of blood groups A, B, and O was obtained from the blood bank of the Middlesex Hospital.

Albumin was removed by treatment with "affi-gel blue" as described in section 2.9.4.1.

2.9.4.13. Other samples

samples from ion exchange chromatography
(see Section 2.6)

samples from affinity chromatography
(see Section 2.7)
RESULTS
3. RESULTS

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3.1.3. PATIENT RECURRENCE STATUS AT THE END OF THE STUDY.

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3.1.4.4. Histological tumour grade.
3.1.4.5. DNA index.
3.1.4.6. S-phase fraction.
3.1. BREAKDOWN OF CLINICAL FOLLOW-UP DATA

The degree of success achieved in obtaining clinical follow-up data on the breast cancer patients in this study is summarised in the following section.

Also included is a summary of patient characteristics -
age at diagnosis
tumour size (tumour cut surface)
degree of lymph node involvement
histological tumour grade
DNA index
S-phase fraction

3.1.1. SUCCESS OF CLINICAL FOLLOW-UP

Total number of patients entering study = 373.
Of these, the number which were - in spite of intensive efforts to trace them (see section 2.1 of Methods) - "lost to follow-up" is summarised in Table 10.

<table>
<thead>
<tr>
<th>Months since beginning patients of study</th>
<th>Number of patients lost</th>
<th>% of total number of entering the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;60 months</td>
<td>9</td>
<td>2.4%</td>
</tr>
<tr>
<td>61-120 months</td>
<td>51</td>
<td>13.6%</td>
</tr>
<tr>
<td>121-180 months</td>
<td>12</td>
<td>3.2%</td>
</tr>
<tr>
<td>181-240 months</td>
<td>5</td>
<td>1.3%</td>
</tr>
<tr>
<td>total patients lost</td>
<td>77</td>
<td>(approx 20%)</td>
</tr>
</tbody>
</table>

Reasons for loss of follow-up
Patients were lost to follow-up for a number of reasons: seven of the nine patients lost during the first five years of the study either returned to their homes in other countries following initial treatment in this country, or emigrated. The majority of "lost" patients, however, having been discharged from hospital follow-up (most usually 7 -10 years after diagnosis if patient remains free of apparent recurrent or residual disease until that time) could not be subsequently traced in spite of extensive enquiries.
3.1.2. PATIENT SURVIVAL STATUS AT THE END OF THE STUDY

Table 11, below, summarises patient survival status at the end of the follow-up period.

<table>
<thead>
<tr>
<th>Survival status:</th>
<th>Number of patients:</th>
<th>% of total number of patients entering study</th>
</tr>
</thead>
<tbody>
<tr>
<td>alive</td>
<td>58</td>
<td>15.5%</td>
</tr>
<tr>
<td>dead (not of breast cancer)</td>
<td>17</td>
<td>4.5%</td>
</tr>
<tr>
<td>dead (breast cancer)</td>
<td>214</td>
<td>57.4%</td>
</tr>
<tr>
<td>dead (? breast cancer)</td>
<td>7</td>
<td>1.8%</td>
</tr>
<tr>
<td>lost to follow-up</td>
<td>77</td>
<td>20.5%</td>
</tr>
</tbody>
</table>

Total number patients dead = 238
Mean total survival time = 80 months

3.1.3 PATIENT RECURRENCE STATUS AT THE END OF THE STUDY

The proportion of patients whose disease recurred at some point during the follow-up period, those who remained disease-free, and those who were lost to follow-up before a recurrence was recorded, is summarised in Table 12.

<table>
<thead>
<tr>
<th>Recurrence status:</th>
<th>Number of patients:</th>
<th>% of total number of patients entering the study</th>
</tr>
</thead>
<tbody>
<tr>
<td>no sign of recurrence</td>
<td>132</td>
<td>35.4%</td>
</tr>
<tr>
<td>total recurred</td>
<td>228</td>
<td>61.1%</td>
</tr>
<tr>
<td>lost</td>
<td>13</td>
<td>3.5%</td>
</tr>
</tbody>
</table>

Mean interval to first recurrence = 60 months
3.1.4. PATIENT CHARACTERISTICS

Patient characteristics - including age at diagnosis, tumour size, lymph node status, histological grade, DNA index and S-phase fraction - are summarised in the following tables.

3.1.4.1. Age at diagnosis

TABLE 13: Age at diagnosis

<table>
<thead>
<tr>
<th>Age at Diagnosis:</th>
<th>Number of Cases:</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>less than 35 years</td>
<td>11</td>
<td>3%</td>
</tr>
<tr>
<td>35-44 years</td>
<td>51</td>
<td>13.7%</td>
</tr>
<tr>
<td>45-54 years</td>
<td>87</td>
<td>23.3%</td>
</tr>
<tr>
<td>55-64 years</td>
<td>120</td>
<td>32.2%</td>
</tr>
<tr>
<td>greater than 64 years</td>
<td>96</td>
<td>25.7%</td>
</tr>
<tr>
<td>unknown</td>
<td>8</td>
<td>2.1%</td>
</tr>
</tbody>
</table>

mean age at diagnosis = 55 years

3.1.4.2. Tumour size at presentation

TABLE 14: Tumour size

<table>
<thead>
<tr>
<th>Tumour Size</th>
<th>Number of Cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1cm</td>
<td>49</td>
<td>13.1%</td>
</tr>
<tr>
<td>2cm</td>
<td>111</td>
<td>29.8%</td>
</tr>
<tr>
<td>3cm</td>
<td>90</td>
<td>24.1%</td>
</tr>
<tr>
<td>4cm</td>
<td>42</td>
<td>11.3%</td>
</tr>
<tr>
<td>5cm</td>
<td>21</td>
<td>5.6%</td>
</tr>
<tr>
<td>6cm</td>
<td>11</td>
<td>3%</td>
</tr>
<tr>
<td>7cm</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>8cm</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>9cm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10cm</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>&gt;10cm</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>

mean tumour size = 2.3cm
For the purpose of later analyses, patients were split into three tumour size groups -
(1) less than the approximate mean of 2cm
(2) greater than 2cm
(3) unknown

3.1.4.3. Lymph node status

TABLE 15: Lymph node status

<table>
<thead>
<tr>
<th>Lymph Node Status</th>
<th>Number of Cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>130</td>
<td>34.9%</td>
</tr>
<tr>
<td>1-3 nodes involved</td>
<td>101</td>
<td>27%</td>
</tr>
<tr>
<td>greater than 3 nodes involved</td>
<td>59</td>
<td>15.8%</td>
</tr>
<tr>
<td>unknown</td>
<td>83</td>
<td>22.3%</td>
</tr>
</tbody>
</table>

3.1.4.4. Histological tumour grade

Histological tumour grade was assessed according to W.H.O. criteria (see section
2.1.2.5.)

TABLE 16: Histological tumour grade

<table>
<thead>
<tr>
<th>Histological Grade</th>
<th>Number of Cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>27</td>
<td>7.2%</td>
</tr>
<tr>
<td>II</td>
<td>135</td>
<td>36.2%</td>
</tr>
<tr>
<td>III</td>
<td>211</td>
<td>56.6%</td>
</tr>
</tbody>
</table>

Examples of tumours designated grade I, II, and III are illustrated in figure 21, overleaf.
FIGURE 21: Examples of histological tumour grades I, II, and III.

**Grade I:** Marked tubule differentiation; very occasional hyperchromatic nuclei or mitoses; highly regular, rounded nuclei.

**Grade II:** Moderate tubule formation; moderate numbers of hyperchromatic nuclei and a few mitoses; some pleomorphism.

**Grade III:** Absence of tubular differentiation - cells growing in sheets; many hyperchromatic nuclei and mitoses; marked pleomorphism.
3.1.4.5. DNA index

Of the 358 tumours analysed by flow cytometry, 221 (62%) gave a coefficient of variation (cv) of less than or equal to 10. These 221 tumours were included in flow cytometric analysis of DNA index; the remaining 137 cases (38%) had to be excluded from this part of the study.

The relatively poor quality of the tissue, as indicated by the rather high proportion of samples in which a cv of >10 was recorded, is perhaps a reflection of tissue age; or may be due to original sub-optimal fixation.

**TABLE 17: DNA index**

<table>
<thead>
<tr>
<th>DNA Index</th>
<th>Number of Cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diploid tumours</td>
<td>67</td>
<td>18.7%</td>
</tr>
<tr>
<td>Aneuploid tumours</td>
<td>154</td>
<td>43%</td>
</tr>
<tr>
<td>not included</td>
<td>137</td>
<td>38.3%</td>
</tr>
</tbody>
</table>

**total =**

358

3.1.4.6. S-phase fraction.

S-phase fraction values could be calculated for 207 cases out of the 221 for which ploidy analysis was available.

The median value for S-phase fraction was 9.0.
3.2. STAINING CHARACTERISTICS - *HELIX POMATIA* LECTIN BINDING TO
SECTIONS OF 373 PRIMARY BREAST CANCERS.

3.2.1. SCORING OF SLIDES.

3.2.2. STAINING PERCENTAGE.

3.2.3. STAINING INTENSITY.

3.2.4. STAINING PATTERN.

3.2.5. *HELIX POMATIA* LECTIN BINDING TO OTHER BREAST
STRUCTURES.

3.2.5.1. Normal breast.
3.2.5.2. Endothelium of blood vessels, and erythrocytes.
3.2.5.3. Benign breast disease.
3.2 STAINING CHARACTERISTICS - *HELIX POMATIA* LECTIN
BINDING TO SECTIONS OF 373 PRIMARY BREAST CANCERS

3.2.1. SCORING OF SLIDES

Slides were scored for a combination of (a) the percentage of cancer cells binding *Helix pomatia* lectin and (b) an estimate of staining intensity.

Positive cases were taken to be those with >5% cells staining with an intensity of +, or >50% +-, as illustrated in Figures 24b-e.

Negative cases were those where 5% or less of cells stained with an intensity of +, or 50% or less +-, as illustrated in Figure 24a.

3.2.2. STAINING PERCENTAGE

The histogram (Figure 22) illustrates the distribution of *Helix* lectin staining percentage amongst 373 primary breast cancers.
80 cases were judged to be negative. While relatively few cases showed only a small percentage (10%-50%) of cells to be Helix-positive, the largest proportion of cases had 80%-100% of cancer cells obviously binding the lectin.

3.2.3. STAINING INTENSITY

The histogram (Figure 23) illustrates distribution of Helix staining intensity amongst 373 cases.

FIGURE 23
Distribution Of Helix Pomatia Lectin Staining Intensity

80 cases were negative; a majority of cases stained with intensity + or ++; fewer +++ or the extreme ++++.

Examples of cases judged to stain with intensity -, +, ++, ++++, ++++ are given in Figure 24, overleaf.
FIGURE 24: Examples of the range of *Helix pomatia* lectin staining intensities seen in this study.

a) negative

b) stain intensity +

c) stain intensity ++

d) stain intensity +++

e) stain intensity ++++

magnification x10
3.2.4. STAINING PATTERN

Cancer cells, if positive for *Helix pomatia* lectin binding, generally showed predominantly cytoplasmic localisation, often with marked staining at the cell border (Figure 25).

**FIGURE 25:** Examples of staining pattern - cytoplasmic and cell border localisation

a) cytoplasmic plus cell border localisation. magnification x20

b) cytoplasmic plus cell border localisation. magnification x40
c) predominantly cytoplasmic localisation. magnification x10

Frequently, a striking heterogeneity of *Helix pomatia* lectin binding was seen - adjacent populations of morphologically indistinguishable cancer cells staining strongly positive and completely negative for lectin binding (Figure 26).

**FIGURE 26:** Adjacent sub-populations of morphologically indistinguishable cancer cells positive and negative for *Helix pomatia* lectin binding.

magnification x10
We noted that sometimes Helix binding positivity was markedly more intense at the invasive front of tumour cell spread into surrounding breast tissue (Figure 27).

FIGURE 27: Markedly increased intensity of lectin staining at the invasive front of tumour infiltration.

magnification x10

tumour cells invading fat.
magnification x40
3.2.5. HELIX POMATIA LECTIN BINDING TO OTHER BREAST STRUCTURES

*Helix pomatia* lectin was noted to bind to numerous structures in breast sections other than malignant cells.

3.2.5.1. Normal breast

The lectin invariably bound strongly to the luminal surface of epithelial cells lining normal ducts and lobules - frequently giving stunningly beautiful staining results - and to breast secretions. (Figure 28.)

**FIGURE 28:** Intense *Helix pomatia* lectin binding to luminal surface of normal breast ducts, and to breast secretions

![Image: magnification x20]
3.2.5.2. Endothelium of blood vessels and erythrocytes

In some, but not all cases, endothelium of blood vessels and red blood cells stained intensely with *Helix pomatia* lectin and in occasional slides, fairly strong, diffuse staining of fibrous stroma (highly suggestive of lectin-binding material diffusing from cancer cells) was also noted. The presence of lectin binding to blood vessels and erythrocytes was quite unrelated to binding to cancer cells. These features are illustrated in Figure 29:

**FIGURE 29: Helix pomatia lectin binding to endothelium and erythrocytes**

endothelium positive, cancer cells positive magnification x10

endothelium positive, cancer cells negative magnification x10
3.2.5.3 Benign breast disease

Examples of benign breast disease were occasionally noted incidentally in sections scored for lectin binding to the predominantly malignant component. No comprehensive survey of Helix binding to these cell populations was undertaken, but it may be of interest that Helix pomatia lectin binding was seen to normal/benign epithelial cells in a majority of cases, and in most examples of carcinoma in situ. (Figure 30)

FIGURE 30: Examples of Helix pomatia lectin binding in benign disease

magnification x20
3.3. CORRELATION OF HELIX POMATIA LECTIN BINDING WITH LONG-TERM PATIENT PROGNOSIS.

3.3.1. OVERVIEW OF CORRELATION.

3.3.2. LOG-RANK LIFE TABLE ANALYSES.

3.3.3. LIFE TABLE ANALYSES OF ALL PATIENTS (n=373).
   3.3.3.1. Survival
   3.3.3.2. Disease free survival (D.F.S).

3.3.4. HELIX POMATIA LECTIN "STAINERS" (n=293) VERSUS "NON-STAINERS" (n=80).
   3.3.4.1. Survival.
   3.3.4.2. Disease free survival (D.F.S.).

3.3.5. OTHER PROGNOSTIC PARAMETERS.
   3.3.5.1. Lymph node status.
   3.3.5.2. Tumour size.
   3.3.5.3. Histological tumour grade.
   3.3.5.4. S-phase fraction.
   3.3.5.5. DNA Index.

3.3.6. DISTRIBUTION OF HELIX POMATIA LECTIN "STAINERS" AND "NON-STAINERS" IN RELATION TO OTHER PROGNOSTIC VARIABLES EXAMINED.
   3.3.6.1. Helix lectin staining by age.
   3.3.6.2. Helix lectin staining by lymph node status.
   3.3.6.3. Helix lectin staining by tumour size.
   3.3.6.4. Helix lectin staining by S-phase fraction.
   3.3.6.5. Helix lectin staining by histological grade.

3.3.7. MULTIVARIATE ANALYSIS
3.3. CORRELATION OF *HELIX POMATIA* LECTIN BINDING WITH LONG-TERM PATIENT PROGNOSIS

3.3.1. OVERVIEW OF CORRELATION

This section describes correlation of *Helix pomatia* lectin binding to tissue sections with clinical follow-up data. Results illustrate the value of the lectin binding as a prognostic indicator in breast cancer.

Log-rank life table analyses were performed to compare the clinical history of *Helix pomatia* "stainers" and "non-stainers". *Helix* lectin binding, as a potential prognostic factor in breast cancer was contrasted with other, established, factors (lymph node status; tumour size; histological grade; S-phase fraction) by calculating analogous life tables on the basis of our data regarding these variables.

The relationship of *Helix pomatia* lectin binding to other prognostic variables was investigated by a simple method of comparison, and by a Cox proportional hazards regression analysis model.

3.3.2. LOG-RANK LIFE TABLE ANALYSES

Log-rank life table analyses were performed for interval between diagnosis of breast cancer and:

(1) the interval to first recurrence of disease (the disease free interval or disease free survival time), and
(2) the time to death (total survival time).

The results were, for the sake of simplicity, recorded in the form of basic graphs. In all cases, disease free survival, or total survival time, was plotted in years from diagnosis on the horizontal (x) axis; against cumulative percentage patients disease free, or cumulative percentage patients surviving, on the vertical (y) axis.

In all survival time analyses, the expected mortality rate of an age matched control population was plotted for comparison (see appendix VI).
N.B. Any p values quoted, refer to survival differences between groups of breast cancer patients only; not to differences in survival between our breast cancer patients and the normal controls.

3.3.3. LIFE TABLE ANALYSES OF ALL PATIENTS (n=373)

3.3.3.1. Survival

The first life-table analysis (Figure 31) merely illustrates the pattern of overall survival in our cohort of 373 breast cancer patients. Overall patient survival being contrasted to that of age matched normal controls (see Appendix VI for calculations regarding expected survival of a normal control population).

FIGURE 31

![Graph showing survival rates for breast cancer patients and age-matched normal controls.](image)

5 years following diagnosis, the cumulative percentage surviving is approximately 62%; at 10 years it is 45%; and 15 years 34%.

This is in sharp contrast to the survival of a hypothetical group of age-matched normal females, where at 5 years from entry into the study, cumulative percentage surviving is 93.5%; at 10 years, 85.5%; and at 15 years, 75.7%
3.3.3.2. Disease Free Survival (D.F.S.)

Figure 32 illustrates the overall pattern of disease free survival in 373 patients.

At 5 years following diagnosis, the cumulative percentage patients free of recurrent disease is approximately 48%; at 10 years it is 40%; but by 15 years only 23% of individuals remain free of disease.
3.3.4. **HELIX POMATIA LECTIN "STAINERS" (n=293) VERSUS "NON-STAINERS" (n=80)**

We then proceeded to analyse the survival pattern of patients whose tumours bound *Helix pomatia* lectin (Helix "stainers") in comparison to those whose tumours did not bind the lectin ("non-stainers").

### 3.3.4.1. Survival

Figure 33 demonstrates a great and obvious difference between survival rates of *Helix pomatia* lectin "stainers" vs. "non-stainers" (p < 0.00001).

![Figure 33](image)

The 5 year cumulative percentage survival of non-stainers is, for example, 85%; that of stainers approximately 55%. At 10 years, the figure for non-stainers is 52% in comparison to, in stainers, 25%.

### 3.3.4.2. Disease free survival (DFS)

The disease free survival of *Helix pomatia* lectin "stainers" versus "non-stainers" was also markedly different as seen in figure 34; "non-stainers" enjoying a considerably extended disease free interval prior to relapse in comparison to "stainers" (p < 0.00001).
At 5 years following diagnosis, for example, the cumulative percentage of "stainers" remaining free of secondary disease is 52% in comparison with 75% "non-stainers". At 10 years, the totals are 32% versus 62%. By 15 years following diagnosis the lines for "stainers" and "non-stainers" are parallel; approximately 35% of "non-stainers" remain disease free in comparison to approximately 18% of "stainers".
3.3.5. OTHER PROGNOSTIC PARAMETERS

Log-rank life-table analyses of disease-free and overall survival time in *Helix* lectin "stainers" and "non-stainers" was then compared with similar analyses based on a range of other, established, prognostic variables.

The results are presented as simple graphs (Figures 35-44) on the following pages, then summarised in Table 18.

3.3.5.1. Lymph node status

**FIGURE 35**

![Survival](image)

Survival
lymph node +ve vs. -ve

age matched normal population

lymph node -ve (n=130)

lymph node +ve (n=160)

*P < 0.00001*

**FIGURE 36**

![Disease Free Survival](image)

Disease Free Survival
lymph node +ve vs. -ve

lymph node -ve (n=130)

lymph node +ve (n=160)

*P < 0.00001*
3.3.5.2. Tumour size

**FIGURE 37**

Survival
tumour size <2.1 cm vs. >2 cm

age matched normal population

small (n=160)
large (n=172)

\[ p < 0.002 \]

Survival time (months)

**FIGURE 38**

Disease Free Survival
tumour size <2.1 cm vs. >2 cm

small (n=160)
large (n=172)

\[ p < 0.03 \]

disease free survival time (months)
3.3.5.3. Histological tumour grade

**FIGURE 39**

Survival
grades I vs. II vs. III

grade I
(n = 27)

age matched
normal population

grade II
(n = 135)

grade III
(n = 211)

Survival time (months)

![Survival Curve Graph](image)

*Survival curve showing grades I, II, and III compared to age-matched normal population.*

**FIGURE 40**

Disease Free Survival
grade I vs. II vs. III

grade I
(n = 27)

grade III
(n = 135)

Disease free survival (months)

![Disease Free Survival Curve Graph](image)

*Disease free survival curve showing grades I, II, and III.*
3.3.5.4 S-phase fraction

**FIGURE 41**

Survival

S-phase fraction <9.1 vs. >9.0

- Age matched normal population
- Low S-phase fraction
  - (n = 110)
- High S-phase fraction
  - (n = 111)

**FIGURE 42**

Disease Free Survival

S-phase fraction <9.1 vs. >9.0

- Low S-phase fraction
  - (n = 110)
- High S-phase fraction
  - (n = 111)

\[ p < 0.00001 \]
3.3.5.5 DNA Index

**FIGURE 43**

Survival
aneuploid vs. diploid tumours

Survival time (months)

Cumulative % surviving

age matched normal population

$\text{p} < 0.03$

$\text{diploid} (n = 67)$

$\text{aneuploid} (n = 175)$

**FIGURE 44**

Disease Free Survival
aneuploid vs. diploid tumours

Disease free survival time (months)

Cumulative % disease free

$\text{p} < 0.3$

$\text{aneuploid} (n = 67)$

$\text{diploid} (n = 175)$
### TABLE 18: Summary of results of life table analyses

<table>
<thead>
<tr>
<th>variable</th>
<th>categories</th>
<th>n=</th>
<th>d.f.s.</th>
<th>survival</th>
<th>p value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helix binding</td>
<td>&quot;stainers&quot;</td>
<td>293</td>
<td>p&lt;0.00001</td>
<td>p&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 33/34)</td>
<td>&quot;non-stainers&quot;</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lymph node status</td>
<td>+ve</td>
<td>160</td>
<td>p&lt;0.00001</td>
<td>p&lt;0.00001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 35/36)</td>
<td>-ve</td>
<td>130</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tumour size</td>
<td>&lt; 2.1cm</td>
<td>160</td>
<td>p&lt;0.03</td>
<td>p&lt;0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 37/38)</td>
<td>&gt; 2cm</td>
<td>172</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>histological grade</td>
<td>I</td>
<td>27</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 39/40)</td>
<td>II</td>
<td>135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>211</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-phase fraction</td>
<td>&lt;9.1</td>
<td>110</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.00001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 41/42)</td>
<td>&gt; 9</td>
<td>111</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Index</td>
<td>diploid</td>
<td>67</td>
<td>p&lt;0.3</td>
<td>p&lt;0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(figs. 42/43)</td>
<td>aneuploid</td>
<td>175</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All parameters examined appear to be of prognostic significance except DNA index.

Simple comparison of life table analyses suggests that the most significant prognostic parameters in our series are: Lymph node status, histological grade, S-phase fraction, and, interestingly, *Helix pomatia* lectin binding.
3.3.6. DISTRIBUTION OF HELIX POMATIA LECTIN "STAINERS" AND "NON-STAINERS" IN RELATION TO OTHER PROGNOSTIC VARIABLES EXAMINED.

293 of the 373 breast cancers included in this study (79%) were *Helix pomatia* lectin "stainers"; the remaining 80 (21%) were lectin "non-stainers".

Having thus classified patients as "stainers" or "non-stainers", we went on to examine the breakdown of these groups in relation to the other variables - degree of lymph node involvement; tumour size; S-phase fraction; histological grade and also with patient age at diagnosis.

The purpose of this exercise was to explore the possibility that *Helix pomatia* lectin was simply binding to a particular sub-set of cancers - for example, those in the very young or old, those which had metastasized to local lymph node; or those of a particular histological grade.

The results are presented on the following pages in a simple diagramatic form. Figures refer to the number of patients in each group.
3.3.6.1. *Helix* lectin staining by age

**FIGURE 45a**: *Helix* "stainers" split by age at diagnosis.

![Helix Staining Age At Diagnosis](image)

**FIGURE 45b**: *Helix* "non-stainers" split by age at diagnosis.

![Helix Staining Age At Diagnosis](image)

It is apparent that a similar distribution of patients classified according to age at diagnosis is seen over the *Helix* "stainers" and "non-stainers" groups.
3.3.6.2 Helix lectin staining by lymph node status

FIGURE 46a: Helix "stainers" split by lymph node status

Helix Staining

Lymph Node Status

FIGURE 46b: Helix "non-stainers" split by lymph node status.

Helix Staining

Lymph Node Status

Helix "stainers" comprise a mixture of lymph node positive, negative and unknown patients. It is interesting, however, that the "non-stainers" are predominantly lymph node negative cases.
3.3.6.3 *Helix* lectin staining by tumour size

**FIGURE 47a:** *Helix* "stainers" split by tumour size.

![Diagram showing Helix Staining and Tumour Size distribution]

**FIGURE 47b:** *Helix* "non-stainers" split by tumour size.

![Diagram showing Helix Staining and Tumour Size distribution]

Tumours of 2cm or less, and of greater than 2cm, are fairly equally distributed between the "stainers" and "non-stainers"; "stainers" perhaps having a slightly higher proportion of the larger tumours than the "non-stainers".
3.3.6.4. *Helix* lectin staining by S-phase fraction

**FIGURE 48a:** *Helix* "stainers" split by S-phase fraction

- Stain: 183
- No-stain: 50

**FIGURE 48b:** *Helix* "non-stainers" split by S-phase fraction.

- Stain: 183
- No-stain: 50

*Helix* staining appears to be unrelated to the S-phase fraction of the tumour.
3.3.6.5. *Helix* lectin staining by histological grade

**FIGURE 49a:** *Helix* "stainers" split by histological grade

![Diagram showing distribution of Helix staining by histological grade.]

**FIGURE 49b:** *Helix* "non-stainers" split by histological grade

![Diagram showing distribution of no-Helix staining by histological grade.]

Tumours of grades I, II, and III are remarkably evenly distributed between *Helix* "stainers" and "non-stainers" groups.
A proportional hazards regression analysis was performed according to the Cox stepwise regression model.

Complete data was available for all prognostic variables to be considered in a total of 160 patients.

**Co-variates**
The following co-variates were initially included in the model:

<table>
<thead>
<tr>
<th>TABLE 19: Multivariate analysis - list of co-variates</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Helix staining</strong></td>
</tr>
<tr>
<td>- &quot;stainers&quot;</td>
</tr>
<tr>
<td>- &quot;non-stainers&quot;</td>
</tr>
<tr>
<td>n=124</td>
</tr>
<tr>
<td>n= 36</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
</tr>
<tr>
<td>- &lt;2cm</td>
</tr>
<tr>
<td>- 2.1-5cm</td>
</tr>
<tr>
<td>- &gt;5cm</td>
</tr>
<tr>
<td>n= 78</td>
</tr>
<tr>
<td>n= 72</td>
</tr>
<tr>
<td>n= 10</td>
</tr>
<tr>
<td><strong>Age at diagnosis</strong></td>
</tr>
<tr>
<td>- &lt;35y</td>
</tr>
<tr>
<td>- 36-50</td>
</tr>
<tr>
<td>- 51-65</td>
</tr>
<tr>
<td>- &gt;65y</td>
</tr>
<tr>
<td>n= 3</td>
</tr>
<tr>
<td>n= 51</td>
</tr>
<tr>
<td>n= 68</td>
</tr>
<tr>
<td>n= 38</td>
</tr>
<tr>
<td>age was also entered as a continuous variable</td>
</tr>
<tr>
<td><strong>Lymph node involvement</strong></td>
</tr>
<tr>
<td>- negative</td>
</tr>
<tr>
<td>n= 69</td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
</tr>
<tr>
<td>- I</td>
</tr>
<tr>
<td>- II</td>
</tr>
<tr>
<td>- III</td>
</tr>
<tr>
<td>n= 6</td>
</tr>
<tr>
<td>n= 67</td>
</tr>
<tr>
<td>n= 87</td>
</tr>
<tr>
<td><strong>DNA index</strong></td>
</tr>
<tr>
<td>dilpoid</td>
</tr>
<tr>
<td>n=54</td>
</tr>
<tr>
<td>aneuploid</td>
</tr>
<tr>
<td>n=103</td>
</tr>
<tr>
<td><strong>S-phase fraction</strong></td>
</tr>
<tr>
<td>entered as a continuous variable</td>
</tr>
<tr>
<td>n=160</td>
</tr>
</tbody>
</table>
Baseline variables:
For each of the discontinuous co-variates listed in Table 19 (ie except for S-phase fraction, and age as a continuous variable) a "baseline variable" was arbitrarily chosen. For example, when considering Helix staining, "non-stainers" were chosen as the baseline variable.

