THE PERIOPERATIVE ASSESSMENT
OF RECTAL NEOPLASIA

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ABSTRACT

Despite technical advances and introduction of adjuvant therapy, mortality from rectal neoplasia remains static. Many patients present with disseminated disease, beyond cure by surgery alone. Even after "curative" excision, recurrence, both local and distant can occur. The objectives of this thesis were twofold, firstly to assess accurately the extent of tumour spread at presentation, and secondly to examine certain biological parameters of these growths. Thus high risk patients could be identified, which would allow a more rational approach to treatment of the individual.

An initial retrospective study demonstrated that patients with local intrapelvic spread of tumour had significantly higher local recurrence and lower survival rates than those without spread. Patients with clinically fixed tumours due to peritumoral fibrosis had recurrence and survival rates comparable with the latter group.

A prospective study revealed that digital rectal examination is inaccurate in assessing local status of the tumour. Pelvic computerised tomography (CT) proved significantly superior in assessing presence or absence of local spread. Serum levels of carcinoembryonic antigen (CEA) and acute phase reactant proteins (APRP) appear able to refine the results of CT, and accurately identified inflammatory fixation. Clinical examination and routine measurement of liver function could not identify the majority of liver secondaries. Hepatic CT proved superior to ultrasound in
detecting macroscopic metastases. Serum CEA correlated with tumour load. "Occult" metastases were not detected by any modality.

Three aspects of biological behaviour were addressed, tumour differentiation, a possible mechanism of tumour infiltration, and tumour growth rate. Degree of histological differentiation of the main tumour could not be determined accurately by examination of pre-operative biopsies, even if multiple. Flow cytometric analysis was significantly better than subjective assessment.

Elevated levels of three proteolytic enzyme, collagenase, collagenase-like peptidase, and cathepsin B were identified in recto-sigmoid tumours. High cathepsin B levels appeared to correlate with local tumour infiltration.

Tumour growth rate was assessed by production of an in vitro model, multicellular tumour spheroids (MTS) from individual carcinomas. Growth rates of MTS in vitro correlated inversely with patient survival.

This perioperative assessment may thus allow a more rational and individual approach to treatment of rectal neoplasia.
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"An operation undertaken for the cure of cancer, if it is to be effective, must be based upon pathological findings in regard to the spread of cancer to the surrounding tissues from the primary growth. If the field of operation does not embrace all the tissues which are pathologically known to be prone to invasion by cancer cells which have become detached from the primary focus, then the operation will be doomed to failure, because it will not prevent recurrence.

The whole question therefore of the operative treatment of cancer of the rectum hinges upon the knowledge we have been able to obtain - first of the manner in which cancer of the rectum spreads, and, secondly, of the paths along which that spread takes place."

W E Miles 1920

With this remarkably perceptive statement, Ernest Miles opened a discussion on the treatment of rectal cancer, at a meeting of the British Medical Association in 1920 (Miles, 1920). The basic principles contained in that address hold as true today as they did in 1920. Unless surgical excision removes all malignant tissue, recurrence is certain.

Despite improvement in surgical technique, anaesthetic practice and postoperative care, overall survival of patients with rectal cancer has remained static over the last 40 years (Dukes, 1957; Eisenberg
et al, 1967; Lockhart-Mummery et al, 1976; Whittaker and Goligher, 1976). This plateau of surgical treatment can largely be explained by the late presentation of many patients in whom the disease had spread locally or by metastasis to an extent where Miles' basic principles cannot be followed. Surgery alone, in these cases thus cannot offer a cure.

The lack of improvement in survival, with overall five year survival figures hovering around 50 per cent, has led in recent years, to clinicians searching for adjuvant forms of treatment. The two main forms of adjuvant therapy employed most commonly are local radiotherapy and systemic chemotherapy.

We do know, however, from the work of Dukes (1957) and others (Eisenberg et al, 1967; Whittaker and Goligher, 1976) that surgery alone is curative in early cases, and that these patients do not require adjuvant treatment, which in itself can be harmful. The logical way forward, in the future management of rectal carcinoma, must therefore lie in careful patient selection, identification of high risk groups, with tailoring of treatment to the stage of disease in each individual. The aim of this thesis is to determine whether by careful perioperative assessment of patients who present with a rectal tumour, we can more clearly define the extent of disease in each individual, and, by a more careful assessment of the biological aggressiveness of the tumour, the most appropriate treatment can be applied to every case.
The Evolution of Surgical Treatment of Rectal Carcinoma

The history of surgical treatment for rectal tumours dates back to the early 18th century when Morgagni proposed removal of the rectum for carcinoma (Rankin, 1937). Faget in 1739, however, was reputedly the first to undertake such a procedure (Rankin, 1937). The first successful rectal excision was probably performed in 1826 by Lisfranc (Lisfranc, 1926). This consisted of amputation of the anus and lower rectum, resulting in an incontinent perineal stoma. A subsequent report of nine patients included three deaths. However, it was not until the end of the 19th century before surgical treatment of rectal carcinoma became widely practised.

Initially the perineal approach to rectal excision was adopted by the majority of surgeons in the late 19th and early 20th century in this country. Allingham (Allingham and Allingham, 1901) made an important advance in this technique by employing inguinal colostomy for the control of sepsis. Another great exponent of the technique was J P Lockhart-Mummery, and up until the early 1930's this operation was the standard procedure at St Marks Hospital. In 1932 perineal excision had an operability rate of 50%, a mortality rate of 11.6% and a five year survival of 40% (Gabriel, 1932).

Other surgeons, however, adopted a different approach to the problem of rectal excision. Amussat in 1839 suggested a posterior approach to the rectum by removing the coccyx (Rankin, 1937), and in 1873 Verneuil reported his experience with technique (Rankin,
1937). In 1874, Kocher (Kocher, 1874) published his posterior approach to the rectum, however it was left to Kraske to popularise the operation in Germany (Kraske, 1885). The posterior approach enabled tumours higher in the rectum to be excised more easily.

Trans-sacral resection became the most popular method of removing rectal tumours on the continent, however this approach had major drawbacks. Most patients were left with a sacral colostomy, which proved extremely difficult to manage, and furthermore, the overall survival following the operation was poor, only 30% of patients surviving five years (Mandl, 1929).

In the early days of rectal surgery, little thought was given to the spread of the disease, the aim being to remove the primary growth. Unfortunately, the results of this type of approach were uniformly poor, with local recurrence occurring in virtually 100% of patients. The first attempt at patient selection, an early form of clinical staging was developed by Harrison Cripps towards the end of the last century. In the fifth edition of his book (Cripps, 1907), he reviewed 380 patients whom he had seen with a rectal carcinoma. All patients were carefully assessed clinically. In just over a quarter, he recognised that surgical treatment was impossible, and recommended symptomatic therapy only. In a further one third, he considered the growth too advanced for local excision, and introduced inguinal colostomy for relief of impending obstruction. Thus only 85 of his patients (22%) were considered suitable for
surgical treatment by way of local approach via a trans-sphincteric approach. The results, however, were impressive. Using this selection procedure, Cripps was able to report a cure