For each co-variates in the list, the relative risk of falling into any category is then compared to that of the baseline variable. For example the prognostic effect of being a Helix "stainer" is compared in the model with being a "non-stainer", the baseline variable.

The baseline variables chosen in this analysis are listed in Table 20.

TABLE 20: Baseline variables

<table>
<thead>
<tr>
<th>Co-variates</th>
<th>Categories</th>
<th>Baseline Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix staining</td>
<td>&quot;stainers&quot;</td>
<td>&quot;stainers&quot;</td>
</tr>
<tr>
<td></td>
<td>&quot;non-stainers&quot;</td>
<td></td>
</tr>
<tr>
<td>Tumour size</td>
<td>&lt;2cm</td>
<td>&lt;2cm</td>
</tr>
<tr>
<td></td>
<td>2.1-5cm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;5cm</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>&lt;35y</td>
<td>36-50y</td>
</tr>
<tr>
<td></td>
<td>36-50y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>51-65y</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;65y</td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>1-3 nodes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;3 nodes</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td>I</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>DNA index</td>
<td>diploid</td>
<td>diploid</td>
</tr>
<tr>
<td></td>
<td>aneuploid</td>
<td></td>
</tr>
</tbody>
</table>
The models calculated, for breast cancer patient survival and disease free survival, are summarised in Tables 21 and 22.

In the models, the baseline variables are assigned an arbitrary relative risk of 1. The relative risk of falling into any other category can then calculated in relationship to the baseline variable. This risk is reflected in the "exponential co-efficient" quoted in the model.

For example, if we consider lymph node status, the baseline variable is lymph node negative. This is assigned a value of 1. If a person has 1-3 nodes involved, the exponential co-efficient in the "survival" model is 2.1835 - in simple terms, this means that they are 2.1835 times more likely to die than if they are lymph node negative.

TABLE 21: Cox regression analysis model - SURVIVAL

<table>
<thead>
<tr>
<th>Variable</th>
<th>co-efficient</th>
<th>standard error</th>
<th>exp. co-efficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodes 1-3</td>
<td>0.7809</td>
<td>0.3002</td>
<td>2.1835</td>
<td>0.0071</td>
</tr>
<tr>
<td>Nodes &gt;3</td>
<td>1.0348</td>
<td>0.3174</td>
<td>2.8145</td>
<td>0.0011</td>
</tr>
<tr>
<td>Age 51-65y</td>
<td>-1.1903</td>
<td>0.4193</td>
<td>0.3041</td>
<td>0.0042</td>
</tr>
<tr>
<td>Age &gt;65y</td>
<td>-1.5938</td>
<td>0.7355</td>
<td>0.2031</td>
<td>0.0278</td>
</tr>
<tr>
<td>S-phase</td>
<td>0.5571</td>
<td>0.2191</td>
<td>1.7455</td>
<td>0.0118</td>
</tr>
<tr>
<td>Helix staining</td>
<td>0.7638</td>
<td>0.3816</td>
<td>2.1465</td>
<td>0.0366</td>
</tr>
<tr>
<td>Size &gt;5cm</td>
<td>0.9099</td>
<td>0.3951</td>
<td>2.4841</td>
<td>0.0366</td>
</tr>
</tbody>
</table>

TABLE 22: Cox regression analysis model - DISEASE FREE SURVIVAL

<table>
<thead>
<tr>
<th>Variable</th>
<th>co-efficient</th>
<th>standard error</th>
<th>exp. co-efficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helix staining</td>
<td>0.5176</td>
<td>0.3446</td>
<td>1.6781</td>
<td>0.0001</td>
</tr>
<tr>
<td>Nodes &gt;3</td>
<td>0.9982</td>
<td>0.2832</td>
<td>2.7134</td>
<td>0.0340</td>
</tr>
<tr>
<td>Nodes 1-3</td>
<td>0.9909</td>
<td>0.3038</td>
<td>2.6937</td>
<td>0.001</td>
</tr>
</tbody>
</table>
The results are very interesting, and very complicated.

**Survival model**
The survival model considers lymph node status as having the greatest prognostic effect, followed by S-phase fraction, age at diagnosis, tumour size, and *Helix pomatia* lectin binding.

Involvement of lymph nodes is related to poor survival (exp. co-efficient 1-3 nodes = 2.1835; >3 nodes = 2.8145) - the greater the number of nodes involved, the greater the relative risk.

Older patients appear to have a slightly better survival (exp. co-efficient age 51-65y = 0.3041; >65y = 0.2031) than younger patients (baseline variable 36-50y).

S-phase fraction ranked third in the survival model. Its prognostic significance in this analysis was slightly greater than that of histological grade, to which it appeared related. When S-phase fraction was included in the model, the effect of histological grade failed to reach statistical significance, and was thus excluded.

Tumour size of >5cm was also included in the model, although the relative effect of tumour size 2-5cm - compared to baseline variable <2cm - did not reach significance.

*Helix pomatia* lectin binding ranked only fifth in the survival model, but its effect was attenuated by the necessary inclusion of lymph node status to which it was shown to be related.

**Disease free survival**
The disease free survival model was much simpler than the survival model.

Only two variables reached statistical significance. These were *Helix pomatia* lectin binding and lymph node status. The two were shown to be related.

Lymph node status had a slightly greater effect on disease free survival (exp.coefficient 1-3 nodes = 2.6937; >3 nodes = 2.7134; *Helix* staining = 1.6781), but p values reflect a greater confidence in the significance of *Helix* staining (1-3 nodes p< 0.001; >3 nodes p<0.03; *Helix* staining p< 0.0001).
3.4. *HELIX POMATIA* LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER.
3.4. *HELIX POMATIA* LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER

A series of breast cancer metastases from a range of different organs were stained to seek binding of *Helix pomatia* lectin.

The results are summarised in Table 23:

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Helix positive</th>
<th>Helix negative</th>
<th>no. cases found / no. possible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>11/15</td>
<td>4/15</td>
<td>15/15</td>
</tr>
<tr>
<td>Liver</td>
<td>16/19</td>
<td>3/19</td>
<td>19/19</td>
</tr>
<tr>
<td>Lymph Node</td>
<td>8/10</td>
<td>2/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Brain</td>
<td>0/2</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Adrenal</td>
<td>3/5</td>
<td>2/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Thyroid</td>
<td>2/2</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2/2</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Uterus</td>
<td>2/3</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Bone</td>
<td>2/2</td>
<td>0/2</td>
<td>2/19*</td>
</tr>
</tbody>
</table>

*Although metastasis to bone was mentioned in reports of 19 of the 46 post mortems included in this study, it was only possible to locate blocks of tissue in 2 cases. All blocks were successfully located for metastases to other sites. Bone is a particularly difficult tissue to process for routine paraffin sectioning, presenting considerable problems even after extensive decalcification. It seems likely that because of this, although obvious bone metastases were observed in 19/46 cases, the pathologist omitted to take samples of tissue for subsequent histology in most cases.*

The primary breast cancers from which these metastases arose were available in 7 of the 46 cases (see section 2.5.2). Sections of all 7 primary breast cancers were stained for *Helix pomatia* lectin binding, and all were strongly positive.

It is unfortunate that the *Helix*-binding status of the remaining primary tumours remained unknown. However, in view of the apparent prognostic significance of *Helix* lectin binding to primary breast cancers, it would appear highly likely that most, if not all
would have shown some degree of positivity.

Overall, approximately 80% of breast cancer metastases bound the *Helix pomatia* lectin. Around 20% did not. No organ specificity with regard to lectin binding was detectable.

Within individual patients, both positive and negative metastases - at different sites - were sometimes observed. Furthermore, when examining lymph node deposits, some positive and some negative metastases were observed in a single individual.

Although it was only possible to locate two blocks of bone metastases, stunningly intense *Helix* staining was seen in both instances (Figure 54).

Examples of *Helix pomatia* lectin binding to a range of metastases are given in the photographs, Figures 50-55, on the following pages.
FIGURE 50: *Helix pomatia* lectin binding - breast cancer metastasis to lymph node

- haematoxylin / eosin
- Helix lectin - positive

- haematoxylin / eosin
- Helix lectin - negative

magnification x10
FIGURE 51: Helix pomatia lectin binding - breast cancer metastasis to lung

haematoxylin / eosin

Helix lectin - positive

magnification x40

haematoxylin / eosin

Helix lectin - negative

magnification x20
FIGURE 52: *Helix pomatia* lectin binding - breast cancer metastasis to liver

haematoxylin / eosin

Helix lectin - positive

magnification x20
FIGURE 53: *Helix pomatia* lectin binding - breast cancer metastasis to brain

Haematoxylin / eosin

*Helix lectin - positive*

Haematoxylin / eosin

*Helix lectin - negative*

Magnification x20
**FIGURE 54:** *Helix pomatia* lectin binding - breast cancer metastasis to bone

haematoxylin / eosin  
Helix lectin - positive

magnification x20

**FIGURE 55:** *Helix pomatia* lectin binding - breast cancer metastasis to uterus

haematoxylin / eosin  
Helix lectin - positive

magnification x10
3.5.1. AIM.

3.5.2. OVERVIEW OF RESULTS.

3.5.3. EXAMPLES OF RESULTS OBTAINED.
3.5.3.1. Metastasis to liver and normal human serum.
3.5.3.2. Liver metastasis and normal liver.
3.5.3.3. Primary breast cancers and breast cancer metastasis to liver.
3.5.3.4. Liver metastasis and human milk and M.F.G.M.
3.5.3.5. Breast cancer metastasis to lymph node.
3.5.3.6. Normal lung / metastasis to lung; normal bone / metastasis to bone.

3.5.4. DETERMINATION OF MOLECULAR WEIGHT.

3.5.5. SUMMARY.
3.5 CHARACTERISATION OF HELIX POMATIA LECTIN-BINDING MATERIAL FROM A RANGE OF HUMAN TISSUES.

3.5.1. AIM

Separation and analysis of Helix pomatia lectin binding molecules was performed on a range of fresh human tissue homogenates, plus serum and milk, by the techniques of polyacrylamide gel electrophoresis with Western lectin blotting.

3.5.2. OVERVIEW OF RESULTS

We had hoped that Helix pomatia lectin would bind to only a very limited number of bands in S.D.S.-P.A.G.E. However, a bewildering range of lectin binding bands were revealed in samples of primary breast cancer, breast cancer metastases, normal control tissues, human milk, milk fat globule membrane (M.F.G.M.) and serum.

Very careful scrutiny of Western blots eventually revealed that one band, of apparent molecular weight approx. 55 kDa., appeared to be of particular interest.

This band was readily detectable in samples of primary breast cancers and their metastases (to lymph node, lung, liver, and bone); barely detectable in human milk and M.F.G.M.; and was not detectable in normal lymph node, lung, liver, bone, or the serum of healthy subjects.

3.5.3. EXAMPLES OF RESULTS OBTAINED

The following photographs represent examples of results obtained by

(1) S.D.S.-P.A.G.E. of crude, highly heterogeneous tissue homogenates and biological fluids

(2) the binding of Helix pomatia lectin to the bands separated by this technique, blotted onto nitrocellulose membrane.

Also included are "interpretations" of the blots - the position of Helix pomatia lectin binding bands are shown on ink tracings of the original stained blot. Such tracings were originally made as a permanent record of results when we discovered that blots often faded and yellowed with age. They are included here as they are frequently much clearer and easier to understand than the photograph of the blot itself.
3.5.3.1. Metastasis to liver and normal human serum

native samples
Figure 56, overleaf, compares the *Helix pomatia* lectin binding bands in a homogenate of breast cancer metastasis to liver with samples of serum from healthy blood group A, B, and O individuals.

It is apparent that most of the *Helix*-binding bands are shared; but valid comparison is rendered impossible by the heavy albumin component of serum causing considerable distortion in the molecular weight region 26.6 - 58 kDa.

**key to figure 56:**

A) Coomassie blue stained gel - total protein  
B) interpretation of blot  
C) blot of gel (A) stained for *Helix pomatia* lectin binding track  
1) liver metastasis  
2) serum, blood group A  
3) serum, blood group B  
4) serum, blood group O
FIGURE 56: Breast cancer metastasis to liver, in comparison to blood group A, B, and O serum - heavy albumin component of serum renders meaningful comparison of Helix lectin binding band impossible.
after removal of albumin

Figure 57, overleaf, compares the same samples after partial removal of the albumin.

Of 9 clear Helix-binding bands present in liver metastasis - six are shared with normal serum, and three are unique.

Two of the three liver metastasis-associated bands run in the 36.5-48.5 kDa. range. The third runs a little in front of the 58kDa. molecular weight marker protein.

**key to figure 57:**

A) Coomassie blue stained gel - total protein
B) interpretation of blot
C) blot of gel (A) stained for *Helix pomatia* lectin binding

track
1) liver metastasis
2) serum, blood group A
3) serum, blood group B
4) serum, blood group O
Figure 57: Breast cancer metastasis to liver, in comparison to blood group A, B, and O serum - the albumin component of the serum samples has been reduced in comparison to that seen in the previous figure.

<table>
<thead>
<tr>
<th>m.w. standards</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>m.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>116</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48.5</td>
<td></td>
<td></td>
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<tr>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(kDa)

---

**Legend:**

A) Controls
B) Interpreted
C) Blot of gel

1) Liver extract
2) Normal liver (controls)
3) Liver extract (diluted)
4) Normal liver (diluted)
3.5.3.2. Liver metastasis and normal liver

Figure 58, overleaf, examines the distribution of the Helix lectin binding bands in homogenates of liver metastasis and normal liver.

Doubling dilutions of normal liver homogenate were run on the gel. It is apparent from Coomassie blue staining (A) that tracks 2 and 3 - corresponding to the more concentrated samples - are actually quite overloaded. In spite of excessive loading of the gel, coupled with extended incubation of the blotted membrane with Helix pomatia lectin, the bands identified as being present in liver metastasis but not in normal serum (two running in the 36.5-48.5 kDa range, and one running around 58kDa), are also undetectable in normal liver homogenate.

This would suggest that these bands do not correspond to normal serum components or molecules strongly expressed by normal liver. They are, however, readily detectable in breast cancer metastasis to liver (track 1).

key to figure 58:

A) Coomassie blue stained gel - total protein
B) interpretation of blot
C) blot of gel (A) stained for Helix pomatia lectin binding

track
1) liver metastasis
2) normal liver (neat)
3) normal liver (diluted 1/2)
4) normal liver (diluted 1/4)
5) normal liver (diluted 1/8)
Figure 58: Normal liver and breast cancer metastasis to liver.

A) Standards

<table>
<thead>
<tr>
<th>m.w.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>180</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>116</td>
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<tr>
<td>84</td>
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<td>58</td>
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</tr>
<tr>
<td>48.5</td>
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<tr>
<td>36.5</td>
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<tr>
<td>26.6</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(kDa)</td>
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<td></td>
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</table>

B) Standards

<table>
<thead>
<tr>
<th>m.w.</th>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
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</tr>
<tr>
<td>58</td>
<td></td>
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</tr>
<tr>
<td>48.5</td>
<td></td>
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</tr>
<tr>
<td>36.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>26.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(kDa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C)
3.5.3.3. Primary breast cancers and breast cancer metastasis to liver

Native samples

Figure 59, overleaf, examines the distribution of Helix binding bands in liver metastasis in comparison to a number of primary breast cancers.

As seen previously with serum samples in Figure 56, the albumin component is again causing some distortion of the run. However, it is clear that the ~58kDa. band is present in most, if not all, primary breast cancers examined. The bands running in the 36.5-48.5 kDa. range are not detectable.

Key to Figure 59:

A) Coomassie blue stained gel - total protein
B) Interpretation of blot
C) Blot of gel (A) stained for Helix pomatia lectin binding

Tracks 1-7 primary breast cancers
Track 8 liver metastasis
FIGURE 59: Primary breast cancer metastasis to liver in comparison to a number of primary breast cancers - heavy albumin component is causing some distortion of the run, making interpretation difficult.

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![Image of gel analysis](image-url)
after removal of albumin
After removal of the albumin, it is apparent (Figure 60, overleaf) that two bands running in the 36.5-48.5 kDa. range are undetectable. The band running at around 58kDa was detectable on the original blot but, unfortunately, by the time the photograph was taken had faded to such an extent that it was quite difficult to detect.

key to figure 60:
A) Coomassie blue stained gel - total protein
B) interpretation of blot
C) blot of gel (A) stained for Helix pomatia lectin binding

tracks 1-6 primary breast cancers
Characterisation of Helix-binding material

FIGURE 60: Primary breast cancer - the albumin component has been reduced to allow easier interpretation.

A) m.w. standards 1 2 3 4 5 6
180
116
84
58
48.5
36.5
26.6 (kDa)

B) m.w. standards 1 2 3 4 5 6
116
84
58
48.5
36.5
26.6 (kDa)

C) m.w. 1 2 3 4 5 6
3.5.3.4. Liver metastasis and human milk and M.F.G.M.

The *Helix* lectin-binding band running at around 58kDa. was sought in samples of human milk and preparations of M.F.G.M. (components of the normal lactating breast epithelial membrane).

Initially, the band was not detectable, although a range of unique *Helix* binding bands was apparent. However, when the gel was overloaded with a concentrated sample, and the blot incubated for extended periods with the lectin, faint traces of the band were observed (figure 61, overleaf).

*key to figure 61:*

A) Coomassie blue stained gel - total protein
B) interpretation of blot
C) blot of gel (A) stained for *Helix pomatia* lectin binding track

1 liver metastasis
2 human milk (concentrated sample)
3 human M.F.G.M. (concentrated sample)
4 human milk (diluted sample)
5 human M.F.G.M. (diluted sample)
FIGURE 61: Breast cancer metastasis to liver, human milk and milk fat globule membrane. The gel must be overloaded with milk and MFGM samples (tracks 2 and 3) before the ~58kDa. Helix binding band is detectable.
3.5.3.5 Breast cancer metastasis to lymph node

Figure 62, overleaf, illustrates the Helix lectin-binding profile of four lymph nodes (three axillary and one dissected from the neck) heavily infiltrated with metastatic breast cancer. The samples have been run in parallel with the original liver metastasis homogenate for comparison. The ~58kDa. band is readily apparent.

The involved nodes were taken at post mortem examinations of patients who had died as a result of disseminated breast cancer. They were grossly distended with tumour, and thus readily recognisable. We were unfortunately unable to obtain normal lymph nodes as a control, as these were not usually sampled at autopsy. Samples of tonsil were tested and the ~58kDa band was not detectable, but we were unhappy about including these as an adequate alternative.

**key to figure 62:**

A) Coomassie blue stained gel - total protein  
B) interpretation of blot  
C) blot of gel (A) stained for *Helix pomatia* lectin binding

track  
1 liver metastasis  
2-9 lymph node metastases
Characterisation of Helix-binding material

FIGURE 62: Breast cancer metastases to lymph nodes

A) Coomassie

B) interpretation

C) hist of

m.w. standards 1 2 3 4 5 6 7 8 9

180
116
84
58
48.5
36.5
26.6
(kDa)

m.w. standards 1 2 3 4 5 6 7 8 9

180
116
84
58
48.5
36.5
26.6
(kDa)

m.w. standards 1 2 3 4 5 6 7 8 9

180
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84
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48.5
36.5
26.6
(kDa)
3.5.3.6. Normal lung / metastasis to lung: normal bone / metastasis to bone

Figure 63, overleaf, illustrates the presence of the ~58kDa Helix lectin binding band in samples of breast cancer metastases to lung and to bone.

The band is not detectable in samples of lung and bone taken at post mortem examination of individuals who had died as a result of non-malignant disease.

**key to figure 63:**

A) Coomassie blue stained gel - total protein  
B) interpretation of blot  
C) blot of gel (A) stained for Helix pomatia lectin binding

track  
1 normal lung  
2 lung metastasis  
3 normal bone  
4 bone metastasis
Characterisation of Helix-binding material

FIGURE 63: Normal lung and bone; breast cancer metastases to lung and bone.

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3.5.4. DETERMINATION OF MOLECULAR WEIGHT

A molecular weight calibration curve was drawn up from relative mobilities of molecular weight standards run in S.D.S.-P.A.G.E. (Figure 64).

From this curve, the apparent molecular weight of the *Helix* lectin binding band identified above as being of interest was calculated. It runs a little in front of the 58kDa marker, and has an apparent molecular weight of 55kDa.

![Molecular Weight Calibration Curve](image)

\[
y = 221.41 - 568.97x + 2013.0x^2 - 1923.8x^3 + 658.67x^4
\]

\[R^2 = 0.999\]

3.5.5. SUMMARY

(1) A bewildering range of *Helix pomatia* lectin binding bands were identified.

(2) One band appeared to be of particular interest. It was readily detectable in primary breast cancers and their metastases (to lymph node, lung, liver and bone), and weakly expressed in human breast milk and M.F.G.M. It was not detected in normal liver, lung or bone; or in the serum of healthy subjects (group A, B, and O).

(3) This band has an apparent molecular weight in S.D.S.-P.A.G.E. of 55kDa.
3.6.1. OVERVIEW.

3.6.2. EXAMPLES OF ION EXCHANGE PROFILES.
3.6.2.1. Primary breast cancer.
3.6.2.2. Breast cancer metastasis to liver.
3.6.2.3. Normal liver.
3.6.2.4. Blood group A serum.

3.6.3. S.D.S.-P.A.G.E.
3.6.ISOLATION/PURIFICATION OF *HELIX POMATIA* LECTIN BINDING MATERIAL -
ION EXCHANGE CHROMATOGRAPHY

3.6.1.OVERVIEW

The technique of ion exchange chromatography was performed-

(1) To further characterise the properties of biological molecules that bind to *Helix pomatia* lectin, in terms of the electrostatic charge they carry.

(2) To explore the possibility of using ion exchange chromatography as a separation / purification step in the isolation of *Helix pomatia* lectin binding material, and in particular, the 55kDa band identified as being of particular interest in the previous section.

Ion exchange chromatography was performed on samples of blood group A serum and pooled homogenates of primary breast cancers, liver metastases, and normal liver.

For each ion exchange chromatography experiment performed, the optical density (measured at a wavelength of 280nm) of each of the fractions collected from the column was plotted against fraction number to give a simple ion exchange profile.

Subsequently, every fraction (in some cases, where a very large number of fractions were collected, every other fraction) was tested by dot-blotting onto nitrocellulose for the binding of *Helix pomatia* lectin. Thus, it was possible to identify which portion(s) of the ion exchange profile corresponded to elution of *Helix pomatia* lectin binding material.

Early investigations clearly demonstrated that the *Helix* binding components bore a strongly negative charge, passing through a cation exchange column unhindered.

In each of the examples illustrated, ion exchange chromatography was performed using a DE52 anion exchange column. Bound components were sequentially eluted by a sodium chloride gradient ranging from 0M (distilled water) to 0.5M NaCl.

3.6.2. EXAMPLES OF ION EXCHANGE PROFILES

Results obtained are illustrated on the following pages (Figures 65-68).
3.6.2.1. Primary breast cancer

Figure 65 illustrates the profile obtained by ion exchange chromatography of pooled homogenate of primary breast cancers.

**FIGURE 65a**

*Ion Exchange Chromatography - Primary Breast Cancer*

Two major peaks were obtained; fractions of the second peak and a large area of the subsequent flattened part of the profile bound weakly to the *Helix pomatia* lectin in a dot-blot test, Figure 65b. Control dots of the original sample bound strongly to the lectin. N.B. *Helix pomatia* lectin-positive dots have in this instance faded considerably with time and are, unfortunately, rather faint.

**FIGURE 65b**: Dot blot corresponding to ion exchange profile 65a

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*control = original sample*
3.6.2.2 Breast cancer metastasis to liver

Figure 66a illustrates an analogous profile obtained with pooled homogenate of liver metastases. 4 major peaks were obtained, the third of which, plus a large portion of the latter flattened portion of the profile, bound strongly to the Helix pomatia lectin in dot-blot, Figure 66b.

**FIGURE 66a**

*Ion Exchange Chromatography - Liver Metastasis*

**FIGURE 66b**: Dot blot corresponding to the ion exchange profile in figure 66a
3.6.2.3. Normal liver

Figure 67a illustrates a quite distinct profile seen with normal liver homogenate.

FIGURE 67a

Ion Exchange Chromatography - Normal Liver

All fractions except those corresponding to the second (unbound) peak and the last part of the profile bound the lectin in dot blot, figure 67b.

FIGURE 67b: Dot-blot corresponding to the ion exchange profile above, Figure 67a.
3.6.2.4 Blood group A serum

Figure 68a shows blood group A serum ion exchange profile.

**FIGURE 68a**

**Ion Exchange Chromatography - Blood Group A Serum**

Fractions corresponding to all peaks bound strongly to the lectin in dot blot, figure 68b.

**FIGURE 68b**: Dot-blot corresponding to the ion exchange profile above, Figure 68a.
3.6.2.5. Summary

One of the most startling impressions gained from examination of the above profiles is that the *Helix pomatia* lectin binding material, in each sample, bears vastly heterogeneous charges, being released from the column by sodium chloride concentration ranging from 0.05 - 0.4M.

3.6.3. S.D.S.-P.A.G.E

S.D.S.-P.A.G.E. was performed on pooled, dialysed, concentrated fractions from the principal peaks of each of the ion exchange separations. The results of S.D.S.-P.A.G.E obtained from the separation of the liver metastasis homogenate, fairly typical of separations performed, is given in Figure 69, overleaf. The gel has been stained with Coomassie blue stain for total protein and illustrates the complexity of components eluted in each peak of the separation. This complexity, in addition to the apparent heterogeneity of charge expressed by *Helix* binding components, illustrates the limitations of ion exchange chromatography as a step in the effective purification of the material in which we are interested.
Characterisation / Isolation of Helix-binding material

**FIGURE 69a**

Ion Exchange Chromatography - Liver Metastasis

---

**FIGURE 69b:** Polyacrylamide gel of fractions from the ion exchange profile given above (Figure 69a) stained with Coomassie blue for total protein.
MATERIAL - *HELIX POMATIA* LECTIN AFFINITY CHROMATOGRAPHY.

3.7.1. OVERVIEW.

3.7.2. AFFINITY CHROMATOGRAPHY RESULTS.
3.7.2.1. First passage.
3.7.2.2. Second passage.
3.7.2.3. Third passage.

3.7.3. YIELD OF SEMI-PURE *HELIX* LECTIN BINDING MATERIAL.

3.7.4. ESTIMATION OF PURITY BY H.P.L.C.
3.7. ISOLATION/PURIFICATION OF HELIX POMATIA LECTIN BINDING MATERIAL-
HELIX POMATIA LECTIN AFFINITY CHROMATOGRAPHY

3.7.1. OVERVIEW

Affinity purification of Helix pomatia lectin binding material was performed on samples of primary breast cancer, liver metastasis, and normal liver homogenates, human milk and blood group A serum.

The purity of separated lectin binding components was assessed by S.D.S.-P.A.G.E. / blotting and by high performance liquid chromatography (H.P.L.C.)

3.7.2. AFFINITY CHROMATOGRAPHY - RESULTS

Affinity chromatography was performed using Helix lectin-coated agarose beads packed into a small column.

A number of problems - illustrated by the results obtained in purification of Helix lectin-binding material from liver metastasis homogenate - were encountered with this method:

3.7.2.1. First passage

A 1ml sample of pooled, homogenised, liver metastases was applied to the column; the column washed with buffer to elute unbound material; and then with a solution of N-Acetylgalactosamine in order to release specifically adsorbed substances.

The optical density (measured at a wavelength of 280nm) of fractions collected from the column was plotted against fraction number to give a simple affinity chromatography profile, Figure 70, overleaf.
Samples of each fraction were tested for the presence of *Helix pomatia* lectin binding material by the technique of dot-blotting onto nitrocellulose membrane. It is apparent (Figure 71) that both the first peak of unbound material; plus the subsequent flattened portion of the curve - the material eluted by N-Acetylgalactosamine - consists of fractions containing *Helix* binding substances. This strongly suggests that the column was more than loaded to capacity, all lectin binding sites on the column having been saturated.

**FIGURE 71**: Dot-blot corresponding to the affinity chromatography profile given in Figure 70, above.
The portion of the curve corresponding to the material eluted by the sugar actually gave negative optical density readings. This curious result can be explained by the discovery that N-Acetylgalactosamine solution actually has a quite strong negative absorbance at 280nm.

Fractions corresponding to (i) the "unbound" portion of the curve, and to (ii) the material eluted by the sugar were pooled, concentrated, and tested by S.D.S.-P.A.G.E. in parallel with the original liver metastasis homogenate. The results of the electrophoresis are given in Figure 72.

FIGURE 72: Coomassie blue stained polyacrylamide gel - fractions from affinity purification of Helix lectin binding material. First passage through the column.

key to figure 72:

- track 1 - liver metastasis - blot stained for Helix binding
- track 2 - original sample - Coomassie blue total protein stain
- track 3 - unbound material - Coomassie blue
- track 4 - GalNAc eluted material

It is apparent, that material "specifically" eluted by the sugar solution is actually extremely impure - presumably, due to material being non-specifically adsorbed along with the lectin binding material.
3.7.2.2. Second passage

The pooled fractions 15 - 30 were thus re-applied to the column, and the affinity chromatography procedure carried out for a second time. Again, fractions were screened for the presence of *Helix* lectin binding material (Figure 73), and *Helix*-binding fractions pooled, concentrated and analysed by S.D.S.-P.A.G.E. (Figure 74)

**FIGURE 73** Second passage through affinity column - dot blot of fractions

**FIGURE 74** Second passage through affinity chromatography column - Coomassie blue stained polyacrylamide gel.

**key**

1: sample
2: unbound material
3: GalNAc eluted material
Material eluted by the sugar contained significantly fewer protein bands than after a single passage through the column (Figure 74); however, bands remained which clearly did not correspond to the *Helix* binding bands present in the original sample. Pooled fractions 25 to 35, after extensive dialysis to remove sugar, were re-applied to the column for a third and final time, and the affinity chromatography protocol carried out once again.

### 3.12.3. Third passage

Dot-blotting of material from the column revealed strong *Helix* lectin binding by fractions 16-19 (Figure 75).

**FIGURE 75:** Third passage through affinity column - dot-blot.

Pooled, dialysed, concentrated samples of unbound components (fractions 1-15); material specifically eluted by the sugar (fractions 16-19) as well as the original liver metastasis homogenate and the actual affinity beads were analysed by S.D.S.-P.A.G.E. and Western lectin blotting.

No bands at all were detectable on a Coomassie blue-stained gel of the sugar-eluted material (Figure 76)! However, on blotting, *Helix*-binding bands were detectable corresponding to those seen in the original sample (Figure 77).
Isolation of Helix-binding material

FIGURE 76: Third passage through affinity column - Coomassie blue stained polyacrylamide gel.

key
1: sample
2: unbound material
3: GalNAc eluted material

FIGURE 77: Third passage - Helix pomatia lectin-binding bands

key
1: sample
2: unbound material
3: GalNAc eluted material
3.7.3 YIELD OF SEMI-PURE \textit{HELIX} LECTIN BINDING MATERIAL

The yield of \textit{Helix} binding material was somewhat disappointing. Bradford protein assay gave an estimated total protein yield of 17-22 \( \mu \text{g/ml} \) when the product of two successive affinity chromatography experiments, as described above, (approximate total volume of sample originally applied to column = 2ml of homogenate) were pooled. Results of the Bradford protein assay are illustrated in Figure 78.

\textbf{FIGURE 78:}

\textit{Bradford's Protein Assay - Standard Curve}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Bradford_curve.png}
\end{figure}
3.7.3 YIELD OF SEMI-PURE \textit{HELIX} LECTIN BINDING MATERIAL

The yield of \textit{Helix} binding material was somewhat disappointing. Bradford protein assay gave an estimated total protein yield of 17-22 \( \mu \text{g/ml} \) when the product of two successive affinity chromatography experiments, as described above, (approximate total volume of sample originally applied to column = 2ml of homogenate) were pooled. Results of the Bradford protein assay are illustrated in Figure 78.

\textbf{FIGURE 78:}

\textit{Bradford's Protein Assay - Standard Curve}
3.7.4. ESTIMATION OF PURITY BY H.P.L.C.

The purity of the eluted material was checked by H.P.L.C.. Eight peaks were detectable, corresponding to eight *Helix*-binding bands in the original sample and in the purified material (Figure 79).

**FIGURE 79:**

HPLC - Gel Filtration.
Affinity Purified Helix Binding Material

Elution by 0.5 M GalNAc

O.D. @ 280 nm

Fraction Number
3.8.1. OVERVIEW.

3.8.2. *HELIX POMATIA* LECTIN STAINING CHARACTERISTICS ON TISSUE SECTIONS.

3.8.3. VALUE OF *HELIX POMATIA* LECTIN AS A MARKER OF PROGNOSIS IN BREAST CANCER.

3.8.4. *HELIX POMATIA* LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER.

3.8.5. CHARACTERISATION OF *HELIX POMATIA* LECTIN-BINDING MATERIAL BY S.D.S.-P.A.G.E. / WESTERN BLOTTING.

3.8.6. CHARACTERISATION / ISOLATION OF *HELIX POMATIA* LECTIN-BINDING MATERIAL - ION EXCHANGE CHROMATOGRAPHY.

3.8.7. CHARACTERISATION / ISOLATION OF *HELIX POMATIA* LECTIN-BINDING MATERIAL - LECTIN AFFINITY CHROMATOGRAPHY.
3.8. SUMMARY OF RESULTS

3.8.1. OVERVIEW

*Helix pomatia* lectin binding was demonstrated to be an interesting and valuable predictor of long term patient prognosis in breast cancer. The physical properties (molecular weight, electrostatic charge) of *Helix* lectin-binding material from a range of normal and malignant tissues was characterised, and a molecule of apparent molecular weight 55kDa was identified as being of particular interest. As a first step in its isolation, affinity chromatography was performed to yield a relatively pure sample of a mixture of *Helix pomatia* lectin-binding components.

3.8.2. *HELIX POMATIA* LECTIN STAINING CHARACTERISTICS ON TISSUE SECTIONS.

(1) The lectin sometimes bound to structures in breast other than invasive cancer - including blood vessels and red blood cells, the luminal surface of normal breast ducts, collagen, carcinoma in situ, and benign breast disease.

(2) 80/373 (21%) of breast cancers were negative for *Helix pomatia* lectin binding to cancer cells; 293/373 (79%) were positive.

(3) Cancer cells, if positive for *Helix* lectin binding, showed either a predominantly cytoplasmic staining, or cytoplasmic staining with marked cell border localisation.

(4) Striking heterogeneity of staining was frequently seen - adjacent populations of morphologically indistinguishable cancer cells staining strongly positive and completely negative for lectin binding.

(5) *Helix pomatia* lectin binding was sometimes noted to be particularly strong at the invasive front of the tumour.

3.8.3. VALUE OF *HELIX POMATIA* LECTIN AS A MARKER OF PROGNOSIS IN BREAST CANCER.

(1) *Helix pomatia* lectin binding to paraffin sections of formalin fixed primary breast cancers appeared to be a very interesting and valuable predictor of long term patient
prognosis: "non-stainers" enjoyed extended recurrence-free survival and overall survival time in comparison to "stainers".

(2) It appeared to be independent of most other, established, physical prognostic markers for which we had data (including tumour size, histological grade, S-phase fraction, DNA index), but was not independent of lymph node status.

(3) Cox's proportional hazards regression analysis ranked *Helix pomatia* lectin binding fourth as a predictor of survival (after lymph node status, S-phase fraction, and patient age at diagnosis), and second as a predictor of disease free survival (after lymph node status).

It is interesting that *Helix* lectin binding plays such a prominent role in the model in view of its close association with lymph node status, a variable which is also included.

3.8.4. *HELIX POMATIA* LECTIN BINDING TO METASTASES ARISING FROM PRIMARY BREAST CANCER.

(1) Approximately 80% of breast cancer metastases to a number of sites (including lymph node, lung, liver, bone, brain, and others) were positive for *Helix pomatia* lectin binding. Approximately 20% were negative.

(2) In 7/46 post mortem cases examined, it was possible to locate and stain sections of the original primary tumour. All seven were strongly positive for *Helix pomatia* lectin binding.

(3) No organ specificity with regard to lectin binding was apparent.

(4) Within individual cases, both positive and negative metastases - at different sites - were sometimes observed. Furthermore, when examining lymph node deposits, positive and negative metastases were often seen which had been taken from the same individual.

3.8.5. CHARACTERISATION OF *HELIX POMATIA* LECTIN-BINDING MATERIAL BY S.D.S.-P.A.G.E. / WESTERN BLOTTING.

(1) A bewildering range of *Helix pomatia* lectin-binding bands were identified.

(2) One band appeared to be of particular interest. It was readily detectable in primary breast cancers and their metastases (to lymph node, lung, liver and bone), and weakly expressed
in human breast milk and M.F.G.M. It was not detected in normal liver, lung or bone, or in the serum of healthy subjects (group A,B, and O).

(3) This band has an apparent molecular weight in S.D.S.-P.A.G.E. of 55kDa.

3.8.6. CHARACTERISATION / ISOLATION OF HELIX POMATIA
LECTIN-BINDING MATERIAL - ION EXCHANGE CHROMATOGRAPHY.

(1) *Helix pomatia* lectin binding material, from several sources (primary breast cancers, metastasis to liver, normal liver, blood group A serum) was shown to bear vastly heterogeneous charges, being released from the ion exchange column by sodium chloride concentration ranging from 0.05 - 0.4M.

(2) All fractions separated by ion exchange contained a very complex, heterogeneous mixture of components.

(3) Ion exchange chromatography was thus judged to be an inappropriate technique for isolation of *Helix* lectin-binding material at this stage.

3.8.7. CHARACTERISATION / ISOLATION OF HELIX POMATIA
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(1) As an initial step toward the purification of the 55kDa molecule, a "cocktail" of *Helix pomatia* lectin binding molecules were successfully isolated from highly complex, heterogeneous tissue homogenates.

(2) Approximately 20μg of *Helix* lectin-binding material was recovered per 1ml of tissue homogenate.

(3) The purity of the isolated material - assessed by S.D.S.-P.A.G.E and gel filtration by H.P.L.C.- was very high. No contaminants were detected by these methods.
3.8. SUMMARY OF RESULTS

3.8.1. OVERVIEW

*Helix pomatia* lectin binding was demonstrated to be an interesting and valuable predictor of long term patient prognosis in breast cancer. The physical properties (molecular weight, electrostatic charge) of *Helix* lectin-binding material from a range of normal and malignant tissues was characterised, and a molecule of apparent molecular weight 55kDa was identified as being of particular interest. As a first step in its isolation, affinity chromatography was performed to yield a relatively pure sample of a mixture of *Helix pomatia* lectin-binding components.

3.8.2. *HELIX POMATIA* LECTIN STAINING CHARACTERISTICS ON TISSUE SECTIONS.

1. The lectin sometimes bound to structures in breast other than invasive cancer - including blood vessels and red blood cells, the luminal surface of normal breast ducts, collagen, carcinoma in situ, and benign breast disease.

2. 80/373 (21%) of breast cancers were negative for *Helix pomatia* lectin binding to cancer cells; 293/373 (79%) were positive.

3. Cancer cells, if positive for *Helix* lectin binding, showed either a predominantly cytoplasmic staining, or cytoplasmic staining with marked cell border localisation.

4. Striking heterogeneity of staining was frequently seen - adjacent populations of morphologically indistinguishable cancer cells staining strongly positive and completely negative for lectin binding.

5. *Helix pomatia* lectin binding was sometimes noted to be particularly strong at the invasive front of the tumour.

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DISCUSSION
4.1 BRIEF OUTLINE.

4.2 COMPARISON OF THE PROGNOSTIC SIGNIFICANCE OF HELIX LECTIN BINDING TO THAT OF OTHER MARKERS.

4.2.1 COMPARISON WITH PHYSICAL PARAMETERS.
4.2.1.1 Earlier work.
4.2.1.2 Recent studies.
4.2.1.3 Direct comparison of different physical prognostic indicators.

4.2.2 HELIX LECTIN IN RELATION TO BIOCHEMICAL TUMOUR MARKERS.
4.2.2.1 HCG.
4.2.2.2 alpha-lactalbumin.
4.2.2.3 CEA.

4.3 CONSIDERATION OF WHAT THE HELIX LECTIN-BINDING MOLECULE MIGHT BE.

4.3.1 OVERALL STAINING PATTERN IN TISSUE SECTIONS.

4.3.2 THE SIGNIFICANCE OF HELIX LECTIN BINDING TO NORMAL BREAST AND BREAST CANCER.

4.3.3 THE EFFECT OF NEURAMINIDASE TREATMENT.

4.3.4 HELIX LECTIN BINDING BANDS IN S.D.S.-P.A.G.E.

4.3.5 HELIX LECTIN BINDING AND BLOOD GROUP A.

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4.4. WHAT IS HELIX LECTIN BINDING TO?

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   4.4.1.1. Human milk.
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   4.5.4.3. Tumour cell glycoprotein binding to endothelial cell lectin.
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4.6.1. AS A PROGNOSTIC INDICATOR.

4.6.2. POSSIBILITIES FOR INTERVENTION.
4.6.2.1. Overview.
4.6.2.2. Limitations of *Helix pomatia* lectin

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4. DISCUSSION

4.1. BRIEF OUTLINE

The aim of this section is to relate the results of our study to the immense wealth of knowledge available in the literature, to consider what role *Helix pomatia* lectin binding may play in the understanding, the management and the treatment of breast cancer, and to suggest new directions that our research should now follow.

It falls into four principal sections:

**Comparison of the prognostic significance of *Helix* lectin binding to that of other markers.**

The efficacy of *Helix pomatia* lectin binding, as a marker of prognosis in breast cancer, is compared with other, established “physical” markers (such as lymph node status, tumour size, histological grade, S-phase fraction) and with various “biochemical” markers (for example, CEA, HCG, alpha fetoprotein etc.) described in the literature.

**Consideration of what the *Helix* lectin-binding molecule might be.**

The staining pattern in tissue sections is discussed, as is significance of lectin binding to breast cancer metastases.

The physical properties of the *Helix* binding material (tissue distribution, molecular weight, charge etc) are compared to those of known breast / breast cancer-associated proteins and glycoproteins.

**What role does the *Helix* binding molecule actually play in the mechanism of metastasis?**

Mechanisms for the involvement of *Helix* binding material in breast cancer progression are proposed with reference to our understanding of the intricacies of the metastatic cascade.

In particular, the possibility that the *Helix* lectin-binding material may correspond to some oncogene product, laminin receptor or type IV collagenase, or an endogenous human lectin or lectin receptor (previously described in Appendix I, and section 1.4) are discussed.
How can this help the patient?

Ways in which our results may be exploited to assist the management and treatment of those suffering from breast cancer are discussed, with reference to ongoing research in our laboratory and future plans.

* * * * * *

4.2 COMPARISON OF THE PROGNOSTIC SIGNIFICANCE OF HELIX LECTIN BINDING TO THAT OF OTHER MARKERS

*Helix pomatia* lectin binding to formalin fixed, paraffin embedded sections of primary breast cancer appeared - in our retrospective series of 373 cases - to be an impressive predictor of long-term patient prognosis.

Patients with tumours negative for lectin binding enjoyed extended disease free interval in comparison to those with lectin-positive tumours, and survival almost comparable to that of age-matched hypothetical controls over a 15-20 year follow-up period.

4.2.1 COMPARISON WITH PHYSICAL PARAMETERS

4.2.1.1 Earlier work

In the small pilot study that preceeded this project, *Helix* lectin binding to primary breast cancers was identified as being of particular interest due, in part, to its close association with the presence of lymph node involvement (Leathem et al 1984; 1985 and unpublished data - see section 1.5.3.). These early results were substantiated by this study in which multivariate analysis revealed no association between *Helix pomatia* lectin binding and other established prognostic factors for which we had data, including S-phase fraction, tumour size, and histological grade - but a close relationship with the presence of lymph node metastases.

The pilot study, involving only 20 breast cancer cases, indicated that *Helix* lectin binding might be a more accurate predictor of patient prognosis than either histological grade or tumour size (Leathem -unpublished data - see section 1.5.3). In this study of 373 patients, simple life table analyses showed *Helix* lectin binding to be superior to histological grade, tumour size, S-phase fraction, or DNA index; but, as a predictor of long term patient prognosis, to show very similar results to lymph node status (see Table 18, in section 3.3).
4.2.1.2. Recent studies

Since the completion of this project, two interesting papers have been published confirming our results -

Fenlon et al (1989):
The first, by Fenlon et al (1987) examined the prognostic significance of *Helix* binding to paraffin sections of 100 breast cancers followed up for a maximum of 8 years after diagnosis. A "P.A.P." (peroxidase-anti-peroxidase - see Appendix V) staining method was used; the percentage of positive cells scored; and cases divided into two groups of 0-50% cells positive and 51-100% cells positive.

The greater proportion of cells staining was closely correlated with lymph node involvement and more advanced disease stage at the time of resection; shorter interval to first recurrence and shorter survival.

*Helix* lectin binding showed a significant relationship with lymph node status, as in our study, but not with other prognostic variables assessed including grade, ER status, monoclonal antibody NCRCII binding, and tumour size. It is very interesting that the prognostic significance of *Helix pomatia* lectin binding was abolished by treatment of the sections with the enzyme neuraminidase, which cleaves sialic (neuraminic) acid, the significance of this observation is discussed later.

Fukutomi et al (1989):
In a second, very recent study, Fukutomi et al (1989) examined 113 primary breast cancers, 63 involved lymph nodes, and 10 resected local recurrences for *Helix* binding using an ABC (avidin-biotin complex, Appendix V) staining technique. When primary breast cancers were grouped as positive or negative for lectin binding, positivity was strongly associated with poorer survival and showed statistically significant correlation with ER status and high nuclear grade (in contrast to Fenlon et al (1986) and this study).

In this study by Fukutomi et al, multivariate analysis evaluated *Helix* lectin binding as *the most indicative prognostic factor in breast cancer* (compared with lymph node status; lymphatic invasion by tumour cells; grade; size; and ER status).

4.2.1.3. Direct comparison of different physical prognostic indicators

In Table 24, the efficacy of *Helix* lectin binding as a prognostic indicator in breast cancer is compared to that of other, well established, prognostic indicators (lymph node status; grade; tumour size) by comparison of 5 and 10 year survival rates of the "good" and "bad"
prognosis groups defined by these parameters.

The studies of Fenlon et al (1987) and Fukutomi et al (1989) were included because they directly address the question of prognostic significance of *Helix pomatia* lectin binding; 5 year survival rates of 45% and 55% for the *Helix* positive patients, and 75% and 90% for the *Helix* negative cases in the two studies respectively agree well with our figures.

The studies of Bloom (1950) and Bloom and Richardson (1957) are chosen for historical interest, and - particularly in the latter survey - because they relate to large series of patients. It is interesting - and very pleasing - that, from the table, the overall survival of breast cancer patients in the modern studies (Weigand et al 1982; Elston et al 1982) appears to be much improved in comparison to the figures of 40 years ago.

The study of Elston et al (1982) is included as a very large, recent survey; and that of Weigand et al (1982), although considering only 175 cases, because it directly addresses the question of prognostic significance of axillary lymph node involvement.

**TABLE 24: Comparison of *Helix pomatia* lectin binding with other indicators of prognosis.**

<table>
<thead>
<tr>
<th></th>
<th>% 5yr survival</th>
<th>% 10yr survival</th>
<th>total no. cases in study</th>
<th>p value</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Helix pomatia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>lectin binding</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>55%</td>
<td>38%</td>
<td>n=</td>
<td>p&lt;0.00001</td>
<td>this study</td>
</tr>
<tr>
<td>-ve</td>
<td>85%</td>
<td>70%</td>
<td>373</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>45%</td>
<td>75%</td>
<td>n=</td>
<td>p&lt;0.02</td>
<td>Fenlon et al 1987</td>
</tr>
<tr>
<td>-ve</td>
<td>75%</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>55%</td>
<td>90%</td>
<td>n=</td>
<td>p&lt;0.0001</td>
<td>Fukutomi et al 1989</td>
</tr>
<tr>
<td>-ve</td>
<td>85%</td>
<td>113</td>
<td>113</td>
<td></td>
<td></td>
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<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>status</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>+ve</td>
<td>48%</td>
<td>38%</td>
<td>n=</td>
<td>p&lt;0.00001</td>
<td>this study</td>
</tr>
<tr>
<td>-ve</td>
<td>87%</td>
<td>70%</td>
<td>373</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>32%</td>
<td>71%</td>
<td>n=</td>
<td></td>
<td>Bloom 1950</td>
</tr>
<tr>
<td>-ve</td>
<td>71%</td>
<td>470</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>~30%</td>
<td>~70%</td>
<td>n=&gt;4000</td>
<td></td>
<td>Harrington 1937</td>
</tr>
<tr>
<td>-ve</td>
<td>~70%</td>
<td></td>
<td></td>
<td></td>
<td>(quoted by Bloom 1950)</td>
</tr>
<tr>
<td>+ve (40% )3yr ()</td>
<td></td>
<td></td>
<td>n=</td>
<td>p&lt;0.001</td>
<td>Weigand et al 1982</td>
</tr>
<tr>
<td>-ve (80% )survival</td>
<td></td>
<td></td>
<td>175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ve</td>
<td>60%</td>
<td>~85%</td>
<td>n=</td>
<td>p&lt;0.001</td>
<td>Elston et al 1982</td>
</tr>
<tr>
<td>-ve</td>
<td>~85%</td>
<td>2800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological</td>
<td></td>
<td></td>
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<tr>
<td>grade</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>I</td>
<td>85%</td>
<td>67%</td>
<td>n=</td>
<td>p&lt;0.0007</td>
<td>this study</td>
</tr>
<tr>
<td>II</td>
<td>70%</td>
<td>50%</td>
<td>373</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>53%</td>
<td>37%</td>
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</table>
The 5 year survival figures for lymph node positive/negative; large/small tumour size and grades I/II/III achieved in this study agree quite closely with those of the much larger published series'(especially the more modern ones) in the Table 22.

Degree of axillary lymph node involvement is generally considered to be one of the most useful prognostic indicators in breast cancer, and in the very large series' of Bloom (1950) and Elston (1982) quoted above, appears to give greater separation of good and bad prognosis cases than either histological grade or tumour size. In our study, Helix pomatia lectin appeared to be as good as lymph node status in predicting long-term prognosis, and considerably better than grade, tumour size or any of the other physical prognostic indicators considered.

4.2.2. HELIX LECTIN IN RELATION TO BIOCHEMICAL TUMOUR MARKERS

Numerous "biochemical tumour markers" have been investigated as potential serum markers for detection of disease/monitoring of treatment success, including carcinoembryonic antigen (CEA), human chorionic gonadotrophin (HCG), fucose, pregnancy associated macroglobulin, breast cyst fluid glycoprotein, placental alkaline phosphatase, and gamma glutamyl transpeptidase. Without exception these markers have proved disappointing in that although serum levels are frequently raised in malignancy, they are not specific or unique to the disease. (see Waalkes and Tormey 1978; Coombes
1978 for review, and section 1.2.6.). Lee et al (1985) and Cohen et al (1987), for example, in 5-10 year retrospective studies found no association between expression of several biochemical markers (including alpha-lactalbumin, pregnancy-specific beta-1-glycoprotein, HCG, and CEA) and breast cancer prognosis.

Although the vast majority of work has concentrated upon the detection of these chemicals in serum, some studies have assessed the prognostic significance their expression in tissue sections, thus enabling us to make a comparison with Helix pomatia lectin binding in this study.

4.2.2.1. HCG

Monteiro et al (1984) compared serum levels of HCG in 570 patients with expression in breast cancer tissue sections. They concluded that measurement of circulating HCG had no clinical utility - raised serum levels carried no prognostic significance in breast cancer, and indeed were common in healthy post-menopausal subjects. Immunocytochemistry demonstrated the expression of HCG by breast cancers to be uncommon, and, again to carry little or no prognostic significance.

4.2.2.2. Alpha-lactalbumin

Walker (1979) showed no correlation between expression of alpha-lactalbumin by 51 of a series of 100 breast cancers, and either histological differentiation or absence/presence of lymph node metastases. Bailey et al (1982) failed to demonstrate alpha-lactalbumin in any of 44 breast cancers studied, although it was readily demonstrable in lactating breast and the breasts of pregnant women. Its usefulness as a prognostic tissue marker appears therefore to be limited.

4.2.2.3. CEA

Many studies have examined the prognostic significance of CEA expression in tissue sections of breast cancer, with very conflicting results: many have found CEA immunoreactivity to be associated with poor prognosis (Shousha and Lyssiotis 1978; Wharen et al 1978; Mansour et al 1983), others have failed to demonstrate any effect (Walker 1979; Persijin and Korsten 1977; van der Linden et al 1985). The explanation for this controversy may lie with variations in methodology, specificity of anti-serum used, and small numbers of cases considered in most studies (van der Linden et al 1985).

Recently, Fukutomi et al (1989) directly examined the prognostic significance of Helix pomatia lectin binding in relation to expression of CEA, which, arguably, still remains the
most promising of all existing biochemical tumour markers - while *Helix* lectin binding showed an extremely strong correlation with survival (p=0.0001), CEA showed only borderline significance (p=0.06).

4.3. CONSIDERATION OF WHAT THE *HELIX* LECTIN-BINDING MOLECULE MIGHT BE.

4.3.1 OVERALL STAINING PATTERN IN TISSUE SECTIONS

In both primary breast cancer and metastases, *Helix pomatia* lectin binding appeared to be predominantly cytoplasmic, but localisation at the cell border, or at the periphery of cell clusters was also frequently observed. In some cases, such staining was dramatic. Staining was either diffuse, or in some cases, finely granular. (See Figures 25, 26 and 27 in Results section 3.2).

The lectin also stained many benign proliferations noted incidentally in tissue sections examined - although no comprehensive survey was undertaken - and gave extremely strong, distinctive staining of the luminal surface of cells lining normal lobules and ducts. (See Figures 28 and 30 in Results section 3.2).

In some cases, binding to the endothelium of blood vessels and to red blood cells was noted. Such staining was unrelated to staining of tumour cells and other structures. (See Figure 29 in Results section 3.2).

4.3.2. THE SIGNIFICANCE OF *HELIX* LECTIN BINDING TO NORMAL BREAST AND BREAST CANCER.

Differences in lectin binding pattern to normal in comparison to malignant breast are well documented in the literature - a switch from discrete cell surface binding in normal breast to a heterogeneous, predominantly cytoplasmic pattern in breast cancer has been reported with many lectins (see, for example, Franklin 1983; Louis et al 1983) including, most commonly, those from peanut (Newman et al 1979; Dansey et al 1988) and wheatgerm (Walker 1984a and b; Dansey et al 1988), as well as *Helix pomatia*.

Leathem et al (1983) reported specific luminal surface staining by a number of galactosyl-binding lectins (*Helix*; peanut; soyabean; and *Bandeirea*) to normal breast which suggests that several different galactosyl-containing groups are expressed at this site. (*Helix pomatia*
lectin gave the most intense and specific staining. This localisation may indicate a normal secretory role for these glycoconjugates, perhaps as components of breast secretions or milk.

*Helix pomatia* lectin binding appears to persist in a majority of breast cancers and their metastases, but is altered to a predominantly cytoplasmic (diffuse or granular) pattern. This switch in lectin binding pattern presumably reflects some aberration in the glycosylation mechanism with malignancy: Accumulation of *Helix* binding material in the cytoplasm, may be indicative of an acceleration in synthesis, or a breakdown in transport mechanism - the material is being produced faster than it can be transferred to the cell surface. That lectin binding sites are still present at the cell surface, as well as in the cytoplasm, of malignant cells has been elegantly demonstrated by Schumacher (1989, personal communication) by incubation of MCF7 cells (a breast cancer cell line) with rhodamine-conjugated *Helix pomatia* lectin, as seen in the photograph (Figure 80).

**FIGURE 80:** rhodamine-conjugated *Helix pomatia* lectin binding to MCF7 cells in culture.

Photograph courtesy of Dr. U. Schumacher, Department of Anatomy, University of Munich.

### 4.3.3 THE EFFECT OF NEURAMINIDASE TREATMENT

Ferlon et al (1987) reported that the prognostic significance of *Helix pomatia* lectin binding was abolished by pre-treatment of the tissue section with the enzyme neuraminidase, which cleaves sialic (neuraminic) acid. They do not state whether the enzyme enhances or
abolishes *Helix* lectin binding, but suggest that this observation "may imply that sialation of lectin binding sites could be important in the production of staining patterns related to prognosis".

This observation is interesting as several studies have reported alterations in sialation with transformation or with metastatic competence. In general, increased expression of sialic acid has been shown to be associated with enhanced metastatic potential (Yogeeswaran and Salk 1980; Altevogt et al 1983; Dennis et al 1984). The reason for this remains unclear, but the strong negative charge carried by sialic acid has been implicated in a "masking" effect, hiding sub-terminal carbohydrate residues which would otherwise be involved in recognition events, and cell adhesion (Lloyd 1975). This concept is illustrated in Figure 81:

**FIGURE 81:** Mechanism by which sialic acid may protect cells from recognition and adhesion. (After Lloyd 1975).

\[\text{oligosaccharide chain} \quad \text{sialic acid}\]

\[\text{asialo recognition site} \quad \text{site "protected" by sialic acid}\]

4.3.4. *HELIX* LECTIN BINDING BANDS IN S.D.S.-P.A.G.E.

In polyacrylamide gel electrophoresis, very many *Helix pomatia* lectin binding bands are seen in human milk, MFGM, in breast cancers and in their metastases. A 55kDa band was identified as being of special interest in that it was expressed weakly by human breast milk and MFGM, and strongly expressed in primary breast cancers and their metastases (but not shared by normal control tissues or the serum of healthy individuals).
It is a tantalising speculation that this band may correspond to the \textit{Helix} binding material localised in tissue sections at the luminal surface of normal breast (perhaps to be released into breast secretions or breast milk), which persists in most cancers of the breast and their metastases.

This hypothesis is currently being actively pursued in our continuing research. Comparison of the 55kDa. molecule to common protein constituents of human milk and MFGM will be considered later (sections 4.4.1.1. and 4.4.1.2.).

\textbf{4.3.5. \textit{HELIX} LECTIN BINDING AND BLOOD GROUP A}

\textit{Helix pomatia} lectin is known to recognise blood group A substance (Prokop et al 1965a and b) and for this reason has been used in blood grouping and in isolation of Forssman and blood group A glycolipids (Torres and Smith 1988). Although studies of blood group antigen expression demonstrate a reduced or absent expression in paraffin-embedded tissues, presumably because blood group antigen-associated glycolipids are extracted during processing with organic solvents (Thorpe et al 1983), we have considered the possibility of a confounding effect of expression of blood group A substance upon the validity of our data.

Our awareness of this potential problem was heightened by the critical response of Grundbacher (1987) to publication of our preliminary results (Leathem and Brooks 1987), which drew our attention to reports of an excess of blood group A individuals amongst certain groups of breast cancer patients in Texas (Anderson and Hass 1984) and Tunisia (Mourali et al 1980).

It is unfortunate that only limited blood group data was available for the patients in this series, (see section 2.1.2.3. of the Introduction), however, in 70 patients for whom blood group data was available, \textbf{no correlation was seen between ABO blood group and \textit{Helix} lectin binding}. Positivity of blood vessels and erythrocytes was seen in some tissue sections - we have assumed that such staining reflects the blood group of the patient. No correlation was seen between the staining of endothelium and red blood cells and that of the breast cancer itself (see section 3.2.5.2. and Figure 29).

In polyacrylamide gel electrophoresis, \textit{Helix} lectin recognised a distinctive pattern of protein bands in the serum of blood group A, as well as B and O subjects. (Figure 57) These bands were quite distinct from the 55kDa. band recognised by the lectin in tissue homogenates of breast cancer primary, metastases, and human milk and milk fat globule membrane. The 55kDa. band was also identified in the milk and milk fat globule membrane
of a healthy, lactating subject known to be of blood group O. These observations, in addition to those of the obviously different electrostatic charge carried by the Helix binding material in group A serum versus breast cancer homogenates as seen in anion exchange chromatography (see sections 3.6.2.1 and 3.6.2.4), strongly suggest that the material recognised by the lectin in breast cancer is not simply a blood group A-related substance.

4.3.6. SIGNIFICANCE OF HELIX LECTIN BINDING TO METASTASES

In this study, we observed Helix lectin binding to around 80% of breast cancer metastases at various sites. This figure is in close agreement with that acheived in a collaborative study between our group and Schumacher et al (unpublished data) in which we examined 16 brain metastases arising from breast cancer primaries, 13 of which showed Helix lectin binding (81%); and by Fukutomi et al (1989) who reported Helix binding to 46/63 (73%) axillary lymph node metastases and 8/10 (80%) locally recurrent tumours. These two studies were based on surgically resected tissue, and thus do not suffer, as does this study, from the potential drawbacks of reliance on autopsy specimens.

It is interesting that as many as 20% of breast cancer metastases do not bind Helix lectin if, as our results suggest, only around 10% of primary cancers are negative, and if Helix-positive breast cancers are more likely to have metastasised than Helix-negative cancers. This would suggest that at some stage following dissemination, the Helix binding material is no longer expressed - a hypothesis supported by our observation that within a single patient metastases at different sites, and even adjacent lymph node deposits, may present a mixture of positive and negative Helix lectin binding results.

It is possible, if the Helix binding material actually plays some functional role in the process of metastasis - and does not merely represent some epiphenomenon (the Helix binding material could, for example, simply be expressed in conjunction with some other substance which itself is involved in the process of metastasis) - that following dissemination of the tumour, expression of Helix binding material ceases to confer a selection advantage upon the cells that express it, and in some tumours it is thus no longer produced.

Alternatively, the relatively benign natural history of Helix-negative primary breast cancers raises the question of potentially altered biological behaviour in Helix-negative metastases. It is interesting to speculate upon the possible alteration of growth characteristics in these tumours, leading, perhaps, to a more benign clinical course.
4.4. WHAT IS *HELIX* LECTIN BINDING TO?

4.4.1. COMPARISON OF THE 55kDa. *HELIX*-BINDING MOLECULE WITH MAJOR COMPONENTS OF MILK AND M.F.G.M.

We have demonstrated by S.D.S.-P.A.G.E. and Western lectin blotting that the 55kDa. *Helix*- binding molecule is present in tissue homogenates of primary breast cancers and their metastases, and also in human milk and MFGM. It does not appear to be expressed by other normal tissues and is not detectable in normal serum. These observations, linked with a switch in *Helix* lectin binding in tissue sections from a discrete localisation at the luminal surface of normal breast duct epithelium to a more diffuse, predominantly cytoplasmic distribution in malignancy, has led us to speculate whether the 55kDa. molecule may be some normal breast protein - perhaps a constituent of the plasma membrane of normal breast cells, or of breast secretions or milk - which is abnormally expressed by aggressive breast cancers.

We have attempted to make a simple comparison between the molecular weight of this molecule and that of known constituents of human breast milk and the major proteins of MFGM.

4.4.1.1. Human milk

The major constituents of human milk, and their reported molecular weights, are listed in Table 25. It is apparent that none obviously corresponds to the 55kDa. *Helix* binding material.

<table>
<thead>
<tr>
<th>milk protein</th>
<th>molecular weight (kDa)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>alpha-lactalbumin</td>
<td>14</td>
<td>Lonnerdal et al 1976</td>
</tr>
<tr>
<td>lactoferrin</td>
<td>80</td>
<td>&quot;</td>
</tr>
<tr>
<td>serum albumin</td>
<td>70</td>
<td>&quot;</td>
</tr>
<tr>
<td>lysozyme</td>
<td>14</td>
<td>&quot;</td>
</tr>
<tr>
<td>IgA</td>
<td>390</td>
<td>Hanson and Johansson 1961</td>
</tr>
<tr>
<td>IgG</td>
<td>170</td>
<td>&quot;</td>
</tr>
<tr>
<td>casein</td>
<td>25</td>
<td>Groves and Townsend 1970</td>
</tr>
</tbody>
</table>
4.4.1.2. Milk fat globule membrane

In secretion of fat from acinar cells of mammary tissue, the fat droplet is first enveloped in plasma membrane, and then pinched off and released (Bargman and Knoop 1959). The milk fat globule membrane therefore represents a unique opportunity to study the plasma membrane of acinar cells of normal, lactating breast.

The principle proteins and glycoproteins of the human MFGM have been described in several studies. These components have not been named, but their apparent molecular weights in SDS-PAGE have been listed.

A summary of the results of three principal studies is given in Table 26.

<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>protein*/glycoprotein**</td>
<td>220</td>
<td>169</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>protein</td>
<td>185</td>
<td>145</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>glycoprotein</td>
<td>115</td>
<td>94.2</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>protein**/glycoprotein*</td>
<td>84</td>
<td>74.6</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>protein**/glycoprotein*</td>
<td>53</td>
<td>55</td>
<td>54.6</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>protein</td>
<td>16</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>glycoprotein</td>
<td>360</td>
<td>148</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>glycoprotein</td>
<td>280</td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>glycoprotein</td>
<td>220</td>
<td>90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The anomalous behaviour of some heavily glycosylated proteins in S.D.S.-P.A.G.E. (Banker and Cotman 1972; Evans and Gurd 1973) - believed to be associated with a decrease in S.D.S.-binding and therefore a decrease in the relative mobility of the protein (Banker and Cotman 1972) - has caused problems in the molecular weight determination of higher molecular weight glycoproteins (Anderson et al 1974). This is seen in the obvious discrepancies between the studies quoted above regarding apparent molecular weights of bands I, II, and A-C.

There is good agreement in molecular weight determination of lower molecular weight bands III-VI, however, and Anderson et al (1974) conclude that molecular weight estimation for these molecules (ie including band V, the 55kDa protein/glycoprotein) are valid.
Band V has an apparent molecular weight equivalent to that of the 55kDa. Helix-binding band identified in this study as being expressed by milk, MFGM, and breast cancers. It appears to be glycoprotein (Anderson et al 1972, 1974 - although Kobylka and Carraway 1972 reported this band as protein rather than glycoprotein) which would be compatible with it binding Helix pomatia lectin. It would seem, therefore, that we might be looking at the same molecule.

Expression of this 55kDa. Helix lectin-binding glycoprotein appears to be in some way involved in the process of breast cancer progression/metastasis - cancers which express the molecule show very much more aggressive biological behaviour than those which do not.

It is, of course, quite possible that what we are detecting is an epiphenomenon - the lectin-binding molecule merely being expressed in conjunction with, and therefore acting as a marker for, some other substance which itself influences prognosis. However, the identification of a 55kDa. Helix-binding molecule apparently associated with the normal breast plasma membrane, and expressed by breast cancers, and their metastases, but not other normal tissues is provocative.

4.4.2. COMPARISON OF THE 55kDa. HELIX-BINDING MOLECULE WITH BREAST / BREAST CANCER-ASSOCIATED GLYCOPROTEINS DEFINED BY MONOCLONAL ANTIBODIES.

We have tried to make a comparison of the 55kDa. molecule with breast / breast cancer associated antigens defined by monoclonal antibodies described in the literature, particularly those believed to carry prognostic significance.

Overall, the 55kDa. material does not appear to obviously correspond with any of these antigens, the majority of which are apparently high molecular weight molecules (eg see, for example, Ceriani et al 1983; Sekine et al 1985; Rind et al 1989; Stacker et al 1989; Kufe et al 1984). A few of the more important and interesting monoclonal antibodies and the molecules they recognise are discussed briefly below:

4.4.2.1. High molecular weight epithelial membrane antigens

HMFG1 and HMFG2

The monoclonal antibodies HMFG1/HMFG2, raised against a >400kDa human milk fat globule membrane component (Taylor-Papadimitriou et al 1981), for example, bind extracellularly to ducts and tubules of normal breast, and heterogeneously to breast cancers.
(Arklie et al 1981), as does *Helix pomatia* lectin. Positive staining of breast cancers appears, however, to be associated with favourable, rather than unfavourable, patient prognosis (Wilkinson et al 1984). Although HMFG2 has been demonstrated to recognise lower molecular weight components, including a glycoprotein of approximately 68kDa. (Griffiths et al 1987), that *Helix* lectin and HMFG1/HMFG2 (a gift from Dr. J.Taylor-Papadimitriou) recognise quite different molecules is also confirmed by our comparison of their binding to both breast cancer sections and Western blots (unpublished results).

*NCRClII and others*

The binding of monoclonal antibody NCRClII (Ellis et al 1984) to sections of primary breast cancer has also been reported to be of prognostic significance, but once again, binding is a marker of good, rather than poor, clinical disease outcome. NCRCI1II recognises a high molecular weight glycoprotein (>400kDa) (Price et al 1985), which exhibits some homology with that recognised by other monoclonals such as HMFG1 and HMFG2 (Price et al 1985), those directed against MAM-6 (Hilkens et al 1984) epithelial membrane antigen, (EMA) (Ormerod et al 1985) and PAS-O (Shimizu and Yamasuchi 1982). Fenlon et al (1987) confirmed that there was no association between *Helix pomatia* lectin and NCRClII binding.

4.4.2.2 Low molecular weight antigens

Lower molecular weight breast epithelial / breast cancer-associated glycoproteins have been described, but each appears distinct from the 55kDa. *Helix* binding material.

*60kDa a "gross cystic disease fluid protein"*

A 60kDa glycoprotein ("gross cystic disease fluid protein") has been described by Toth et al (1988); it is, however, composed of four 15kDa. monomers distinct in S.D.S.-P.A.G.E. performed under denaturing conditions - no such sub-units are apparent in our characterisation of the *Helix* binding material.

*68kDa MMTV-associated glycoprotein*

Kevdar et al (1989) described a mouse mammary tumour virus-associated glycoprotein, detectable in the culture fluid of a breast cancer cell line (T47D) and in pleural effusions of breast cancer patients, and of apparent molecular weight 68kDa. Immunoperoxidase staining revealed the presence of this substance in 91% of 812 breast carcinomas tested, but - unlike *Helix*-binding material - not in normal breast or benign disease.

*43kDa glycoprotein*

Similarly, a monoclonal antibody recognising a 43kDa. glycoprotein has been
demonstrated to bind to most primary breast cancers (59%) and lymph node metastases (75%), plus some (20%) benign lesions. Normal breast, however - contrary to results obtained with *Helix pomatia* lectin binding - was negative (Edwards et al 1986).

**52kDa. oestrogen-regulated protein**
Much attention has recently been focussed upon an oestrogen-dependant 52kDa. glycoprotein, believed to be a proenzyme precursor of a lysosomal cathepsin-D-like protease (molecular weight 34/48kDa). Vignon et al (1983;1986) presented interesting data to suggest that this substance may possess autocrine mitogenic effects. It is thought to be associated with tumour proliferation and/or invasion, rather than hormone responsiveness (Garcia et al 1987), and expression by breast cancer cells is correlated with poor patient prognosis (Thorpe et al 1988). The glycoprotein is expressed by a majority of breast cancers, some benign disease, but, again unlike the *Helix* lectin binding material, not by normal breast (Garcia et al 1984; 1986).

"gp52"
An antigen immunologically related to the mouse mammary tumour virus (MMTV) and its envelope glycoprotein, "gp52", has been identified in human breast cancers. Expression of gp52 has been shown to be associated with larger tumour size, but appears to be unrelated to histological tumour grade, lymphocyte infiltration of the primary tumour, or nodal status (Lloyd et al 1983). *Helix pomatia* lectin binding, in contrast, does not appear to be associated with tumour size, but shows significant correlation with lymph node status.

4.5. WHAT ROLE DOES THE HELIX LECTIN-BINDING MOLECULE ACTUALLY PLAY IN THE MECHANISM OF METASTASIS?

4.5.1. OVERVIEW

Our study indicates that expression of *Helix* lectin-binding material, and particularly a 55kDa. lectin-binding molecule, is associated with aggressive biological behaviour of breast cancers.

At this stage in our investigations we know far too little of the characteristics of this *Helix* binding material to understand what role it actually might play in the mechanism of metastasis.

Some of the very diverse molecules believed to play critical roles in this highly complex
and still ill-understood mechanism are discussed below, with comparison, where appropriate, to the little we know about the *Helix* binding material.

The molecules to be considered are listed below-

(a) the products of various **oncogenes** implicated in breast cancer growth and differentiation.

(b) molecules believed to be directly involved in the metastatic cascade, in particular, *laminin receptor*, *type IV collagenase*, and integrins.

(c) **endogenous human lectins** believed to be implicated in cell recognition events.

**4.5.2. ONCOGENE PRODUCTS**

Currently, much interest is being shown in the involvement of oncogenes and their products in tumour growth and differentiation (see section 1.2.7.). It is quite possible that *Helix pomatia* lectin is binding some oncogene product associated with enhanced breast cancer growth or metastasis.

That lectins can be used as probes to detect oligosaccharide changes associated with oncogene expression is illustrated by the study of Dennis and Laferte (1989a) who used L-PHA (a lectin from *Phaseolus vulgaris*) to detect increased B1-6 branching of oligosaccharides associated with transformation and metastatic potential after c-H-ras and v-fps oncogene transfection. Over-expression of the c-erbB-2 oncogene, of particular interest in breast cancer (Slamon et al 1987), could - at least theoretically - be detected in the same way, as over-expression of c-erbB-2 may similarly result in an increase in B1-6 branching (Dennis and Laferte 1989a).

Many oncogenes appear to code for growth factor-related proteins or their receptors, such as the c-sis gene and the polypeptide B-chain of platelet derived growth factor (Waterfield et al 1983), c-erbB gene and an epidermal growth factor receptor-like molecule (Downward et al 1984) and c-fms gene and the receptor for colony stimulating factor (Sherr et al 1985), with the implication that over-expression may confer enhanced growth potential upon malignant cells. This surmise is supported by the observation that the epidermal growth factor and related peptides appear to act as mitogens for many tissue types, including normal breast, epithelial cells in culture, and malignant breast cell lines (eg. Stroker et al 1976; Stampfer et al 1980; Fitzpatrick et al; Osborne et al 1980), and Cline et al (1987),
have actually reported a greater incidence of c-myc oncogene expression in tumours of >4cm diameter.

If the *Helix pomatia* lectin were to be marking some growth factor, growth factor receptor, or related oncogene product, cancers binding the lectin might be expected to exhibit enhanced growth characteristics over those which do not bind the lectin. In our study there was absolutely no correlation between *Helix* binding and tumour size (section 3.3.6.3. and 3.3.7.), perhaps suggesting that this is not the case. Similarly, a rapidly dividing tumour, containing many mitoses, would most likely be of high grade; and in flow cytometric analysis would give a high S-phase fraction - no correlation was seen between histological grade or S-phase fraction values and *Helix pomatia* lectin binding (section 3.3.6.4., 3.3.6.5. and 3.3.7.).

Most of the initial research on the role of oncogene expression in human cancer has simply provided a catalogue of the expression of different oncogenes in different tissues - this is a very new field, and one where we still have much to learn! However, there are several reports in the literature which begin to suggest a direct link between cellular oncogenes and actual metastatic competence (see Ormerod and Hart 1987 for review), but, perhaps owing to the relatively small number of such studies, and the conflicting results obtained, there remains very little direct support for such an association. It is thus very difficult to directly compare the prognostic significance of *Helix pomatia* lectin binding with that of oncogene expression in order to look for a direct link between the two.

The apparent molecular weight of the *Helix* binding band identified as being of interest in S.D.S.-P.A.G.E. does not obviously correspond to reported molecular weights of growth factors/receptors or oncogene products as yet described in the literature. The c-H-ras GTP-binding protein, for example, has a reported molecular weight of 21kDa. (McGrath et al 1984); the phosphoprotein associated with c-scr expression has a molecular weight of 60kDa. (Sefton et al 1980), and the product of many oncogenes, such as c-erbB-2, remain uncharacterised.

4.5.3. MOLECULES INVOLVED IN INVASION AND METASTASIS

In order for metastasis to take place, tumour cells from the primary cancer must first invade the basement membrane and endothelial layer of blood vessel walls, before being dislodged, singly or as clumps, into the circulation. Circulating tumour cells must then arrest in capillaries of the target organ by attachment to endothelial cell surfaces, or exposed basement membranes. They must once again invade the basement membrane to initiate metastatic colony formation (Liotta et al 1986). This sequence of events is depicted in the diagram overleaf (Figure 82).
FIGURE 82: Tumour cells invade epithelial basement membrane to reach interstitial stroma where they gain access to blood vessels. They invade the basement membrane and endothelium of the blood vessel wall and are dislodged into circulation. Circulating tumour cells arrest in the precapillary venules of the target organ by adherence to endothelium or exposed basement membrane. They must then again invade the basement membrane to form a metastatic colony.

Clearly, tumour cell interaction with (a) the basement membrane, and (b) endothelial cells are critical steps in the metastatic cascade. We must consider whether the Helix lectin-binding material could be implicated at either stage.

4.5.3.1 Tumour cell interaction with the basement membrane

Basement membranes act as an interface between histologically disimilar tissues that arise from different primary germ layers; major components include a special basement membrane-specific collagen, collagen type IV; and a large complex glycoprotein, laminin. The interaction of cancer cells with these components may represent an important step in the escape of cancer cells from primary tumour into circulation, and colonisation of the target organ. It is believed that metastasising cancer cells, in common with some non-neoplastic cells, possess the ability to synthesize enzymes which degrade these and other basement membrane components.
4.5.3.2. Laminin receptor

High-affinity receptors for laminin have been demonstrated on the surface of both normal and neoplastic cells (Malinoff and Wicha 1983; Liotta et al 1985; Rao et al 1983; Terranova et al 1983). Tumour cells selected for their ability to attach to the basement membrane through such receptors show markedly increased ability to form metastases when injected intravenously into experimental animals (Terranova et al 1982). In addition, the treatment of tumour cells with the receptor-binding fragment of laminin at very low concentrations has been demonstrated to markedly inhibit or even abolish lung metastases in a murine model system by blocking the adhesion of circulating tumour cells to sub-endothelial basement membrane (Barsky et al 1984).

The laminin receptor has been isolated and characterised - it appears to contain interchain di-sulphide bonds, have an isoelectric point of 5.2, and a molecular weight of slightly less than 70kDa. (Malinoff and Wicha 1983; Terranova et al 1983; Rao et al 1983; Liotta et al 1985) - it is thus much larger than the 55kDa. Helix binding material identified in this study as being potentially associated with aggressive breast cancer.

4.5.3.3. Type IV collagenase

An enzyme which degrades the basement membrane-specific type IV collagen has been isolated from highly metastatic cancer cells (Kalebic 1983). Antibodies raised against the enzyme have been shown to react with invading breast cancer cells and breast cancer lymph node metastases by immunohistochemistry (Barsky et al 1983).

The molecular weight of type IV collagenase is 62-68kDa. (Salo et al 1982), again a slightly larger molecule than the 55kDa. Helix binding material.

4.5.3.4. Plasminogen activator

Plasminogen activators, a family of proteases which are active blood coagulants, have also been implicated in the process of intracellular matrix degradation. Tissue invasion by endothelial cells, macrophages, or regenerating nerves is usually associated with elevated plasminogen activator activity, and plasmin generated from plasminogen by tumour cell-associated plasminogen activator may be crucial for matrix hydrolysis (Tokes 1986).

Colombi et al (1984) reported high levels of plasminogen activator in breast cancer tissue, suggesting, perhaps, a role in peritumoral tissue destruction. It is interesting that lowered plasminogen activator levels in the serum of some of their breast cancer patients was strongly associated with the presence of lymph node metastases - perhaps some flaw in
blood coagulation mechanism enhancing metastatic potential.

Two principle types of plasminogen activator are of special interest - tissue type \( t-PA \), which has a molecular weight of approximately 100kDa. or 70kDa. (Pohl 1984), and urokinase type \( u-PA \) which exists in a low molecular weight form of 33kDa., and, provocatively, a higher molecular weight form of reported molecular weight 55kDa. (Gunzler et al 1982). Furmanski (1984) demonstrated a strong correlation between the expression of the 55kDa. \( u-PA \) in the original tumour tissue and subsequent breast cancer recurrence: at 2 years post-operatively, no recurrences were reported in patients where neither plasminogen activators had been detectable; where only \( t-PA \) was detectable, the recurrence rate was 7%; where \( t-PA \) and \( u-PA \) were present it was 36%; and where \( u-PA \) was present it was 46%.

Immunohistochemical studies have demonstrated \( u-PA \) to be produced at rather discrete sites in normal tissues - such as, in the mouse, in the lamina propria of gastrointestinal mucosa, and in larynx, trachea and bronchi. Kidney tubules, decidual cells of the placenta, and involuting mammary gland also stained strongly (Larsson et al 1984).

The active form of \( u-PA \) consists of two chains of molecular weight 30kDa. and 20kDa. linked by a disulphide bridge. Under the denaturing conditions of S.D.S.-P.A.G.E. performed in the presence of mercaptoethanol, these chains would, clearly, become dissociated, and it thus appears unlikely that our 55kDa. \textit{Helix} binding molecule corresponds to \( u-PA \).

\subsection*{4.5.3.5 Integrins and RGD peptides}

A family of cell surface glycoproteins termed "integrins" has been identified which bind to peptides bearing the Arg-Gly-Asp (RGD) sequence, which is present in many adhesion molecules including fibronectin, fibrin, type I collagen and thrombospondin (Liotta 1988).

Experimentally, co-injection of tumour cells and large quantities of RGD peptides inhibits metastasis in animal models. (Furcht 1986). RGD peptides may interfere with the adhesion of tumour cells to the endothelial surface; this action is believed to be directly or indirectly mediated through integrins (Liotta 1988).

The integrins are a complex of alpha and beta subunits of molecular weight 140kDa. and 95kDa. respectively. - clearly quite distinct from the 55kDa. \textit{Helix} lectin-binding glycoprotein.
4.5.3.6. Helenx binding material - potential role in invasion?

In staining of tissue sections for Helenx pomatia lectin binding we often noted more intense staining at the edge of tumour cell groups, and particularly at the invasive edge of the tumour mass as it infiltrated fat or collagen or muscle. (see Figure 27) This observation may, of course, be of no significance, but may carry implications regarding the potential role of Helenx binding material in local tissue invasion.

Parish et al (1987) have described differences in cell membrane associated proteins between infiltrative and non-infiltrative mouse cells in culture. Antibodies directed against a glycoprotein of molecular weight 37kDa. (called gp37) showed dramatic effect in completely inhibiting invasive behaviour. The Helenx binding material identified by this study as being of interest does not correspond to gp37, but it is conceivable that it could be involved in a similar mechanism.

4.5.4. ENDOGENOUS LECTINS

4.5.4.1. Tumour cell-endothelial cell interactions

The presence of cancer cells in the blood stream is not an inevitable indication of metastasis - although most cancer patients appear to have very large numbers of circulating cancer cells (see Stewart 1960 for overview), not all develop metastatic disease. Tarin and colleagues, for example, have described how the artificial introduction of millions of cancer cells into the circulation through peritoneo-venous shunts, usually fail to produce metastases in patients with existing malignant disease, even over long time periods (Tarin et al 1984; Tarin 1986). Following escape into circulation, cells must then go on to become entrapped in small blood vessels at the site of metastasis, and then invade the organ and proliferate.

A simple "anatomical/mechanical" hypothesis of tumour dissemination, such as that originally proposed by Ewing (1928), predicts that distribution of metastases will be determined solely by the number of tumour cells reaching the microcirculation of each organ. Metastasis simply requires the mechanical entrapment of malignant cells. Although evidence - the preponderance of natural metastases found in lung, the first organ encountered by most circulating cancer cells and in draining lymph node adjacent to primary tumour (Weiss 1983; Lindberg 1972), and animal tumour models in which injected tumour cells yield primarily lung colonies (Weiss 1983; Roos and Dingemans 1979) - exists to support such a theory, the wealth of evidence points to a more complex mechanism.
Paget (1889) first proposed, as a result of his observations of the quite different patterns of spread of micro-organisms and breast cancer, a "seed/soil" hypothesis whereby metastatic development was a consequence of particular tumour cells ("seeds") finding a suitable environment ("soil") in order to develop and grow. This idea is supported by the natural organ selectivity of metastasis in many cancers - primary breast cancer, for example, commonly metastasises to lung, liver, brain, bone, but rarely to kidney, gut or spleen (Romsdahl et al 1970) - and by a wealth of experimental evidence - numerous animal tumour cell lines, for example, show well-defined organ preference for metastatic colonisation (see Nicolson 1988 for review).

The "seed/soil" hypothesis requires there to be some recognition system whereby circulating cancer cells are selectively retarded in specific organs - several studies have demonstrated the binding of tumour cells with known organ-colonising preference to cultured endothelial cells of that organ, but not of inappropriate organs (Nicolson 1988), and this little-understood recognition system may represents one of the most important events in organ-specific metastasis.

Components of organ endothelial cell surface have been identified which bind to tumour cells. These molecules appear, by their binding to lectins, to be glycoprotein in nature, and an organ specific pattern has been demonstrated - brain endothelial cells, for example, are characterised by increased display of a 180kDa wheat-germ agglutinin binding glycoprotein and lung endothelium by a 55kDa. *Ricinus communis* lectin-binding glycoprotein (Belloni and Nicolson 1988).

Tumour cell surface components involved in endothelial cell interactions are less well characterised, although monoclonal antibodies raised against cell surface components have been demonstrated to block tumour cell-endothelium interactions in vitro and experimental metastasis formation in vivo (Shearman et al 1980; McGuire et al 1984).

4.5.4.2. The idea of a role for endogenous lectins

Understanding the mechanism of normal lymphocyte homing has provided further insight into endothelial cell adhesion process. Lymphocytes recirculating between blood and lymphoid organs enter lymph nodes through a process which may be analogous to tumour cell-endothelial interactions mediated through specific cell surface receptors (Gallatin et al 1983; 1986).

A lymphocyte "homing" receptor for lymph node epithelium has been described which seems to be a cell surface lectin - lymphocyte attachment to lymph node endothelium is inhibited experimentally by mannose-6-phosphate (Stoolman et al 1984; Rosen and...
Yednock 1986), the polysaccharide fucoidin (Rosen and Yednock 1986) and by sialidase digestion (Rosen et al 1985).

Recently, much interest has been shown in "endogenous" human lectins (see section 1.4 and Appendix I), and a rapidly increasing body of evidence supports their ubiquitous distribution and tentative roles in all manner of cell-cell recognition phenomena. A particularly interesting and exciting idea is that the material expressed by breast cancer cells which binds to the Helix lectin in tissue sections and Western blots could, in the same way, bind some, perhaps as yet unidentified, human endogenous lectin in vivo.

4.5.4.3. Tumour cell glycoprotein binding to endothelial cell lectin

The evidence forwarded above suggests the idea of a tumour cell-surface lectin, perhaps analogous to the lymphocyte lectin, recognising specific endothelial glycoproteins at the site of potential metastasis.

Our observation is that a glycoprotein which binds Helix pomatia lectin is expressed by cancer cells of tumours showing aggressive biological behaviour. Although this substance binds to a lectin, this does not necessarily preclude it from actually acting as a lectin in its own right (most lectins are glycosylated molecules). This hypothesis is illustrated in Figure 83:

**FIGURE 83:** Cancer cell bearing carbohydrate moiety binds to endogenous lectin expressed at the endothelial cell surface.
4.5.4.4 Tumour cell lectin binding to endothelial cell glycoprotein

The presence of *Helix pomatia*-like lectin at the site of potential metastasis linked with the expression of a specific lectin-binding molecule by some cancer cells could provide an alternative mechanism for the recognition of circulating cancer cells by their target organs, as illustrated in Figure 84.

![Figure 84: Lectin on cancer cell surface recognises carbohydrate moiety expressed at the endothelial cell surface](image)

A number of mammalian and human lectins have been described and, although their function is, as yet, little understood, their existence illustrates the feasibility of such a system. For example, a hepatic mannose/N-Acetyl-glucosamine binding lectin has been shown to be present on the sinusoidal cells of rat liver (Achord et al. 1978; Schlesinger et al. 1978; Kawasaki et al. 1978; Townsend and Stahl 1981), and subsequent work suggests its presence on endothelial cells of other organs (Schlesinger et al. 1980; Sun-Sang et al. 1983), it has been shown to mediate uptake of glycoproteins from circulation (Stahl et al. 1976). In chicken, a liver GlcNAc-binding lectin (Lunney and Ashwell 1976) has been described which also appears to be present in several other organs (Kawasaki and Ashwell 1977), and again appears to be involved in the clearance of circulating glycosylated molecules (Lunney and Ashwell 1976).

4.5.4.5 Tumour cell-parenchymal cell interactions

Once cancer cells have become extravasated, adhesive interaction with organ parenchymal cells becomes important, and, indeed, recent experimental evidence suggests that similar
adhesive mechanisms as described above apply (Belloni and Nicolson 1988).

It is interesting that in one of the few systems where the mechanism responsible for such adhesion is understood, the interaction of endogenous lectin and carbohydrate appears to play a key role: Springer et al (1983) have shown that ESb lymphoma cells bear a surface oligosaccharide which recognises the mammalian asialoglycoprotein receptor (Schwartz et al 1981). Highly metastatic variants of the cell line express much more oligosaccharide than the non-metastatic parent line (Springer et al 1983).

4.5.4.6. Endogenous lectins in tumour cell clumping

The enhanced metastatic potential of clumps of cancer cells over single cells is well documented (Fidler 1973; Liotta et al 1976; Updyke and Nicolson 1986). This phenomenon is generally accepted to be the result of physical factors - emboli lodging more readily than single cells in the confined space of small capillaries/lymphatics.

An intriguing possibility is the possible role of lectin in cell clumping. Tumour cells released into circulation could be cross-linked by soluble lectin in the same manner as erythrocytes are agglutinated by lectins in blood typing. This idea is illustrated in Figure 85:

![Diagram](image)

**FIGURE 85:** Cancer cells express carbohydrate moieties which can become cross-linked by soluble, circulating lectin.
Lectins expressed at the surface of circulating tumour cells may, alternatively, be crosslinked in a similar manner by free carbohydrate molecules.

Clumps of cells would be more liable to arrest in small vessels than single cells, thus primary tumours expressing the Helix-binding material would be characterised by enhanced metastatic potential.

4.6. HOW CAN THIS HELP THE PATIENT?

Helix pomatia lectin binding to formalin-fixed, paraffin-embedded sections of primary breast cancer appears to provide an accurate indicator of long term patient prognosis. Although this in itself is an interesting finding, we must still ask the question "How can this actually help the patient with breast cancer?"

4.6.1. AS A PROGNOSTIC INDICATOR

Many groups (for example, see Fuster et al 1983; Blamey et al 1983; Stoll 1986; Haybittle et al 1982) have attempted to formulate an individualised patient "prognostic index", attributing various weightings on the basis of complex multivariate analysis to a selection of principle prognostic features, with a view to tailoring the selection of optimal therapy to any individual patient. Standing alone, Helix pomatia lectin binding provides valuable information regarding likely clinical disease outcome; it could also form a valuable addition to such a prognostic index.

Helix lectin binding appears to be very closely associated with axillary lymph node involvement, hitherto the most powerful prognostic indicator available in breast cancer. This close association may prove particularly valuable in assessing the most appropriate treatment for patients whose lymph node status is unknown. With current interest in mammographic screening of asymptomatic women for early disease, and the increasing popularity of conservative surgery where practicable, small tumours of unknown nodal status are becoming more common, and pose considerable problems in choice of appropriate therapy. Helix lectin binding may provide a useful tool in the staging of disease in these women, and an aid in treatment decisions.

The value of accurately predicting breast cancer patient prognosis is, sadly, of little benefit as long as the most appropriate treatment for any given patient group remains largely unknown. However, one obvious - and for the women concerned, very valuable - application of Helix pomatia lectin binding - is the selection of patients likely to enjoy a
favourable prognosis, as they can be offered considerable reassurance and be spared aggressive, debilitating and hopefully unnecessary adjuvant treatment. For those with Helix-positive tumours, close follow-up, and aggressive therapy may be more appropriate.

4.6.2. POSSIBILITIES FOR INTERVENTION

4.6.2.1. Overview

Our results indicate that a 55kDa molecule, expressed by the normal breast is shared by a majority of (poor prognosis) primary breast cancers, plus around 80% of their metastases, but not by other normal tissues. This intriguing idea of a breast/breast cancer-related substance, expressed by aggressive primary tumours and most of their metastases, although still to be substantiated in our continuing work, is indeed provocative.

A fuller characterisation of the 55kDa Helix binding molecule will, hopefully, help us to understand what role it may be playing in breast cancer disease progression, and may thus suggest ways in which this knowledge could be usefully adapted to diagnosis and treatment strategies.

4.6.2.2. Limitations of Helix pomatia lectin

The Helix pomatia lectin itself may be of only limited value in a potential diagnosis/treatment context owing to its rather broad, basic carbohydrate-binding specificity: it binds the monosaccharides GalNAc and GlcNAc (Hammarstrom 1973), and will thus recognise, with varying degrees of avidity, a very wide range of glycosylated molecules bearing these basic residues. This broad selectivity is seen in tissue sections where in breast and other organs staining of normal, benign and malignant components is seen; and in Western lectin blots where countless Helix-binding bands are observed in a range of tissues. Such broad binding specificity limits the lectin's potential as a receptor for the 55kDa breast/breast cancer marker molecule, but can be overcome, at least in theory, by production of a specific monoclonal antibody raised against pure antigen.

With this aim in mind, our priorities in this continuing project include:

(1) Further purification of the 55kDa material
(2) The production of a specific monoclonal antibody raised against it
4.6.3 POTENTIAL APPLICATIONS OF A MONOCLONAL ANTIBODY

A monoclonal antibody specific for a marker of breast cancer metastasis would have several obvious applications in diagnosis and treatment, including -

(a) serum assay for diagnosis / monitoring of treatment response
(b) imaging
(c) specific cytotoxic therapy

these possibilities are discussed briefly below.

4.6.3.1 Serum assay for diagnosis / monitoring of treatment response

The secretion of human chorionic gonadotrophin (HCG) by choriocarcinoma provides, perhaps, the finest example of a useful tumour marker. The sensitivity of the test for serum HCG is such that a tumour burden of as little as $10^4 - 10^5$ malignant trophoblastic cells can be detected and serum levels of HCG closely mirror tumour burden and provide a convenient and accurate means of monitoring treatment. Such a test represents a very valuable tool in the staging and management of this disease (Bagshawe 1973).

Other examples of the use of tumour markers in cancer management include the use of HCG measurements in the management of testicular tumours; alphafetoprotein (AFP) in management of hepatomas and testicular tumours (Abelev 1971) and the use of calcitonin and other peptide hormones in the management of neuroendocrine tumours (Clarke et al 1969; Rees 1975).

Biochemical tumour markers have been of most use, as in the examples given above, in cancers arising from specialised tissues whose main functions and products are well characterised. Breast cancer has posed a much more difficult problem, as it arises from a such a heterogeneous organ consisting of stromal, ductal, alveolar and myoepithelial components. Normal breast and some breast cancers - in common with other organs of the reproductive system and their neoplasms - express hormone receptors and possess steroid hormone metabolising activity; some breast cancers synthesise normal milk proteins; and others produce a wide range of substances including CEA, ferritin and enzymes, as do other malignant tissues and even normal breast (Coombes 1981). The search for a specific and useful biochemical tumour marker in breast cancer has thus proved very difficult.

Although further confirmation is required, the 55kDa. molecule identified by this study
appears to be specific to breast, breast cancers, and their metastases. It may thus have great potential in this context. We have shown in this study that it is not detectable in the serum of healthy individuals, but we have yet to screen the blood of breast cancer patients (this work is underway). We must also screen other normal tissues, and other cancers.

If such an assay were to be developed, it would have great application in the rationalisation of breast cancer treatment. At present, for example, patients are selected for adjuvant chemotherapy on the basis of histological evidence of axillary lymph node involvement (Coombes 1981) - however, lymph node involvement does not give an accurate indication of risk of early recurrence, in that 35% of node positive patients will remain disease free for >5 years, and 18% of node negative patients will relapse in this time (Fisher 1977). An accurate marker of early metastatic disease, sensitive enough to detect tumour deposits earlier than conventional imaging techniques (bone scan, liver scan, ultrasound, x-ray, clinical symptoms) would hopefully allow systemic therapy to be administered at an early enough stage for it to be maximally effective, while sparing healthy patients the ordeal of undergoing unnecessary and harmful regimes.

Such a marker would also be of assistance in early clinical assessment of response to treatment - unnecessary and potentially harmful treatment could be suspended if no fall in marker level were observed - and drug dose could be adjusted in response to fluctuations in marker level.

4.6.3.2. Imaging

The coupling of a specific monoclonal antibody to a radio-nucleotide has enormous, as yet largely unrealised potential, in diagnosis and localisation of occult disease.

In breast cancer, one of the principal values of such imaging might be the non-invasive assessment of lymph node involvement (not simply axillary nodes, but - potentially - also supraclavicular nodes, interpectoral nodes, and those of the internal mammary chain) - of particular value in cases treated by limited surgery without axillary lymph node sampling - and, of course, early detection of distant metastatic disease.

Experimentally, it has been demonstrated that labelled monoclonal antibodies raised against tumour markers can specifically localise human cancer xenografts in animal model systems, and some have already progressed to the clinical trial stage (see Primus et al 1984; Larson 1985; Baldwin and Byers 1985 for review). Mach et al (1981), for example, successfully localised colorectal cancer sites in patients scanned with $^{131}$I-anti CEA antibody - one of the first reports of this kind - and Siccardi et al (1986) reported the results of a multicentre trial in which radio-labelled F(ab')$_2$ fragments of the 225.28S anti-melanoma antibody were
employed in radioimmunodetection assay of 254 patients.

In spite of intensive research in this field, many technical problems are still to be satisfactorily resolved including (1) high blood background (2) non-specific uptake in normal tissues (3) low absolute levels of labelled monoclonal antibody in the tumour itself (4) the fact that not all antigen positive tumours can be imaged by a given antibody directed against that antigen (Wright and Cox 1987). In addition much controversy exists regarding the best radio-isotope to use, and whether intact antibodies or active fragments are preferable (McGuire et al 1985). However, such problems are unlikely to be insurmountable, and generally there seems to be great optimism regarding the future of this type of approach (McGuire et al 1985).

In breast cancer, as in other malignancies, the potential of such an approach is mostly limited by the availability of useful monoclonal antibodies directed against specific cancer-associated antigens. As a marker substance, the 55kDa. Helix-binding molecule would carry the advantage that it does not appear to be expressed by normal tissues or normal serum; but the disadvantages that it seems to be present in normal breast and its secretions - perhaps problematic for the imaging of primary tumour- and that it is only expressed in around 80% of breast cancer metastases. This latter problem, could be at least partially overcome by the eventual inclusion of any potential monoclonal into a panel of such antibodies to maximise the likelihood of successful imaging (McGuire et al 1985).

4.6.3.3. Specific cytotoxic therapy

The logical next step from the use of specific monoclonal antibodies in imaging, is their use in selectively targetting toxins to kill cancer cells.

Cytotoxic monoclonal antibodies

Some monoclonals have the intrinsic ability to kill target cells expressing their antigen in vivo, either directly or by activation of host effector mechanisms. Regression of metastatic malignant melanoma lesions were, for example, observed in 4/21 patients treated with a complement-fixing IgG3 monoclonal antibody (Houghton 1986), due, it is surmised, to its activity in the mechanism of antibody-dependant cell-mediated cytotoxicity, and its interaction with a subpopulation of lymphocytes (Hersey et al 1986).

Most immunotherapeutic strategies, however, rely upon conjugation of specific monoclonal antibody to cytotoxic drugs, poisonous lectins or radionucleotides.
Toxin-conjugated monoclonal antibodies

Monoclonal antibodies conjugated to the toxic lectin ricin have been intensively investigated, and have been shown to be active both in vitro and in animal models. One overwhelming problem with this approach is, however, that the intact ricin molecule is intensely toxic to normal as well as malignant cells, and the subunit A-chain when conjugated to monoclonal antibody is unstable and is not readily delivered to target cells (Wright and Cox 1987).

Cytotoxic drug conjugates

Many monoclonal antibody-cytotoxic drug conjugates have been prepared and analysed both in vitro and in animal model systems. For example, the drug vindesine conjugated to an anti-osteogenic monoclonal antibody, an antimelanoma monoclonal, and two anti-CEA monoclonals (Rowland et al 1985), as well as methotrexate-linked anti-prostatic acid phosphatase (Deguchi et al 1986) have each been demonstrated to retard / suppress growth of the appropriate tumour xenografts in nude mice.

Problems with drug-monoclonal antibody conjugates include (1) very high levels of antigen binding are required to produce an effect (2) non-specific uptake by normal tissues (3) surface binding, but lack of drug internalisation (4) antigenic heterogeneity resulting in sub-total tumour cell killing and leading to emergence of drug resistant populations (Wright and Cox 1987). These problems are not insurmountable, however - one recent and very exciting advance is the recent work of Bagshawe et al (1989) on the development of a "prodrug", where antibody targets an enzyme-activated toxic alkylating agent actually at the site of the cancer.

Radionucleotide conjugates

Monoclonal antibody-radionucleotide conjugates appear to have the greatest potential in immunotherapy at present - so much so, in fact, that small clinical trials are already underway. Objective response have been seen in melanoma (Carrasquillo et al 1984), lung, ovarian and breast cancer patients (Pectasides et al 1986) treated with $^{131}$I-conjugated monoclonals.

The great advantage of this approach is that radionucleotides produce toxicity over several cell diameters, which effectively negates the need for internalisation, and even for direct binding. In addition, radioimmunodetection facilitates ready tracing of monoclonal antibody localisation, and monitoring of treatment efficacy (Wright and Cox 1987; McGuire et al 1985).
4.7. FURTHER WORK

4.7.1 PROSPECTIVE STUDY

One obvious criticism of this study is that it is based on a retrospective series of patients. We are at present beginning to analyse data collected over the past three years as part of a blind prospective study.

Sections of all primary breast cancers excised at University College and The Middlesex Hospitals since January 1987 have been stained for binding of Helix pomatia lectin. Follow-up on these patients is now complete. Although the maximum follow-up period is only three years, a "computer prediction" based on the results presented in this thesis suggests that we should be beginning to see statistically significant difference in prognosis between "stainers" and "non-stainers".

Collection of tissues and staining of sections will continue indefinitely as part of the ongoing project.

4.7.2. COLLABORATIVE WORK WITH OTHER GROUPS

As a result of this project, many groups have shown interest in the potential significance of Helix pomatia lectin binding. We are thus collaborating with a number of other centres.

Principal studies include those in association with:

(1) Dr. G. Jundt, Department of Pathology, University of Giessen, West Germany. We are seeking a possible association between expression of oestrogen / progesterone receptors and binding of Helix pomatia lectin.

(2) Dr. P. Horny, Department of Pathology, University of Tubingen, West Germany. Collaborative project to assess the prognostic significance of Helix pomatia lectin binding on their series of breast cancers.

(3) Dr. U. Schumacher, Department of Anatomy, University of Munich, West Germany. Isolation / characterisation of Helix pomatia lectin binding material.

(4) Dr. W. Natrath, Department of Morbid Anatomy and Histopathology, University of Munich, West Germany. Collaborative project to assess the prognostic significance of Helix pomatia lectin binding on their series of breast cancers.
4.7.3. PURIFICATION OF HELIX POMATIA LECTIN BINDING MATERIAL

The main thrust of our project at present is further isolation/purification of Helix pomatia lectin binding material, and in particular, of the 55kDa glycoprotein.

We are approaching this with a combination of lectin affinity chromatography (Helix pomatia and other lectins), gel filtration chromatography, ion exchange chromatography and chromatofocussing.

"Pure" material can then be further characterised, in order that we may further understand its role in the process of breast cancer metastasis, and will be used as an immunogen for the production of a specific monoclonal antibody.

4.7.4. MONOCLONAL ANTIBODIES

A specific monoclonal antibody raised against the 55kDa glycoprotein (or against other Helix pomatia lectin binding components) could have a number of potentially valuable applications, as discussed in section 4.6.3. Briefly, these include:

(1) As a prognostic marker (more specific than the lectin itself, therefore perhaps more accurate).
(2) In the screening of sera - early diagnosis, monitoring of treatment success, early detection of metastatic disease.
(3) Imaging.
(4) Specific cytotoxic therapy (as a "magic bullet").
5. APPENDICES

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APPENDIX I
ENDOGENOUS LECTINS OF HIGHER VERTEBRATES

A.11. SOLUBLE LECTINS OF HIGHER VERTEBRATES
A.11.1. The chicken lactose lectins (CLLI and CLLII)

A soluble lectin, called "chicken lactose lectin I" (CLLI) has been identified in extracts of chicken embryonic muscle (Den and Malinzak 1977; Nowak et al 1977), embryonic and adult liver (Kobiler et al 1978; Gremo et al 1978), retina, spinal cord (Eisenbarth et al 1978; Kobiler et al 1978), pancreas and intestine (Beyer et al 1979). The molecular weight of CLLI is reported as around 15kDa. (Den and Malinzak 1977).

A second chicken lactose binding lectin, CLLII, has subsequently been described in a number of other chicken tissues, notably embryonic kidney, skin, and adult intestine. It is monomeric, of molecular weight 14kDa. (Beyer and Barondes 1982a; Oda and Kasai 1983).

A lactose-specific lectin, similar to CLLI has been described in rat lung (Powell 1980; Cerra et al 1984; 1985; Whitney et al 1985). Whitney (1985) has suggested that there may actually be two rat lactose lectins, both having a sub-unit molecular weight of around 14.5kDa. - the first lectin having an isoelectric point of 5.2; the second, rarer, pulmonary macrophage lectin having a PI of >9.0. The lectin has also been described in human muscle (Childs and Feizi 1979; Franklin et al 1980); heart (Childs and Feizi 1979); lung (Powell 1980); brain (Bladier et al 1989); and in the tissues of newborn mice (Ohara and Yamagata 1986).

In human placenta, a monomeric lectin has been described, subunit molecular weight 14kDa. and showing sequence homology with CLLII (Hirabayashi et al 1987).

A.1.1.2. Chicken heparin lectin

A third, and rather fascinating chicken lectin has been described which appears to bind exclusively to aged or damaged erythrocytes (Mir-Lechaire and Barondes 1978; Kobiler and Barondes 1979; Ceri et al 1981). Since this agglutinin is readily inhibited by heparin, it has been named "chicken-heparin-lectin". It also binds dermatan sulphate and heparan sulphate, but no other glycosaminoglycans (Kobiler and Barondes 1979; Ceri et al 1981). Interestingly, rather similar lectins have been purified from rat lung (Roberson et al 1981) and human muscle (Franklin et al 1980). The apparent molecular weight of the lectin sub-units is 13kDa. and 16kDa.
A.1.1.3 Rabbit erythroid developmental lectin (lactose-binding)

A monomeric lactose-binding lectin has been isolated from the bone marrow of rabbits (Harrison and Chesterton 1980; Godsave et al 1981; Harrison et al 1984). Its apparent molecular weight is approximately 12-13kDa.; it has been named "erythroid developmental agglutinin".

A.1.1.4 Soluble lectins of bovine origin

DeWaard et al (1976) purified a soluble β-galactoside binding lectin by the affinity chromatography of calf heart and lung extracts. Subsequent studies suggest that the lectin is dimeric with subunit molecular weights of 13kDa. (Briles et al 1979; Montelione et al 1981) or 16,500 (Levi and Teichberg 1981).

Carding et al (1984) has confirmed that similar lectins in man and monkey are antigenically related to the bovine lectin.

A lectin has also been described in bovine serum by Loveless et al (1989). It has at least 12 subunits of molecular weight 48kDa., and binds N-Acetyl-glucosamine, mannose, and fucose.

A.1.1.5 β-galactose binding soluble lectins from rat and mouse

Three β-galactose binding lectins have recently been isolated from young rat lung. The predominant lectin, molecular weight ~14.5kDa. has been designated RL-14.5; two others of molecular weights ~18kDa. and 29kDa. are known as RL-18 and RL-29 (Cerra et al 1985). Possibly related to these rat lectins are the three β-galactose binding mouse lectins of molecular weights 13.5kDa.; 16kDa.; and 35kDa. described in Triton-X extracts of cultured mouse-derive fibroblasts (Roff and Wang 1983; Roff et al 1983; Critteden et al 1984).

A.1.1.6 Human serum galactose binding lectin

Recently Hamazaki (1986) has described an octameric lectin in human serum, subunit molecular weight 29.5kDa. which binds penultimate galactose.

A.1.1.7 Rat cerebellar soluble lectins CSLI and CSLII

Two soluble lectins have been described in rat brain. The first, called "cerebellar soluble
lectin I" (CSLI) binds mannose, has a sub-unit molecular weight 33kDa., and forms aggregates of up to 40 subunits which localise to brain ependyma (Zanetta et al 1987; Perraud et al 1988). This lectin is closely related to a second, "cerebellar soluble lectin II" (CSLII), which also binds mannose and has a subunit molecular weight of 31.5kDa. The two appear to share a common precursor (Zanetta et al 1987).

A.1.2. Proposed functions of soluble lectins of higher vertebrates

It is most interesting that the actual quantity of mammalian soluble lectin detectable within a given tissue varies markedly throughout the development of the animal. This phenomenon is most striking in the case of CLLI which becomes prominent, for example, at different stages in embryonic chick muscle development (Nowak et al 1976; Den et al 1976); and levels of CLLII (Beyer and Barondes 1982a) and chicken heparin lectin (Mir-Lechaire and Barondes 1978) also change with developmental stage.

The rat lectin RL-14.5 appears with development in a myogenic cell line (Nowak et al 1976; Gartner and Podleski 1976) and in embryonic rat lung (Powell and Whitney 1980). It is most striking that RL-18 and RL-29, which are readily detectable in embryonic liver, completely disappear in young adult life (Cerra et al 1985).

Those lectins which are detectable in embryonic tissues but disappear in the adult may perhaps be assumed to play some organisational function during embryogenesis. Lectins detectable in the adult tissues may, on the other hand, play some regulatory role in the mature organism. Interestingly, some mammalian lectins, notably CLLI and CLLII are abundant in different organs in the embryo compared to the adult (Barondes 1986) suggesting a facility for recruitment to different physiological roles at different stages in development.

The addition of rabbit erythroid developmental agglutinin to erythroblasts results in strong agglutination; however, mature erythrocytes and an erythroid tumour cell line are hardly affected at all (Godsave et al 1981). This suggests changes with differentiation or transformation of erythroblasts which alter or abolish an interaction with the purified lectin. Godsave et al suggest that the lectin in vivo may function to aggregate erythroblast cells.

Immunohistochemical experiments have localised CLLI in the extracellular matrix of muscle and pancreas suggesting, perhaps, a role in the organisation of extracellular materials; maybe cross-linking them via β-galactoside residues. The rat lectin RL-14.5 has also been localised in the extracellular matrix. CLLII is localised in secretory granules of the goblet cells in intestinal mucosa and its mucins.
The ligands which naturally bind these lectins have proved difficult to identify. Beyer and Barondes (1980) have localised an as yet unidentified endogenous CLLI-binding ligand by histochemical techniques, and the same authors (Beyer and Barondes 1982b) have suggested that CLLII may interact with intestinal mucins and secretions. Powell and Whitney (1984) have identified several glycoproteins in immature rat lung which bind the rat lectins RL-14.5 and RL-29 and localised in a specific sub-set of ganglion neurons which also express lactoseries glycolipids; the biological significance of these observations remains to be determined.

A.1.3. INSOLUBLE MEMBRANE-BOUND LECTINS OF HIGHER VERTEBRATES

A.1.3.1 Mammalian hepatic lectin
(asialoglycoproteins/galactose/and N-Acetyl-galactosamine-binding)

The first membrane-associated mammalian lectin to be described, and by far the most intensively studied, is the mammalian hepatic lectin.

A hepatic lectin was first isolated from rabbit liver by Hudgin et al (1974); it comprised two glycoprotein sub-units of molecular weight ~40kDa. and 48kDa. which are believed to associate to form a complex total molecular weight 260kDa. (Kawasaki and Ashwell 1976).

A similar lectin has been isolated from the liver of the rat. There appears to be some disagreement in the literature as to the molecular weight of this lectin, but it seems to consist of a major component of molecular weight 40kDa. or 43kDa. plus either two (Tanabe et al 1979) or three (Schwarz et al 1981b) larger sub-units of possible molecular weight 55kDa.; 65kDa. and 100-120kDa., although it has been suggested that the 100-120kDa. molecular weight species may simply be an artefact of dimerisation produced by the harsh conditions used in sodium-dodecyl-sulphate polyacrilamide gel electrophoresis.

Drickmar et al (1984) have determined the amino acid sequence of the predominant polypeptide of rat liver lectin. It has a molecular weight of 41,500 and consists of 283 amino acid residues.

It is interesting that a lectin has been described in human liver by Baenziger and Maynard (1980). This lectin appears to consist of a single sub-unit of molecular weight 41kDa. which associates to form a complex of very high molecular weight. Its amino acid and carbohydrate composition are very similar to that seen in rabbit (Kawasaki and Ashwell 1976).
This group of liver lectins bind asialoglycoproteins with terminal galactose and, perhaps with slightly greater avidity, N-Acetyl-galactosamine residues (Sarker et al 1979; Baenziger and Maynard 1980).

The most likely function of this lectin is a mediating role in the uptake of asialoglycoproteins with terminal galactose residues by the mammalian liver. Stockert et al (1980) perfused rat liver with antibody directed against the lectin prior to injection of radiolabelled orosomucoid. The antibody effectively blocked uptake of orosomucoid by more than 80%.

The lectin is expressed by the hepatocytes of the liver (Steer and Clarenburg 1979; Hubbard et al 1979; Wall and Hubbard 1981; Geuze et al 1982). It seems that it may cycle between the cell surface and the intracellular compartments; delivering around 1000 molecules of ligand to the lysosome to be degraded before it is itself destroyed (Warren and Doyle 1981). The lectin has been located on the luminal membrane surface of the Golgi apparatus and on the cytosolic surface of the lysosome (Tanabe et al 1979) or endocytic vesicle (Debanne et al 1982). It appears, however, that the endocytosed ligand may not always be degraded; but may sometimes be released (Tolleshaug et al 1980; Connolly et al 1982) or modified and released (Regoeczi et al 1982).

It is possible that the lectin may play a number of quite different roles in the organism. Several authors have suggested, for example, that the liver lectin is so abundant on the cell surface that it may play some important role in cell-cell interaction (eg Baenziger and Maynard 1980).

A.1.3.2. Hepatic mannose / N-Acetyl-glucosamine binding lectin

A considerable amount of work has been aimed at the purification and characterisation of a mannose/N-Acetyl glucosamine binding lectin from rat liver. This work was originally stimulated by studies on the uptake of glycosides by rat liver; clearance of these molecules was inhibited by agalacto-orosomucoid (which terminates in N-Acetyl galactose) (Stahl et al 1976) and yeast mannan (Achord et al 1977). The site of action was subsequently demonstrated to be on the sinusoidal cells, rather than hepatocytes (Achord et al 1978; Schlesinger et al 1978) and an analagous system of binding and uptake has been demonstrated on the reticuloendothelial cells of other tissues (Schlessinger et al 1980; Sun-Sang et al 1983).

A mannose and N-Acetyl glucosamine binding protein of molecular weight 31kDa. has been isolated from detergent/acetone extracts of rabbit liver (Kawasaki et al 1978;
Townsend and Stahl (1981). Both the uptake system of intact liver and the isolated liver lectin appear to be calcium dependent.

Maynard and Baenziger (1982) isolated a mannose and N-Acetyl glucosamine binding lectin from rat liver; it gave an apparent molecular weight of 24kDa. in S.D.S.- P.A.G.E., but migrated exactly with, and may be identical to, Townsend and Stahl’s (1981) rabbit liver lectin. They concluded, however, that it could not be the same molecule responsible for mannose uptake in the reticuloendothelial system - principally because it was localised in hepatocytes rather than endothelial cells. An antibody raised against the lectin failed to inhibit uptake of carbohydrate by reticuloendothelial cells of the liver (Maynard and Baenziger 1982; Townsend and Stahl 1981). Maynard and Baenziger's (1982) rat liver lectin is probably not a true membrane-bound lectin, as it was initially extracted quite readily by a 0.2M NaCl solution (although the addition of detergent was necessary for stability and satisfactory yield later in the purification process).

Ezekowitz et al (1988) have isolated a mannose binding protein from human liver which shares some sequence homology with the rat mannose-binding protein.

A.1.3.3. Chicken N-Acetyl glucosamine lectin

Chicken liver appears to lack the asialoglycoprotein receptor of the rabbit system (Regoezi et al 1975) but does contain an N-Acetyl glucosamine receptor (Lunney and Ashwell 1976) as does, to a lesser extent, chicken kidney and other organs (Kawasaki and Ashwell 1977).

The chicken liver N-Acetyl glucosamine receptor has been studied in some depth. It is composed of sub-units of molecular weight 26kDa. which aggregate in eights (Kawasaki and Ashwell 1977). Drickmar (1981) has determined the entire amino acid sequence of the molecule: it comprises 207 amino acid units with a proposed membrane interaction region between residues 25-48. It is glycosylated at residue 67. This chicken liver lectin shows some (28%) homology with the rat liver asialoglycoprotein receptor (Drickmar 1984).

A.1.3.4. Rodent liver fucose receptor

Prieels et al (1978) very neatly demonstrated the uptake of serum-borne fucose- containing glycoproteins by hepatocytes of the murine liver. Subsequent studies have shown that the purified receptor has a fairly broad range of sugar specificities, binding in addition to D-fucose; L-fucose, N-Acetyl glucosamine, galactose, mannose, and mannose-6-phosphate (Lehrman and Hill 1983).

A fucose-binding protein is also expressed by rat liver Kupffer cells (Lehrman and Hill
1986; Hoyle and Hill 1988). It shows some sequence homology with other lectins and has a subunit molecular weight of 77kDa and 88kDa.

A.1.3.5. Mammalian phosphomannosyl receptor

A number of studies have suggested the presence of a mannose-6-phosphate receptor in human cultured fibroblasts (see Neufeld and Ashwell 1980; Sly et al 1981 for review). Fischer et al (1980) has demonstrated phosphomannyl-residue binding activity in rat liver. The receptor appears to be 90% intracellular, 10% cell surface, the intracellular component facing the interior of the endoplasmic reticulum, Golgi, and lysosomes. Its presumed function is transport of mannose-6-phosphate bearing glycoproteins to the lysosome. A mannose-6-phosphate receptor has been purified from cow liver cell membranes by Sahagian et al (1981) - in S.D.S.-P.A.G.E.. It has an apparent sub-unit molecular weight of 215kDa. - and similar receptors have been demonstrated in human skin fibroblasts, Chinese hamster ovary cells, and rat hepatocytes.

A.1.3.6. Thyroid N-acetylglucosamine binding lectin

A membrane-bound N-acetylglucosamine-specific receptor, subunit molecular weight 45kDa, has been described in thyroid of pig (Miquelis et al 1987).
APPENDIX II
EXAMPLE OF A FOLLOW-UP FORM

The following three pages contain an example of the form used in this study to record clinical and follow-up data regarding our breast cancer patients (section 2.1).
**CLINICAL HISTORY**

6. Family History Breast Disease  
   0 = NIL  1 = Benign  2 = Malignant  
   Details ________________________________  

7. Number of Pregnancies ____________________  

8. Age at First Pregnancy ____________________  

9. Were the children breast fed?  
   0 = No  1 = Yes  2 = partly (details)  

10. Sex of each child (in order) ________________________________  

11. Time since last pregnancy to symptoms/lump first noticed ________________  

12. Age at Menarche ________________________  

13. Menstrual periods  
   1 = regular  2 = irregular  0 = not known (details)  

14. Age at Menopause _______________________  

15. Menopausal status at operation:  
   0 = not known  1 = Pre-  2 = Peri-  3 = Post-  4 = art.  

16. History of Contraceptive (or similar hormone) pills;  
   0 = Nil  1 = Yes  
   Details: when, type, dose ________________________________  

17.  

18.  

19.  

**PERI OPERATION**

20. Time from appearance of Lump/Symptoms until operation ________________________  

21. Age at operation ______________________  

22. Date of operation ______________________  

23. Stage of menstrual cycle at operation (take start of menstrual flow as Day 1) ______________________  

24. Site of PRIMARY ______________________  

25. SIZE of PRIMARY  
   Clinical estimate ____________________________  
   Measured cut surface ________________________  

26. Type of Operation:  
   0 = Nil  1 = biopsy only  2 = lump excision  
   3 = simple mastectomy  4 = Radical mastectomy  
   Details of operation type ____________________________
27. Clinical Stage at op. T = ___ N = ___ M = ___
28. Pathological Stage at op. T = ___ N = ___ M = ___
29. Stage 1 2a 2b 3a 3b 4
30. Disease Spread at operation: 1 = breast only 2 = local LN 4 = loco-regional
8 = Bone 16 = Bone marrow 32 = Lungs 64 = Liver 128 = CNS 256 = Peritoneum
512 = other (details________)
31. BLOOD GROUP 1 = A 2 = B 3 = AB 4 = O 5 = other
32. Blood Group Secretor status: 1 = Secretor 2 = Non-Secretor
33. PATHOLOGY
34. Intravascular carcinoma______
35. Lobular carcinoma-in-situ ______
INVASIVE
36. Invasive Ductal Ca.______
37. Invasive Ductal Ca. with predominant intraductal component____
38. Invasive Lobular Ca.____
39. Mucinous Ca.______
40. Medullary Ca.______
41. Papillary Ca.______
42. Tubular Ca.____
43. Adenoid cystic Ca.______
44. Secretory (juvenile) Ca.____
45. Apocrine Ca.____
46. Ca with metaplasia (specify squamous, spindle, cart, oss, mixed)____
47. other Ca.____
48. Tubule formation_____ Nuclear regularity_______ Mitotic rate________
49. GRADE I (1) II (2) III (3)
50. Cellular reaction intensity: 0 1 = slight 2 = intense
51. Type of cellular reaction:, lympho / plasma / macrophage / other
52. Cell Type Markers
53. Vascular invasion 0 1 = slight 2 = conspicuous
54. Lymphatic invasion 0 1 = slight 2 = conspicuous
55. Neural invasion 0 1 = slight 2 = conspicuous
56. NECROSIS 0 1 = slight 2 = conspicuous
57. Circumscription 1 = poorly 2 = well
58. AVERAGE DIFFERENTIATION : 1 = well 2 = mod. well 3 = poorly 4 = very poorly
59. Least Differentiated percentage of total_______%
60. Axillary Nodes; number involved/total = / . ( %). (including apical)
61. Apical node involved = 1 not involved = 2.
62. LN micrometastases = 1. macrometastases = 2.
### Clinical Disease State

**Code:**
- 0 = a.w., no recurrence
- 1 = local recurrence
- 2 = glandular
- 4 = regional
- 8 = distant
- 16 = visceral
- 32 = lost to follow-up
- 64 = died

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### Clinical Response

**Code:**
- 1 = Advancing
- 2 = ISQ in status quo
- 3 = Regression

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### Site of First Recurrence

**Code:**
- 1 = local skin/chest wall
- 2 = axillary LN
- 3 = cervical LN
- 4 = Bone
- 5 = Bone marrow
- 6 = Liver
- 7 = Lung
- 8 = Brain/CNS
- 9 = visceral
- 10 = ascites
- 11 = pleural
- 12 = other

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### Sites of Recurrence

**Code:**
- 1 = local skin/chest wall
- 2 = axillary LN
- 4 = cervical LN
- 8 = Bone
- 16 = Bone marrow
- 32 = Liver
- 64 = Lung
- 128 = Brain/CNS
- 256 = visceral
- 512 = ascites
- 1024 = pleural eff.
- 2048 = other

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### Evidence of Recurrence

**Code:**
- 1 = Symptomatic
- 2 = Clinical exam
- 4 = CXR
- 8 = Skeletal X-ray
- 16 = Bone scan
- 32 = Liver scan
- 64 = biopsy
- 128 = bone marrow
- 256 = serum markers

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### Active Treatment

**Code:**
- 1 = Radiotherapy
- 2 = Chemotherapy
- 3 = Hormones
- 4 = other (specify)

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Normal saline
A 0.9% solution of NaCl (BDH) in distilled water.

Formol Saline
A 4% solution of formaldehyde in normal saline
(1 part of 40% formaldehyde solution plus 9 parts normal saline)

Eosin
A 1% solution of eosin (BDH) in tap water.

Harris's Haematoxylin
1g haematoxylin (Raymond A.Lamb)
10ml absolute alcohol
20g aluminium potassium sulphate (potash alum)
200ml distilled water
0.5g mercuric oxide

1) Haematoxylin is dissolved in the alcohol.
2) Potash alum is dissolved in the water, with gentle heating.
3) The two solutions are mixed and brought rapidly to the boil.
4) Removed from heat, and mercuric oxide added.
5) Solution reheated until it becomes deep purple in colour; then immediately removed from heat and plunged into a vessel of cold water.

Mayer's Haematoxylin
1g haematoxylin
0.2g sodium iodate
50g aluminium potassium sulphate (potash alum)
1 litre distilled water
50g chloral hydrate
1g citric acid

1) Haematoxylin, sodium iodate, and potash alum are dissolved in distilled water, overnight, at room temperature.
2) Chloral hydrate and citric acid are added, and the mixture boiled for 5 minutes.

Acid Alcohol
1% hydrochloric acid in 95% alcohol
Tris Buffered Saline (TBS)

60.57g Tris (Sigma)
85g NaCl (BDH Analar)
dissolved in 10 litres distilled water and adjusted to pH 7.6
with concentrated HCl (BDH).

Acetate Buffer, pH 4.5

0.1M sodium acetate (Sigma) 100ml
0.1M glacial acetic acid (BDH) 100ml

0.025M Tris-HCl Buffer, pH 7.5
(for H.P.L.C.)

0.025M Tris base (Reagent grade - Sigma)
in purified water.

pH to 7.5 with concentrated HCl (BDH.)
APPENDIX IV

PRODUCTION OF A POLYCLONAL RABBIT ANTISERUM AGAINST HELIX POMATIA LECTIN

A polyclonal rabbit antiserum was raised against the Helix pomatia lectin. This antiserum was used in staining of tissue sections for Helix pomatia lectin binding (section 2.3 and Appendix V)

A.IV.1. Immunisation schedule

Animal: New Zealand white rabbit; 3 months old.
Immunogen: Helix pomatia lectin (Sigma; Pharmacia).

The immunisation schedule for production of a polyclonal rabbit antiserum against Helix pomatia lectin is detailed in Table 27.

TABLE 27: Immunisation schedule:

<table>
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<th>Event</th>
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<tr>
<td>day 1:</td>
<td>primary immunisation</td>
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<tr>
<td>14-21 days</td>
<td>second immunisation</td>
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<tr>
<td>14-21 days</td>
<td>first bleed; third immunisation</td>
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<tr>
<td>21-28 days</td>
<td>second bleed; fourth immunisation</td>
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bleeding/immunisation then repeated at intervals of 21-28 days for as long as necessary.

A.IV.2. Detoxification of the lectin

Some lectins are intensely toxic; for others, including Helix pomatia lectin, data regarding possible toxic effects is not available. For this reason, prior to immunisation into an animal, potential toxicity of the lectin may need to be destroyed by fixation in formol saline.

1mg lectin incubated overnight, at room temperature, in 1ml formol saline (see appendix III for formula); the mixture then dialysed for several hours or overnight against normal saline (appendix III) to remove formaldehyde.
A.IV.3. Preparation of the immunogen

The detoxified lectin emulsified in Freund's complete adjuvant (Sigma; Difco) in a ratio of 2 parts lectin: 3 parts adjuvant.

A.IV.4. Primary immunisation

Subcutaneous injection into multiple sites along the animal's back. 100-200μl injected at 10-20 separate sites.

A.IV.5. Preparation the immunogen for secondary immunisation

Detoxified lectin emulsified with Freund's incomplete adjuvant (Sigma; Difco) in a ratio of 2 parts lectin: 3 parts adjuvant.

A.IV.6. Second immunisation

14-21 days following primary immunisation.

Subcutaneous injection into multiple sites along the animal's back. 100-200μl injected at 10-20 separate sites.

A.IV.7. First bleed (from marginal vein of ear)

2-3 weeks after second immunisation.

1) Hair plucked from marginal vein of ear.
Area wiped with xylene (BDH), an irritant to encourage dilation of vein.

2) After 5 minutes, the marginal vein was flicked, again to encourage dilation of vein.

3) A butterfly scalp vein infusion needle with attached tubing (from hospital pharmacy), size 19 or 21 depending on size of animal, inserted into vein against the direction of blood flow.

4) A 10 ml syringe attached to the end of the tubing and blood slowly drawn out.

5) A total of 20-50 ml blood taken, transferred into clean glass tubes.
A.IV.8. Subsequent bleeds

At intervals of 2-3 weeks, the animal was bled again, and following each bleed additional immunisations of de-toxified lectin in incomplete adjuvant was administered.

A.IV.9. Preparation of sera

The blood was allowed to stand at room temperature for several hours, or overnight, to allow clotting and full clot retraction. The serum was then drawn off, centrifuged, and 0.01% sodium azide (Sigma) added as an anti-bacterial agent. Serum stored at 4°C.
APPENDIX V

DETERMINATION OF OPTIMAL METHODOLOGY FOR HISTOCHEMICAL STAINING TO DEMONSTRATE HELIX POMATIA LECTIN BINDING.

AV.1. INTRODUCTION

A number of histochemical techniques exist which may be adapted to effectively demonstrate the binding of Helix pomatia lectin to sections of formalin-fixed, paraffin embedded material. Each method varies in the number of steps involved and in the ultimate level of sensitivity of the test. Each method carries its own advantages and disadvantages. The methodology for each technique was first adjusted to give optimal staining results - maximum clean specific staining coupled with minimum non-specific background staining.

Once the methodologies had been optimised for use with Helix pomatia lectin staining on formalin-fixed, paraffin-embedded sections of breast cancer, the techniques were then compared in order to determine which would demonstrate the greatest difference in staining intensity between breast carcinomas associated with a particularly good or poor patient prognosis.

AV.2. CHOICE OF PATIENTS

40 patients diagnosed as suffering from primary carcinoma of the breast were chosen from the Histopathology reports of the Middlesex Hospital of January 1967 - June 1972.

The patients were selected on the basis that they fell into one of two broad groups - those who were shown on follow-up to have a "good prognosis", and those who had a "bad prognosis". The groups were arbitarily defined as follows:

"good prognosis": patient alive and well with no sign of metastatic disease more than 10 years after primary diagnosis.

"bad prognosis": patient dead as a result of widespread metastatic disease less than 1 year after primary diagnosis.

We have assumed, in subsequent investigations that the histochemical staining method which most efficiently distinguishes carcinomas associated with such extremes of biological behaviour will also be optimally effective in distinguishing differences between tumours of less extreme natural history.
AV.3. TISSUES

Blocks of formalin-fixed paraffin-embedded tissue taken from the original surgical specimen were retrieved from the archives of the Middlesex Hospital for each case.

AV.4. HISTOLOGY

100 x 5 μm serial sections were cut by microtome from the tissue blocks. The first and last section from each block was stained with haematoxylin and eosin, by the method described in section 2.4.3.1.

Sections were examined using a microscope to confirm the presence of malignant cells.

AV.5. HELIX POMATIA LECTIN BINDING

Duplicate sections were then stained to demonstrate *Helix pomatia* lectin binding by a range of histochemical methods.

AV.5.1. Principles

Lectin staining of routinely processed, formalin-fixed, paraffin-embedded human tissues can be extremely successful, as many of the carbohydrates or glycoproteins which lectins bind appear to remain largely unaltered, although glycolipids may be lost during processing.

Sections of our 20 "good-" and "bad-prognosis" breast cancers were stained with variations of a number of immunohistochemical techniques:

AV.5.1.1. The "direct" method and enzyme "labels"

The simplest, a "direct" method, involves incubating tissue sections with lectin labelled by a substance which can be visualised under the microscope. This method is illustrated in Figure 86, overleaf.
1) Direct Method - Labelled lectin

Commonly used fluorescent labels include fluorescein, which fluoresces green/yellow, or rhodamine, which fluoresces red, and which can be detected by examining the section under ultraviolet light. These labels can give stunningly beautiful results, but carry the disadvantage that the preparation is somewhat ephemeral and must be photographed immediately if a permanent record of results is desired. Enzyme labels offer an attractive alternative. Perhaps the most commonly employed enzyme label is horseradish peroxidase - it splits hydrogen peroxide, and the released oxygen produces a permanent, granular, deep brown oxidation deposit with 3-3'-diaminobenzidine (after Graham and Karnovsky 1966). An alternative enzyme label is alkaline phosphatase which gives a bright red or deep blue reaction product by a simultaneous azo-coupling reaction (after Burstone 1962).

The same labels used in the direct method can be utilised in a number of more complex "sandwich" techniques for visualising lectin binding to tissue sections. These techniques each carry their own advantages and disadvantages, but are generally more sensitive than the simple "direct" approach.

AV.2.1.2. Labelled secondary antibody technique

The section is incubated with native lectin, then with an antibody directed against it, and finally with a labelled secondary antibody raised against the species in which the primary antibody was raised. This method is illustrated in Figure 87.

FIGURE 86

1) Direct Method - Labelled lectin

Commonly used fluorescent labels include fluorescein, which fluoresces green/yellow, or rhodamine, which fluoresces red, and which can be detected by examining the section under ultraviolet light. These labels can give stunningly beautiful results, but carry the disadvantage that the preparation is somewhat ephemeral and must be photographed immediately if a permanent record of results is desired. Enzyme labels offer an attractive alternative. Perhaps the most commonly employed enzyme label is horseradish peroxidase - it splits hydrogen peroxide, and the released oxygen produces a permanent, granular, deep brown oxidation deposit with 3-3'-diaminobenzidine (after Graham and Karnovsky 1966). An alternative enzyme label is alkaline phosphatase which gives a bright red or deep blue reaction product by a simultaneous azo-coupling reaction (after Burstone 1962).

The same labels used in the direct method can be utilised in a number of more complex "sandwich" techniques for visualising lectin binding to tissue sections. These techniques each carry their own advantages and disadvantages, but are generally more sensitive than the simple "direct" approach.

AV.2.1.2. Labelled secondary antibody technique

The section is incubated with native lectin, then with an antibody directed against it, and finally with a labelled secondary antibody raised against the species in which the primary antibody was raised. This method is illustrated in Figure 87.

FIGURE 87

2) Indirect Method - Labelled second antibody
AV.2.1.3. PAP or APAP method

In the peroxidase-anti-peroxidase (PAP) or alkaline phosphatase-anti-alkaline phosphatase (APAP) method, the binding of the lectin is detected by layering of an unlabelled antibody directed against it, followed by an unlabelled secondary antibody in excess as a link, and, finally, a peroxidase-anti-peroxidase (PAP) or alkaline phosphatase-anti-alkaline phosphatase (APAP) complex formed from antisera raised in the same species as the original primary antisera. This method is highly sensitive owing to the large aggregates of chromogen ultimately developed. This method is illustrated in Figure 88.

FIGURE 88

(3) Peroxidase-anti-peroxidase (PAP) method

rabbit anti-peroxidase

peroxidase

rabbit anti-peroxidase

swine anti-rabbit

rabbit anti-lectin

lectin

carbohydrate

AV.2.1.4. Direct Avidin-Biotin technique

Other histochemical methods utilise the remarkable affinity of avidin (a glycoprotein found in the white of eggs) which carries four binding sites for biotin (one of the group of B vitamins). Avidin-biotin techniques are usually regarded as carrying the advantage of great sensitivity and specificity. The simplest avidin-biotin technique is where the lectin is coupled to biotin; its binding to tissue sections can then be revealed by incubation with avidin linked to an enzyme label, as illustrated in Figure 89, overleaf.
Biotinylation of Helix pomatia lectin:

Helix pomatia lectin was linked to biotin by the method described by Goding (1983). By using the N-hydroxysuccinimide (NHS) ester of biotin, protein reaction with the ester (involving nucleophilic attack of the unprotonated epsilon-amino group of lysine) displaces N-hydroxysuccinimide, linking the biotin. This reaction is illustrated in Figure 90.
1) To prevent condensation of water vapour, the NHS-biotin, stored frozen, is allowed to thaw to room temperature in its sealed bottle (the half-life of the succinimide ester of biotin is in the order of minutes from the point of hydrolysis, so water must be excluded for as long as possible).

2) NHS-biotin dissolved in dimethyl-sulphoxide (Sigma) at a concentration of 1mg/ml.

3) Lectin dissolved in 0.1M NaHCO₃ solution at a concentration of 1mg/ml.

4) NHS-biotin and lectin mixed in a ratio of 1ml lectin solution : 120µl biotin solution, at room temperature for 2 hours.

5) Dialysed overnight against Tris/HCl buffer, pH7.6, and stored at 4°C.

AV.2.1.5. Avidin-biotin sandwich technique

In a slightly more complex and sensitive avidin-biotin technique, the lectin is layered with, firstly, an unlabelled antibody directed against it, then a biotinylated secondary antibody, followed by labelled avidin. This method is illustrated in Figure 91.

FIGURE 91

(5) Multi-step avidin-biotin sandwich technique
AV.2.1.6. The "ABC" (avidin-biotin-complex) method

Avidin-biotin interaction is employed in the ABC technique where the tissue section is incubated with lectin, then sandwiched with a polyclonal antibody directed against it; a biotin-conjugated secondary antibody; and finally a labelled pre-formed complex of avidin and biotin mixed in specific proportions so that each avidin molecule has only three of the possible four biotin-binding sites saturated, leaving an extra site free to couple with the biotin label on the secondary antibody, as illustrated in Figure 92.

FIGURE 92

(6) ABC technique

AV.2.2. OPTIMISATION OF CONCENTRATIONS

Variants of all of these methods were tested, using horseradish peroxidase as a label. In each case the optimal concentrations of the principal reagents used in each step were first determined by performing a series of trials wherein combinations were tested against each other according to a simple "chequerboard" plan.

As a simple example, in the "direct" method, in order to determine the most effective concentration of peroxidase labelled lectin, a range of concentrations between 0.01µg/ml and 100µg/ml were tested; sections were incubated with the reagent at 0.01µg/ml; 0.1µg/ml; 1µg/ml; 10µg/ml; 100µg/ml and the results compared for maximum specific
staining of cancer cells in positive cases coupled with minimum non-specific background.

In the more complex, multi-layer procedures, "chequerboards" of dilutions of different reagents were tested against each other. For example, in the simple avidin-biotin method, dilutions of biotinylated lectin ranging from 0.01\mu g/ml - 100\mu g/ml and of avidin-peroxidase from 0.01 - 100\mu g/ml were tested in combination according to the chequerboard plan illustrated in Table 28:

<table>
<thead>
<tr>
<th>Concentration of lectin (\mu g/ml)</th>
<th>Concentration of avidin peroxidase (\mu g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.01, 0.1, 1, 10, 100</td>
</tr>
</tbody>
</table>

When slides were examined through the microscope, every combination was assessed for maximum intensity of specific staining combined with minimum non-specific background.

Once optimum conditions had been determined for each method, the efficacy of the various techniques for discrimination of "good-" vs. "bad-prognosis" tumours was assessed.

AV.2.2.1. METHODS TESTED

For all methods, sections were first:

(a) De-waxed in xylene, re-hydrated through graded alcohols (100\% ; 95\% ; 60\%) and brought to water.

(b) Endogenous peroxidases blocked by incubation in a 3\% solution of hydrogen peroxide in methanol for 20 minutes, then washed for 5 minutes in tap water.

(c) Trypsinised for 20 minutes [concentration 1mg trypsin : 1mg calcium chloride : 1ml
All dilutions and washes between stages were performed in Tris buffered saline (TBS), pH 7.6. The binding of many lectins requires the presence of heavy metal ions, and for this reason salts such as magnesium chloride and calcium chloride may be added to the basic Tris buffered saline recipe to give a "lectin buffer"; however, for Helix pomatia lectin binding, the addition of such salts appears to be unnecessary.

Then:

**AV.2.2.1.1. Direct - labelled lectin**

(d) Sections incubated with horseradish peroxidase labelled Helix pomatia lectin (Sigma) at concentrations ranging from 0.01µg/ml - 100µg/ml in TBS for 60 minutes.

(e) Sections incubated with diaminobenzidine (DAB) 0.1 mg/ml and hydrogen peroxide (30 vols) 0.6µl/ml in TBS for 10 minutes.

**AV.2.2.1.2. Indirect - labelled secondary antibody**

(d) Sections incubated with unlabelled Helix pomatia lectin at concentrations ranging from 0.01µg/ml-100µg/ml for 60 minutes.

(e) Sections incubated with polyclonal antisera raised in rabbit against Helix pomatia lectin at dilutions ranging from 1/50 to 1/500 for 60 minutes.

(f) Sections incubated with peroxidase-conjugated antisera raised in swine against rabbit immunoglobulins at dilutions between 1/100-1/1000 for 60 minutes.

(g) Sections incubated with diaminobenzidine (DAB) at 0.1 mg/ml and hydrogen peroxide (30 vols) 0.6µl/ml in TBS for 10 minutes.

**AV.1.2.1.3. P.A.P. - peroxidase-anti-peroxidase**

(d) Sections incubated with unlabelled Helix pomatia lectin at concentrations ranging between 0.01µg/ml and 100µg/ml for 60 minutes.

(e) Sections incubated with polyclonal antisera raised in rabbit against Helix pomatia lectin at dilutions ranging from 1/50-1/500 for 60 minutes.
(f) Sections incubated with antisera raised in swine against rabbit IgG (Dako) at a dilutions between 1/10-1/100 for 60 minutes.

(g) Sections incubated with rabbit peroxidase-antiperoxidase (PAP) complex (Dako) at a dilutions between 1/50 and 1/500 for 60 minutes.

(i) Sections incubated with diaminobenzidine (DAB) at 0.1 mg/ml and hydrogen peroxide (30 vols) 0.6μl/ml in TBS for 10 minutes.

AV.2.2.1.4 Direct Avidin-Biotin

(d) Sections incubated with biotin-labelled *Helix pomatia* lectin at concentrations of 0.01μg/ml- 100μg/ml for 60 minutes.

(e) Sections incubated with avidin-conjugated peroxidase at concentrations of 0.01μg/ml-100μg/ml for 30 minutes.

(h) Sections incubated with diaminobenzidine (DAB) 0.1 mg/ml and hydrogen peroxide (30 vols) 0.6μl/ml in TBS for 10 minutes.

AV.2.2.1.5 Avidin-Biotin Sandwich Technique

d) Sections incubated with unlabelled *Helix pomatia* lectin at concentrations of 0.01μg/ml- 100μg/ml for 60 minutes.

(e) Sections incubated with polyclonal antisera raised in rabbit against *Helix pomatia* lectin at dilutions of 1/50-1/500 for 60 minutes.

(f) Sections incubated with biotin-conjugated antisera raised in swine against rabbit immunoglobulins at dilutions of 1/100-1/1000 for 60 minutes.

(g) Sections incubated with avidin-conjugated peroxidase at concentrations of 1μg/ml-100μg/ml for 30 minutes.

(h) Sections incubated with diaminobenzidine (DAB) 0.1 mg/ml and hydrogen peroxide (30 vols) 0.6μl/ml in TBS for 10 minutes.
AV.2.2.1.6. ABC Method

(d) Sections incubated with unlabelled *Helix pomatia* lectin at concentrations of 1µg/ml-100µg/ml for 60 minutes.

(e) Sections incubated with polyclonal antisera raised in rabbit against *Helix pomatia* lectin at dilutions of 1/50-1/500 for 60 minutes.

(f) Sections incubated with biotin-conjugated antisera raised in swine against rabbit immunoglobulins at dilutions of 1/100-1/1000 for 60 minutes.

(g) Sections incubated with ABC complex (Dako), prepared according to the manufacturers instructions, for 1 hour.

For all methods, sections were then washed thoroughly in running tap water to remove all traces of DAB, counterstained in Mayer’s haematoxylin, dehydrated through graded alcohols (60%; 95%; 100%), cleared in xylene and mounted in Depex resinous mountant.

AV.2.3. ASSESSMENT

AV.2.3.1. Optimisation of staining method

Different combinations of dilutions of principle reagents were assessed visually for each method on the basis of strength of positive, specific lectin staining in combination with minimal non-specific background staining.

AV.2.3.2. Selection of "good-" vs "bad-prognosis" cancers

For each technique, slides stained under optimal conditions for each of the "good-" and "bad" prognosis cases were compared to see which method gave the most obvious difference in staining intensity/distribution between the two prognosis categories.

Staining was recorded simply as an estimate of the intensity of the staining reaction, scored as either negative, or as +, + to ++++ (for example, 80% +++; 100% +; 25% +-- etc).

Statistical evaluation of results was performed by means of a simple chi squared test.
**AV.3. RESULTS**

Generally, the simpler, more direct approaches were less sensitive, producing weaker specific staining coupled with higher levels of non-specific background staining than the more complex "sandwich" techniques.

**Positive staining** was gauged as being either (a) more than 5% of tumour cells staining strongly (intensity + to ++++), or (b) more than 50% of tumour cells staining weakly (intensity +-).

**Negative**, (a) less than 5% tumour cells strongly positive, or (b) less than 50% weakly positive

Table 29 lists the number of good prognosis and bad prognosis breast cancer cases staining positively for *Helix pomatia* lectin binding by the different methods tested.

<table>
<thead>
<tr>
<th>Method</th>
<th>Good Prognosis</th>
<th>Bad Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>direct</td>
<td>0/20</td>
<td>15/20</td>
</tr>
<tr>
<td>labelled secondary</td>
<td>0/20</td>
<td>17/20</td>
</tr>
<tr>
<td>PAP</td>
<td>2/20</td>
<td>20/20</td>
</tr>
<tr>
<td>avidin-biotin direct</td>
<td>0/20</td>
<td>18/20</td>
</tr>
<tr>
<td>avidin biotin sandwich</td>
<td>0/20</td>
<td>20/20</td>
</tr>
<tr>
<td>ABC</td>
<td>3/20</td>
<td>20/20</td>
</tr>
</tbody>
</table>

It is apparent from the table that, when applied under optimal conditions, the six histochemical staining techniques tested actually produce quite different results regarding the detection of *Helix pomatia* lectin binding to breast cancers - these differences reflect the varying levels of sensitivity afforded by the different methods screened.

The method that was adopted for this study was chosen on the basis that maximum discrimination between groups of breast cancers showing such extreme biological behaviours are likely to optimally discriminate between tumours of less extreme clinical course.
For the purposes of this study, the avidin-biotin sandwich technique provided the clearest discrimination between breast cancers of an aggressive and those of a relatively benign clinical course. All of our good prognosis cases were negative by this method; all the bad prognosis cases were positive. It was, therefore, this method that was ultimately adopted to stain the 373 cases included in this project.
APPENDIX VI.
CALCULATION OF EXPECTED SURVIVAL IN A NORMAL POPULATION

AVI.1. AIM

To calculate the expected survival of a hypothetical age-matched female control group, on the basis of overall mortality rates in the general population.

AVI.2. STARTERS

Age at diagnosis was recorded for 350/358 breast cancer patients in our study (see section 2.1.2.1.). Age groups were defined according to the divisions employed by the O.P.C.S. (Office of Population Censuses and Surveys), and the number of patients in each age group at diagnosis was recorded. The hypothetical age-matched control population has an identical composition. The number of individuals in each age group were identified as \(n_{1}\) (the number of starters in the 25-29 year age group) to \(n_{13}\) (the number of starters in the 85+ years age group).

AVI.3. CHOICE OF YEARS FOR CALCULATION OF Spx VALUES

Probability of survival of individuals of the hypothetical normal control cohort will be calculated at 5 year intervals (reflected in the Spx values, see section AV.4.). Our recruitment period was from 1967-1972. The method by which choice of years for calculation of Spx values was made is explained in Table 30, overleaf.
TABLE 30: Choice of years for calculation of $5p_x$ values

<table>
<thead>
<tr>
<th>Recruitment period</th>
<th>1967 - 1972</th>
</tr>
</thead>
<tbody>
<tr>
<td>67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85  86  87  88  89</td>
<td></td>
</tr>
<tr>
<td>^</td>
<td></td>
</tr>
<tr>
<td></td>
<td>^</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mid-year of recruitment period is 1969/1970

AVI.4. PROBABILITY OF SURVIVING ONE QUINQUENNIA: CALCULATION OF $5p_x$ VALUES

At each quinquennia, the death rate/1000 females is abstracted from O.P.C.S. tables for each age group to be considered (For 1987 figures, O.P.C.S. statistics are not yet available; values were taken instead from the Central Statistical Office Annual Abstract of Statistics 1988 in which 10 year rather than 5 year age groupings are quoted). The value $5p_x$ can then be calculated as follows:

**Example:**

For the 60-64 year age group in 1972, death rate = 12.63 / 1000

= 0.01263

Probability of surviving through 1 year = 1 - 0.01263

= 0.98384

Probability of surviving through 5 years = (0.98384)$^5$

= 0.9384
Alternatively, one might calculate $5px$ from the individual death rates for each of the years making up the quinquennia

$$5px = (1-0.1264)(1-0.01227)(1-0.01262)(1-0.01230)(1-0.01237)$$

$$= 0.9393$$

The two methods give almost identical results, but the former approach was used as it is slightly simpler to calculate.

AVI.5. $N5px$ VALUES: NUMBER OF INDIVIDUALS SURVIVING EACH QUINQUENNIUM

The number of individuals of each age group to survive the quinquennium is given by the product of the number of starters and the $5px$ values. ie: $N5px$. The sum of products for all age groups gives the total number of individuals surviving the quinquennium. The number of survivors of the first quinquennium is designated $N5'$. These individuals will be the starters for the beginning of the next quinquennium, their total now called $N5$, and so on.

AVI.5.1. Correction for South-East England

The method of correction for differing regional population structures employed in the O.P.C.S. publications is that of an indirect standardisation. Standardised Mortality Rates (S.M.R.'s) are calculated as a ratio of observed deaths in a region to those expected if the England and Wales total death rates had been in operation.

$$S.M.R. = \frac{\text{number of observed deaths}}{\text{number of expected deaths}}$$

From O.P.C.S. tables for the appropriate years, the Standardised Mortality Rates were recorded for females in South East England in comparison with the whole of England and Wales. The South East region encompasses London, South East and Southern regions. Unfortunately, S.M.R. values are not yet available for the year 1987. Probability of five, ten, and fifteen year survival for our population could then be calculated for the part of England from which our breast cancer patients had been recruited. (For 1987, uncorrected figures were used as the closest possible approximation).
From O.P.C.S. tables, in 1972, the S.M.R. for females in South East England was 0.95 for that of the whole country.

**Probability of Surviving Each Quinquennium:**
The probability of survival for the first quinquennium $P_{5'}$ can therefore be calculated as follows:

$$P_{5'} = \frac{(1-0.95)N_0 + 0.95N_5'}{N_0}$$

Values of $P_{10'}$ and $P_{15'}$ can be calculated for subsequent quinquennia in a similar manner.

$P_{20}$ is calculated without correction for the South East England region by the formula

$$P_{20} = \frac{N_{20'}}{N_{15}}$$

The probability of surviving 5 years $P_5$, 10 years $P_{10}$, 15 years $P_{15}$, and 20 years $P_{20}$ are:

- $P_5 = P_{5'}$
- $P_{10} = P_{5'} \times P_{10'}$
- $P_{15} = P_{5'} \times P_{10'} \times P_{15'}$
- $P_{20} = P_{5'} \times P_{10'} \times P_{15'} \times P_{20'}$
AVI.6. CALCULATIONS

AVI.6.1. Calculation of $n_{5px}$ values

First Quinquenium: 1972

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Starters</th>
<th>$5px$ (1972)</th>
<th>product $n_{5px}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-29</td>
<td>n1 1</td>
<td>0.9976</td>
<td>0.9976</td>
</tr>
<tr>
<td>30-34</td>
<td>n2 9</td>
<td>0.9966</td>
<td>8.9694</td>
</tr>
<tr>
<td>35-39</td>
<td>n3 13</td>
<td>0.9942</td>
<td>12.9246</td>
</tr>
<tr>
<td>40-44</td>
<td>n4 36</td>
<td>0.9899</td>
<td>35.6364</td>
</tr>
<tr>
<td>45-49</td>
<td>n5 45</td>
<td>0.9827</td>
<td>44.2215</td>
</tr>
<tr>
<td>50-54</td>
<td>n6 36</td>
<td>0.9733</td>
<td>35.0388</td>
</tr>
<tr>
<td>55-59</td>
<td>n7 71</td>
<td>0.9595</td>
<td>68.1245</td>
</tr>
<tr>
<td>60-64</td>
<td>n8 45</td>
<td>0.9384</td>
<td>42.228</td>
</tr>
<tr>
<td>65-69</td>
<td>n9 43</td>
<td>0.9016</td>
<td>38.7688</td>
</tr>
<tr>
<td>70-74</td>
<td>n10 27</td>
<td>0.8344</td>
<td>22.5288</td>
</tr>
<tr>
<td>75-79</td>
<td>n11 18</td>
<td>0.7281</td>
<td>13.1058</td>
</tr>
<tr>
<td>80-84</td>
<td>n12 6</td>
<td>0.5818</td>
<td>3.4908</td>
</tr>
<tr>
<td>85+</td>
<td>n13 0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Total = 350

$NO = 350$

$NS' = 326.035$
Second Quinquennium: 1977

<table>
<thead>
<tr>
<th>Age Group</th>
<th>5 year survivors</th>
<th>$5p_x$ (1977)</th>
<th>product $n5p_x$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-29</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30-34</td>
<td>$5n1$ 0.9976</td>
<td>0.9968</td>
<td>0.9944</td>
</tr>
<tr>
<td>35-39</td>
<td>$5n2$ 8.9694</td>
<td>0.9947</td>
<td>8.9219</td>
</tr>
<tr>
<td>40-44</td>
<td>$5n3$ 12.9246</td>
<td>0.99118</td>
<td>12.8106</td>
</tr>
<tr>
<td>45-49</td>
<td>$5n4$ 35.6364</td>
<td>0.9844</td>
<td>35.0805</td>
</tr>
<tr>
<td>50-54</td>
<td>$5n5$ 44.2215</td>
<td>0.9754</td>
<td>43.1337</td>
</tr>
<tr>
<td>55-59</td>
<td>$5n6$ 35.0388</td>
<td>0.9621</td>
<td>33.7108</td>
</tr>
<tr>
<td>60-64</td>
<td>$5n7$ 68.1245</td>
<td>0.9406</td>
<td>64.0779</td>
</tr>
<tr>
<td>65-69</td>
<td>$5n8$ 42.228</td>
<td>0.9074</td>
<td>38.3177</td>
</tr>
<tr>
<td>70-74</td>
<td>$5n9$ 38.7688</td>
<td>0.8494</td>
<td>32.9302</td>
</tr>
<tr>
<td>75-79</td>
<td>$5n10$ 22.5288</td>
<td>0.7541</td>
<td>16.9890</td>
</tr>
<tr>
<td>80-84</td>
<td>$5n11$ 13.1058</td>
<td>0.6002</td>
<td>7.8661</td>
</tr>
<tr>
<td>85+</td>
<td>$5n12$ 3.4908</td>
<td>0.3517</td>
<td>1.2277</td>
</tr>
</tbody>
</table>

$\text{total} = 326.035$  \hspace{1cm} $\text{total} = 296.0605$

$N5 = 326.035$  \hspace{1cm} $N10 = 296.0605$

Third Quinquennium: 1982

<table>
<thead>
<tr>
<th>Age Group</th>
<th>10 year survivors</th>
<th>$5p_x$ (1982)</th>
<th>product $n5p_x$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-29</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>30-34</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>35-39</td>
<td>$10n1$ 0.9944</td>
<td>0.9954</td>
<td>0.9397</td>
</tr>
<tr>
<td>40-44</td>
<td>$10n2$ 8.9219</td>
<td>0.9918</td>
<td>8.8487</td>
</tr>
<tr>
<td>45-49</td>
<td>$10n3$ 12.8106</td>
<td>0.9860</td>
<td>12.6313</td>
</tr>
</tbody>
</table>

$\text{total} = 296.0605$
<table>
<thead>
<tr>
<th>Age Group</th>
<th>10n4</th>
<th>50.0805</th>
<th>0.9766</th>
<th>total = 296.0605</th>
</tr>
</thead>
<tbody>
<tr>
<td>55-59</td>
<td>10n5</td>
<td>43.1337</td>
<td>0.9627</td>
<td>41.5248</td>
</tr>
<tr>
<td>60-64</td>
<td>10n6</td>
<td>38.3177</td>
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Fourth Quinqueval: 1987*

15 year survivors

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AVI.6.2. Correction for South-East England

First Quinquenium: 1972

S.M.R. (standardised mortality rate for S.E. England) = 0.95

\[ \rho_5' = N_5' \]
\[ = \frac{(1 - 0.95)N_0 + 0.95N_5'}{N_0} \]
\[ = \frac{(0.05 \times 350) + (0.95 \times 326.035)}{350} \]
\[ = 17.5 + 309.7333 \]
\[ \rho_5' = 0.9350 \]

Second Quinquenium: 1977

S.M.R. = 0.93

\[ \rho_{10'} = N_{10'} \]
\[ = \frac{N_{10'}}{N_5} \]
\[ \rho_{10'} = (1 - 0.93)N_5 + 0.93N_{10'} \]
\[ = \frac{(0.07 \times 326.035) + (0.93 \times 296.0605)}{326.035} \]
\[ = 22.8225 + 275.3363 \]
\[ \rho_{10'} = 0.9145 \]
Third Quinquennium: 1982

S.M.R. = 0.94

\[ P1S' = \frac{N1S'}{N10} \]

\[ P1S' = (1 - 0.94)N10 + 0.94N1S' \]

\[ = (0.06 \times 296.0605) + (0.94 \times 260.5515) \]

\[ = 17.7636 + 244.9184 \]

\[ = 262.682 \]

\[ P1S' = 0.8856 \]

Fourth Quinquennium: 1987

Standardised mortality rates for 1987 are not yet available; it is not possible at present to correct the 1987 figures for the South East England region.

\[ P20'(uncorrected) = \frac{N20'}{N1S} \]

\[ P20'(uncorrected) = 220.58 \]

\[ 260.5515 \]

\[ P20'(uncorrected) = 0.8466 \]
AVI.6.3. Probability of survival of the normal population

First Quinquenium: 1972

At 5 years, $P_5 = P_5'$

$P_5 = 0.9350$

Second Quinquenium: 1977

At 10 years, $P_{10} = P_5' \times P_{10}'$

$P_{10} = 0.9350 \times 0.9145$

$P_{10} = 0.8551$

Third Quinquenium: 1982

At 15 years, $P_{15} = P_5' \times P_{10}' \times P_{15}'$

$P_{15}' = 0.9350 \times 0.9145 \times 0.8856$

$P_{15} = 0.7572$

Fourth Quinquenium: 1987

At 20 years, $P_{20} = P_5' \times P_{10}' \times P_{15}' \times P_{20}'$

$P_{20} = 0.9350 \times 0.9145 \times 0.8856 \times 0.8466$

$P_{20} = 0.6410$
AVI7. RESULTS

The survival of a hypothetical, age-matched normal population, based upon the above calculations, is given in Figure 93.

All figures taken from:


My thanks to Dr. J. Haybittle for his invaluable help and advice in the preparation of these calculations.
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Telex: 96113
Fax: 0622 691425
PUBLICATIONS AND PRESENTATIONS

Below are listed a number of publications and presentations relating to this project:


LEATHEM A.J. and BROOKS S.A "Predictive value of lectin binding on breast cancer recurrence and survival". The Lancet. May 9, 1987. 1054-1056. *(Paper enclosed at the end of this section).*


LEATHEM A.J. BROOKS S.A. 1987 "Enzyme binding to detect carbohydrate expression in tissue sections. I Native and cross-linked glucose oxidase." Histochemical J. 19 405-412. *(Paper enclosed at the end of this section).*
BROOKS S.A. and LEATHEM A.J. Oral presentation at The Royal Marsden Hospital, Fulham Road. Breast cancer research meeting, Summer 1988. "Helix pomatia lectin binding as a prognostic indicator in breast cancer".


BROOKS S.A. Departmental research seminar, February 1989. "Carbohydrate receptors in human tissues - possible role in breast cancer metastasis?"


SCHUMACHER U.; KRETZSCHMAR H.; BROOKS S.A.; LEATHEM A.J. (in preparation) "Helix pomatia lectin binding pattern of brain metastases originating from primary breast cancers."
"Prognostic value of S-phase fraction and DNA index in breast cancer."
in other signs of fluid overload, at the time when diuresis and
natriuresis follow relief of the obstruction. Patients with high pressure chronic renal failure who constitute a homogeneous group of patients in whom the clinical features suggest circulatory overload. The peripheral oedema is unlikely to be due to either the pressure of the large bladder on the pelvic veins or venous thrombosis since signs of these are not seen in patients with other forms of chronic retention in which the bladder volume may be equally great but in which hydrenephrosis does not occur.

During obstruction the absolute amount of sodium excreted was within normal limits and urinary volume, as calculated from frequency-volume charts, did not differ from that of normal men of comparable age. The increase in fractional excretion of sodium was in keeping with the reduced glomerular filtration rate, and the relation between fractional excretion of sodium and glomerular filtration rate (as measured by creatinine clearance) lay within the expected range. It would thus appear that the obstructed kidney is partly able to adapt to a reduced filtered load to maintain sodium and water homeostasis. Under these circumstances the development of sodium and water retention remains to be explained.

One possibility is that the obstructed kidney—despite relative well-preserved renal function—is particularly susceptible to occasional extremes of dietary sodium intake exceeding its excretion capacity, leading in the long term to accumulation of salt and water. Thus patients ingesting a diet rich in sodium might be expected to have signs of extracellular fluid overload sooner than those with a low salt intake, and this would explain why some patients in this study presented with hypertension and oedema at an early stage of the disease process—mean pre-drainage creatinine clearance was far from critical at 32.5 ml/min. However, such theories cannot be proven without knowledge about the time course of HPCR and the precise mechanisms of tubular adaptation to a reduced filtered load.

After relief of obstruction absolute and fractional sodium excretion and urinary volume showed a significant increase, greatest at 24 h. Diuresis and natriuresis, which were essentially complete by 2 weeks, showed a striking correlation with improvements in cardiovascular state, providing compelling evidence that the presenting symptoms and signs were due to volume overload. Although recent excretion also increased after relief of obstruction, the electrolytes always constituted the major part of the osmolar load, which indicated that the fluid excretion was primarily a salt diuresis.

About 10% of men will eventually require prostatectomy and 5% of these will be found on investigation to have upper urinary tract obstruction. Patients with HPCR usually have a typical prostate symptom complex and will be missed by casual clinical enquiry. Hydrenephrosis and painless bladder distension were not suspected in 24% of the patients in this series, who were referred to medical clinics for control of cardiovascular symptoms that had failed to respond to first-line therapy. Attention to history and careful examination of the lower abdomen are important if what is almost certainly the most common form of surgically correctable hypertension is not to be missed.

This work was supported by the Manchester and North West Region Kidney Research Association.

Correspondence should be addressed to D. A. J.

References at foot of next column
Patients and Methods

Paraffin sections from blocks of 179 primary cancers, removed between 1967 and 1972 by mastectomy, were dewaxed and stained by an immunoperoxidase method. The sections were reacted with HPA (Pharmacia, Sigma, and isolated by ourselves: 10 µg/ml) at room temperature for 1 h; then added rabbit antiserum to HPA (Leda and our own, 1 in 200 for 1 h), biotinylated swine anti-rabbit immunoglobulin (Dako, 1 in 400 for 30 min, avidin-peroxidase [Sigma, 5 µg/ml for 30 min], and then hydrogen peroxide-diaminobenzidine.

Staining of cancer cells was recorded as: negative = no staining or very weak staining (±) of occasional cells or positive = definite, strong staining of most cancer cells (to ++ ++ ++). Details of binding to normal structures (epithelium, endothelium) and distribution on cancer cells will be published elsewhere.

The clinical progress of patients was followed by means of patients' notes, the Cancer Registry, and the Cancer Death Registry. For recurrence we needed to know the time between surgery (ie, histological confirmation of malignant disorder) and the onset of the first symptom of recurrence. If that symptom (for example, bone pain was later confirmed as disease spread [for example, by bone scan, biopsy, or at necropsy]), the time to that first symptom was taken as time to first recurrence. If there was no later confirmation, that symptom was disregarded. Times were recorded in months. These recurrences included local (chest wall and glandular), regional (including pleural effusion, skin elsewhere, and other breast), and distant (bone, liver, lung, and brain) recurrences. We also recorded time to death. Menopausal status was initially recorded from notes, but the information was lacking for many patients; we therefore assumed menopausal status from age (assuming premenopausal before 50 years and younger were assumed to be premenopausal, those over 50 years postmenopausal).

Log-rank analysis and life-tables were prepared by the Kaplan-Meier method, by means of a program written by David Bradlev, Queens University, Belfast, for the Department of Radiotherapy, Middlesex Hospital.

Results

HPA staining of cancer cells was seen as two main patterns—one or occasional cells staining (fig 1, A), moderate staining (B, intensity + ), or strong staining (C, intensity ++ ++ ).

Patients A had no axillary-node metastases or other recurrences, but survival was long. Patients B and C had axillary-node metastases and short survival.

Discussion

We have previously shown a strong relation between HPA binding to breast-cancer cells and local axillary lymph-node metastasis. Fenlon et al used a similar approach in 126 patients and found that HPA binding was associated with lymph-node stage (p = 0.01), locoregional recurrence (p = 0.01), and survival (p = 0.01). Our results suggest that, regardless of histopathological features, the metastatic potential of an individual breast cancer might be assessed at the time of operation, at least in premenopausal patients. In our premenopausal group, survival (cumulative percentage) at 5 years was 90% for patients without staining and 40% for those with staining; at 10 years the respective percentages were 75% and 35%.
HPA appears to recognise an as yet undefined biological marker associated with both high metastatic potential and aggressive tumour behaviour in young women. If we are detecting the same glycoconjugates in cancers of older patients, presumably the effects may be attenuated by the different hormonal status. The recurrence rate of non-staining tumours was similar in premenopausal (20% 5 year) and postmenopausal (20% 5 year, 30% 10 year) groups. In the older patients, however, positive-staining tumours was much lower recurrence rate (recurrence at half the premenopausal rate) and behaved less aggressively (5-year survival, premenopausal 40%, postmenopausal 60%). Although we have grouped together patients with different types of tumour and with metastases to different sites, HPA does seem to recognise a marker of aggressive behaviour in younger patients. It is not clear what influence menopausal status has on aggressive behaviour and survival, and clearly many factors are involved; however, if by aggression we mean short survival after recurrence, the postmenopausal tumours in this study showed less aggressive behaviour than premenopausal tumours.

HPA reacts with a terminal N-acetyl-galactosaminyl residue. An oligosaccharide with terminal galactose residues has been identified on the surface of a rat mammary carcinoma (13762 NF) associated with clones of high metastatic potential. The most accurate assessment of prognosis is by means of a prognostic index, whereby several features are taken into consideration—for example, lymph-node involvement, tumour size, and tumour grade. Because of the trend towards more conservative forms of breast surgery in many centres, one of the most important prognostic features, lymph-node status, remains unknown for most patients. HPA binding may provide a useful adjunct to a prognostic index, since even alone it appears valuable. The value of HPA lies partly in its ability to predict the prognosis independently of tumour grade and lymph-node status, and partly in its simple application to routinely fixed paraffin-embedded sections of primary breast cancer. It may be invaluable in staging of disease, facilitating stratification of patients into different treatment groups, and aiding decisions on early adjuvant therapy. The analysis of such carbohydrate markers might contribute to the development of new approaches to understanding biological mechanisms of behaviour and metastasis in breast cancer.

We thank the Cancer Research Campaign for funding to Susan Brooke, the Breast Cancer Research Trust for continued support, Teresa Young for advice and data analyses, Jane Fallows for preparing figures, and Prof N. Wood and Prof F. Lanceit for their encouragement.

Correspondence should be addressed to A. J. L.

REFERENCES


HELIX POMATIA LECTIN-BINDING AND PREDICTIVE VALUE IN BREAST-CANCER

Sir,—Dr Leatham and Susan Brooks (May 9, p.1055) report that binding of agglutinin from Helix pomatia (HPA) to sections of primary tumour was useful in the assessment of long-term prognosis of breast-cancer patients. However, the A, B, and O blood-types of the patients do not seem to have been considered. This is relevant because HPA displays blood-type A specificity, and human A and B antigens are not only erythrocyte but also non-erythrocyte tissue antigens. Individuals of blood-type A and AB are therefore expected to carry the A antigen on practically all tissue cell membranes, presumably including the sections studied by Leatham and Brooks. Thus it is essential to assess the potential confounding effects of the A antigen. Tumours on blood-type O and B patients might give information on the value of the lectin for the purpose stated.

An excess of blood-type A patients was detected in certain groups of breast-cancer patients in Texas and in a population displaying a rapidly progressing form of breast-cancer in Tunisia. Because the blood-types were not stated, it is unclear what extent these reports might have bearing on the data of Leatham and Brooks.

Department of Basic Sciences.
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Downers Grove, Illinois 60515, USA.

F. J. GRUNDBACHER


"This letter was shown to Dr Leatham and Susan Brooks, whose reply follows.—Eo L.

Sir,—We agree with Dr Grundbacher that, since a lectin from Helix pomatia (HPA) displays blood group A specificity, we should consider blood group antigen (BGA) expression in these breast cancer patients to see if we are simply demonstrating secreted blood group A. BGA are expressed in a wide variety of epithelial tissues. Although all the patients in our 20-year retrospective study had metastasized, only a minority had been cross-matched and blood-tested. None had been tested for secretor status. In 70 patients, there was no association between positive or negative HPA binding and ABO blood group. HPA-positive tumours stained regardless of blood group type (antitypy and proportion of cells staining) were not influenced by tumours, from A, AB, and B, and O patients showed positive staining of stromal stroma between the negative tumour cells. We do not know the significance of this. Although we have isolated A-type glycoproteins from these tumours, the specific carbohydrate sequences preferentially or specifically reactive with HPA are not yet known and may well occur in glycoproteins from cells, other than erythrocyte and epithelial, not recognized as carrying BGA. The binding of HPA appears to some complex oligosaccharides, such as GalNAcα2-3GalNAcβ1-3Galβ1-4GlCα1-4GlCα1-6A linked with BGA (GalNAcα1-3Galβ1-4GlCα1-6A GlCα1-3Galβ1-4GlCα1-6A). Hence we could expect binding to structures other than to accepted BGA.

Changes in BGA expression have been described in several epithelial cancers and may be of prognostic significance—for example, loss in superficial bladder tumours has been reported to precede invasion. However, studies of BGA expression often show reduced or absent BGA in paraffin sections, presumably because BGA associated with glycoproteins is extracted during processing with organic solvents. Reproducible BGA detection in tissue sections probably requires cryostat sections without any organic solvents. Even though we find no association between patient blood group and HPA binding to tumour cells and that binding may be too weak to structures other than BGA, we may still be detecting blood group related oligosaccharides in our sections. They are not reproducibly detected in paraffin sections suggesting they are linked to peptide rather than to lipid, and analysis of extracts supports this.

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A. LEATHAM

SUSAN BROOKS


OVERWHELMING MYOCARDITIS DUE TO CRYPTOCOCCUS NEOFORMANS IN AN AIDS PATIENT

Sir,—Cohen et al have discussed the problem of cardiac involvement in AIDS. However, life-threatening myocarditis due to fungal infections has been rarely reported in AIDS patients. A 20-year-old bisexual man was admitted to our intensive care unit in acute respiratory failure (dyspnea and cyanosis). He had had fever, cough, and diarrhea for the past five months. His serum contained antibodies against human immunodeficiency virus (HIV). Two months before admission cryptococcal antigens in serum were positive at a dilution of 1 in 8. On examination of the chest there were crackles in both lungs and a gallop rhythm. X-ray revealed pulmonary edema, electrocardiogram showed sinus tachycardia, and echocardiogram showed major atherosclerosis of the left ventricle without cardiac dilation. A flow-guided pulmonary artery catheter was inserted. The right atrial mean pressure was 15 mm Hg, the pulmonary arterial pressure 66/45 (mean 54) mm Hg and the pulmonary capillary wedge pressure 20 mm Hg. The cardiac index was 2.85 (normal) despite large doses of dopamine. These patients were consistent with acute myocarditis and pulmonary hypertension with right ventricular failure. The cryptococcal antigen in serum was positive at a dilution of 1 in 10 000. Two blood cultures and the tracheal aspirates yielded Cryptococcus neoformans. Treatment with amphotericin and flucytosine was started but the patient died on the fifth day. At necropsy C neoformans was found in the lungs, liver, spleen, pericardium, lymph-nodes, and thymus. Microscopic examination of myocardium revealed multiple inflammatory cell infiltrates containing numerous organisms. In addition there was a disseminated Mycobacterium avium intracellulare infection without cardiac involvement and few cysts of Pneumocystis carinii in the lungs.

In this case, clinical, echocardiographic, and hemodynamic findings were those of fulminant myocarditis in a patient with disseminated cryptococcal infection. Heart failure was the cause of
Enzyme binding to detect carbohydrate expression in tissue sections.

1. Native and cross-linked glucose oxidase

A. LEATHEM and SUSAN BROOKS

Histopathology Department, Joint Middlesex and University College Hospitals, Riding House St., London W1P 7PN, UK

Received 22 August 1986 and in revised form 3 February 1987

Summary

Enzymes may be useful as highly specific histochemical probes to identify and localize macromolecular substrates in tissue sections. We have used glucose oxidase, a double-headed enzyme, to demonstrate β-glucosyl groups in paraffin sections.

Native glucose oxidase has two active sites per molecule. Soluble polymers formed by glutaraldehyde combine many active binding sites on to one molecule. Some of these bind to glucose in tissue sections, leaving others free to react with chromogenic substrate. The intensity of staining is directly related to the concentration of enzyme, duration of incubation with enzyme, temperature and pH. Polymeric forms of enzyme are about 100 times more effective than native.

Glucose oxidase, particularly in a polymeric form, appears a simple reagent for the identification of glucose-containing structures. The use of native and polymerized enzymes as a histochemical probe has enormous potential in the analysis of normal tissues and in the detection of aberrant carbohydrate deposition in pathological tissues; this system serves as a useful model.

Introduction

Glucose oxidase offers several attractive features as an alternative label to horseradish peroxidase in immunohistochemistry. It is not present in animal tissues, it is highly active (and is thus a sensitive label) and it produces a stable finely grained coloured product.

Whilst producing conjugates of antibodies to glucose oxidase by glutaraldehyde cross-linkage for indirect immunohistochemical staining, we noticed that although no endogenous enzyme could be detected histochemically in animal tissue sections, conjugated antibodies bound to certain tissues even when primary antiserum was omitted. This suggested that either the antibody was binding (but this was excluded by other immunoperoxidase staining), or the glucose oxidase was binding. This raises the question as to why would the glucose oxidase bind. As two of the tissues which had stained, liver and striated muscle, are rich in glycogen (a polymer of glucose), it was thought that glucose oxidase might be binding to structures containing fixed glucose. We have investigated this further by incubation of tissue sections with glucose oxidase, detecting bound enzyme by a glucose oxidase, to demonstrate β-glucosyl groups in paraffin sections.

Nitro BT phenazine chromogen.

The binding of an enzyme to its substrate is a necessary step in enzyme kinetics. Such binding may be tight and exact to give the complex the necessary geometry, and may be irreversible if the substrate is in a form which can be recognized but not altered, such as pepstatin (Miyanor et al., 1972), a potent inhibitor of acid proteases, or deoxycoformycin (Woo et al., 1974), which binds to adenosine deaminase irreversibly and blocks its function.

Many enzymes have several substrate-binding sites (as in double-headed enzymes and multi-enzyme complexes), such as aspartate carbamoyltransferase which has at least four specific binding sites for substrate per molecule (Changeux et al., 1968; Weber, 1968). Such enzymes, in excess, may be able to bind their specific substrate and still display free binding sites (see Figs. 1 and 2).

With these two features in mind, we have sought binding of glucose oxidase to substrate sites in tissue sections and demonstrated such binding using free active sites to produce a histochemical reaction. To
increase the number of available binding sites, we have attempted to produce active soluble polymers of glucose oxidase by cross-linking with glutaraldehyde (Figs. 3 and 4).

**Materials and methods**

Glucose oxidase (from *Aspergillus niger*) was obtained from Sigma (150 units mg, type X). Solutions were made up freshly at 1000 μg, 100 μg, 10 μg and 1 μg/ml of buffer. Dilutions and washing were performed either in Tris-buffered saline (TBS: 0.05 M Tris, 0.15 M NaCl, pH 7.6) or, for determination of the effect of pH variation on binding, in 0.15 M citric acid-phosphate buffer of pH 4.0, 5.0, 6.0 or 7.0. Glutaraldehyde (25% solution) was obtained from Emscope.

**Formation of multienzyme complexes**

Polymeric forms of glucose oxidase were made by slowly adding 10 μl of 25% glutaraldehyde ml to 1 mg ml glucose oxidase solution over 60 min. and 5 min before diluting and applying to tissue sections. Unreacted glutaraldehyde was blocked after this time by addition of 10 μl of 1 M ethanolamine ml of reactants.

**Tissues and staining**

Human, rabbit, mouse and chicken tissues were fixed in 10% unbuffered formalin-saline and paraffin sections cut from composite blocks. Sections were dewaxed in xylene, brought to water and incubated with 10-fold dilutions of glucose oxidase in a humid box at room temperature for 1, 5, 25 and 60 min. After incubation, the sections were washed in three changes of buffer to remove unbound enzyme and then incubated in substrate for 30, 60, 120 or 180 min in the dark either at room temperature or at 37°C.

**Enzyme-disclosing reagent**

The method described by Sufin et al. (1979) was used: β-D-glucose, p-Nitro Blue tetrazolium chloride (NBT) and phenazine methosulphate (PMS) (obtained from Sigma) were freshly dissolved in 0.1 mM monosodium dihydrogen phosphate buffer, pH 6.9 or 5.6 to give a final solution containing 7.5 mg/ml glucose, 0.5 mg/ml NBT and 0.1 mg/ml PMS.

Incubation was performed in the dark (as PMS is light sensitive) at room temperature or at 37°C (for this the buffer was prewarmed).

Sections were post-incubation fixed in 10% formalin, washed in tap water, and then mounted in glycerine jelly or dehydrated, clarified and mounted in Eukit.

**Competitive blocking**

Glycogen (liver/muscle), glucose and methyl glucoside, were obtained from Sigma. Competitive inhibition was performed either by dilution of glucose oxidase in 0.5 mM glucose, 0.5 mM α- and β-methylglucoside or 10% glycogen or by washing sections in sugar solution, after incubation in glucose oxidase, to seek competitive displacement.
Glucose oxidase binding to tissue glucosyl groups

Negative controls
(a) The enzyme step was omitted and sections incubated in substrate.
(b) Sections of liver and muscle were pretreated with salivary amylase for 10 min to digest glycogen, washed, then incubated with glucose oxidase.

Positive controls
Enzyme solutions (native and polymerized) were incubated in substrate.

Results

Negative controls
(a) No endogenous enzyme activity was detected in any tissues.
(b) Salivary amylase treatment reduced but did not abolish binding of glucose oxidase.

Positive controls
All enzyme preparations in solution gave positive colour reactions. The colour ranged from pale-violet at lower concentrations, short incubations, neutral pH and at room temperature to dense blue-black at high concentrations, longer incubation, acid pH and 37°C.

Staining
Histochemical staining was seen in a variety of different tissues when sections were preincubated in glucose oxidase (see Table 1), but no staining was seen when the enzyme was omitted. The strongest staining was seen with adrenal cortex (see Figs. 5 and 6) and in muscle, cartilage (chondrocytes) (Figs. 7 and 8) and liver (Fig. 9). After treatment first with salivary amylase, there was a reduction in glucose oxidase binding but not complete abolition.

Nuclei of many tissues stained strongly positive (such as adrenal, Figs. 5 and 6, kidney and liver, Fig. 9), with prominent nucleoli, but nuclei of other tissues, such as the gastrointestinal system, were negative.

Kidney showed a strong diffuse staining of cytoplasm in most tubules, which persisted in renal adenocarcinomas.

The intensity of staining was directly related to:

1. Concentration of glucose oxidase used (strongest with 1000 μg/ml).
2. Duration of incubation in glucose oxidase (strongest at 60 min).
3. Temperature (greater at 37°C than at room temperature).

Table 1. Tissue staining with glucose oxidase.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney, normal (human, mouse, rabbit, chicken), tubule cytoplasm</td>
<td>+++</td>
</tr>
<tr>
<td>Kidney, carcinoma (human), cytoplasm</td>
<td>+++</td>
</tr>
<tr>
<td>Breast, normal (luminal surface and secretion only)</td>
<td>+</td>
</tr>
<tr>
<td>Breast, infiltrating cancer cells</td>
<td>++</td>
</tr>
<tr>
<td>Thymus, some Hassal’s corpuscles</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
</tr>
<tr>
<td>Prostate</td>
<td>-</td>
</tr>
<tr>
<td>Liver (human, mouse, rabbit) some cells –, many</td>
<td>+++</td>
</tr>
<tr>
<td>Muscle (striated, smooth, cardiac)</td>
<td>+++</td>
</tr>
<tr>
<td>Fat (human, mouse, rabbit), some cell groups</td>
<td>+++</td>
</tr>
<tr>
<td>Stomach, some basal epithelial cells</td>
<td>++</td>
</tr>
<tr>
<td>Small intestine, some basal epithelial cells</td>
<td>++</td>
</tr>
<tr>
<td>Thyroid</td>
<td>–</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>+++</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>–</td>
</tr>
</tbody>
</table>

Fig. 5. Human adrenal gland. × 100. Native glucose oxidase 100 μg/ml, 1 h, pH 6. Cytoplasm of zona reticularis cells showing weak binding to enzyme, absent binding to other cells. Note binding to nuclei.

Fig. 6. Human adrenal gland. × 100. Polymerized glucose oxidase 100 μg/ml, 1 h, pH 6. Stronger binding of glucose oxidase to zona reticularis cells, some binding to zona fasciculata, zona glomerulosa and to collagen in capsule. Absent binding to cells of medulla.

Fig. 7. Human tracheal cartilage. × 32. Polymerized glucose oxidase as in Fig. 6. Strong binding to many chondrocytes but not to others. Adjacent lung negative.

Fig. 8. Human tracheal cartilage. × 200. Polymerized glucose oxidase as Fig. 6. Intense staining of cytoplasm in some chondrocytes. Nucleoli of others positive. Matrix mostly negative.
Glucose oxidase binding to tissue glucosyl groups

Fig. 9. Human liver. × 320. Polymerized glucose oxidase as Fig. 6. Strong staining of nuclei and nucleoli in hepatocytes and Kupffer cells. Patchy granular cytoplasmic staining of parenchymal cells.

4. Duration of incubation in disclosing reagent (greatest at 180 min).
5. pH (increasing with acidity to a maximum at pH 4).
6. Native or polymerized enzyme (polymerized much stronger).

Each of these was cumulative, and thus the strongest staining was achieved by using polymerized enzyme at 1000 μg/ml for 60 min at 37°C and at pH 4 and then incubating in chromogenic substrate at pH 5.6 for 180 min at 37°C. The main variables are shown in Table 2.

Table 2. Effect of varying conditions on staining of muscle and adrenal cortex.

<table>
<thead>
<tr>
<th>Condition</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polymerized</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Duration of enzyme incubation (min)</td>
<td>1</td>
<td>5</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>Native</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polymerized</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Duration in substrate (min)</td>
<td>30</td>
<td>60</td>
<td>120</td>
<td>180</td>
</tr>
<tr>
<td>Native</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polymerized</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>pH of enzyme solution</td>
<td>7.6</td>
<td>7</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Native</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polymerized</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Distribution of enzyme binding
Positive tissues (Table 1) showed a diffuse and granular cytoplasmic distribution but some tissues also showed a conspicuous reaction with nuclei and nucleoli. This nuclear pattern was conspicuous and reproducible for different tissues.

Concentration of native and polymerized enzyme
The intensity of staining increased from very weak at 1 μg/ml to very strong at 1000 μg/ml. The latter was too strong, obscuring cell detail. Comparing the intensity of staining at each dilution, the polymer appears to give 100 times stronger staining than does native enzyme. See Table 2.

Polymers or glutaraldehyde-enzyme complexes
Preformation of the complexes 60 or 5 min before incubation on the sections did not make any appreciable difference, neither did using some complexes prepared 24 h previously and stored at 4°C, or stored for over a week at room temperature. Varying the concentration of glutaraldehyde (1 μl-100 μl, 2.5 or 25% per mg of enzyme) did not make a significant difference. No precipitate was detected on centrifugation.

Duration of incubation
Polymerized enzyme produced a similar pattern of staining to that of the native enzyme; however, the intensity of staining was much higher and was detectable at lower dilutions and shorter incubations. Incubations in chromogenic substrate were eventually terminated earlier than with native enzyme (e.g. within 10-30 min) as the blue-black staining was so intense and obscured details.

Effect of pH
Using a pH range of 4.0, 5.0, 6.0, 7.0 and 7.6, an increase in staining was observed with progressive acidity to a maximum at pH 4.0 (see Table 2). Varying the pH of the chromogenic substrate gave a peak at pH 5 to 6.

Effect of blocking excess glutaraldehyde
Blocking unreacted aldehyde groups with ethanolamine did not alter the binding of glucose oxidase, suggesting that no free aldehyde groups remained after complexing and that sufficient enzyme was present to saturate the glutaeraldehyde.

Sugar inhibition and displacement
Binding of enzyme to tissue sections was reduced by incorporation of the small sugar methyl glucoside and the polymer glycogen into diluting buffer. The inhibition was much greater with glycogen than with methyl glucoside. At high concentration of glucose oxidase (1000 μg/ml) and using long incubations, the inhibition by glycogen was slight, but low concentrations of enzyme (10 μg/ml) were completely blocked by glycogen. Glucose failed to inhibit binding of enzyme unless used at 1 in and incubation of the enzyme on the section was for only 1 min. Displacement of bound enzyme by soluble sugar was only achieved with glycogen.

Discussion
The colour reaction obtained on incubating sections with glucose oxidase, and its absence when glucose oxidase was omitted, indicates that there is binding of enzyme to particular tissues. If bound enzyme can still react with soluble chromogenic substrate, more than one active site might exist on each glucose oxidase molecule (Figs. 1 and 2), although the substrate-binding site and the active site of an enzyme may be separate. Possibly, also, an enzyme with one active site could bind with fixed substrate through that combining site but continue to show activity for exogenous substrate. The existence of more than one binding site on an enzyme molecule has been well-documented, some enzymes having multiple sites such as aspartate carbamoyltransferase which has at least four binding sites. The work of O'Malley & Weaver (1972), Tsuge et al. (1975) and of Jones et al. (1982) on the structure of glucose oxidase demonstrates the presence of two identical active subunits. These two subunits could provide a two-headed enzyme structure which might bind to tissues but still react with chromogenic substrate (see Figs. 1 and 2).

Polymers versus native
The dramatic increase in binding shown by glutaraldehyde-glucose oxidase complexes (see Figs. 3 and 4, 5 and 6) over native enzyme suggests that multi-enzyme complexes with many binding sites are being created. No attempt at separation of these complexes into sizes was attempted, but because there was no visible precipitate it was felt unnecessary. By comparing the staining intensities (Table 2), the polymeric form appears to give 100 times stronger staining at the same concentration.

Cross-linking of glucose oxidase did not appear to diminish the activity of the enzyme. Indeed glutaraldehyde has been used previously to produce cross-linking of glucose oxidase for structural studies (Solomon et al., 1977). The resulting glucose oxidase reportedly exhibits high stability, high enzymic activity (95% of native enzyme) and is water-soluble. Solomon et al. (1977), using glutaraldehyde on glucose oxidase from a different source from ours, have produced soluble mostly intramolecular cross-linked products. Although our product and that of Solomon et al. (1977) was soluble and stable,
from the microheterogeneity described by Havashi & Nakamura (1981) there may be differences according to sources. The complexes prepared by us appear usable after at least a week at room temperature.

**pH**

The effect of pH on binding appears to be related to the optimum pH for glucose oxidase activity. Bright & Appleby (1969) studied the pH-dependence of native glucose oxidase over a range of 3 to 8 and using glucose as substrate found a sigmoid pH profile for $k_1$, with an optimum at about pH 5.6. Solomon et al. (1977) determined the pH-activity profile of native glucose oxidase over a range of 2-10 and found that over a range of 3-9 there was considerable activity, with two maximum peaks at pH 5.6 and 6.2. Their cross-linked enzyme showed a similar profile with slightly diminished activity. However, this material showed high stability after 1 month at room temperature, suggesting that glutaraldehyde confers structural rigidity: The increased binding that we have found at a more acid pH than the published optimum pH might suggest the optimum pH for binding is different from that for enzymic activity, and that the optimum pH for binding requires investigation for each enzyme.

**Binding specificity and inhibition**

The specificity of binding to glucosyl groups in tissue sections was partly confirmed by the inhibition obtained through preincubation with glycogen and the part inhibition obtained with methylglucoside. The greater inhibition achieved with glycogen might result from tighter interaction between the polymeric glucose chains in glycogen. The weaker inhibition by the simple sugar methyl glucoside and very weak inhibition by glucose, particularly after prolonged preincubation, suggests that the simple sugars were being oxidized rapidly. The very high rates of oxidation of glucose by glucose oxidase render this substrate as unsuitable for blocking.

**Other types of binding**

The binding of an enzyme to its substrate for its detection at electron microscope level has been used to detect nucleic acids in cytochemistry (see Moyne, 1980; Bendayan, 1985). Bendayan (1985), using enzyme-gold complexes, has done extensive work on nucleic acid localization, both RNA and DNA, also collagen, elastin, glycogen and xylans in plants, and has shown how this can be done quantitatively.

The binding of an enzyme to tissues may be mediated through quite a different system, such as that described by Straus (1981), who demonstrated binding of horseradish peroxidase to lymphoid cells via mannose receptors present on the tissue sections. Peroxidase is rich in mannose and appears to bind to tissue receptors or lectins which recognize mannose. Glucose oxidase has a reported carbohydrate content of over 10%, most of which is mannose (about 100 residues per molecule), with some galactose and glucosamine (Hayashi & Nakamura, 1981). Thus tissue receptors or lectins binding to these sugars might bind glucose oxidase.

**Applications**

Carbohydrates are important structural and functional components of cells, where they confer individuality to cell groups, and of extracellular material. Aberrant glycosylation may play important roles particularly in infection and in the control of normal and new growth; thus detection of changes in carbohydrates may be helpful in identification of alterations of cell and tissue behaviour. Enzymes have evolved to interact with highly specific and generally small sequences of ligand. They could thus be very specific probes in tissue analysis. The immediate application of carbohydrate-reactive enzymes is perhaps in the investigation of carbohydrate-storage diseases but first it is necessary to evaluate enzyme-binding sites in normal tissues.

**Problems of enzymes as labels**

The binding of glucose oxidase, particularly after glutaraldehyde 'activation' as is used in antibody conjugates, poses the problem of binding of antibody-glucose oxidase conjugates to tissues not through the antibody but through the enzyme, to produce staining unrelated to the antibody specificity. We are investigating this as a cause of 'non-specific binding' of other antibody-enzyme conjugates.

The specificity of this approach for the identification of enzyme substrates is determined by the method used and the purity of the enzyme. If a histochemical chromogenic method is used, the purity of the enzyme preparation is less critical than if a mixture of labelled enzymes is used (where everything that binds is detected).

The simple procedure described here suggests that enzymes, particularly in a polymeric form, will provide an interesting tool for the analysis of carbohydrate composition of tissues.

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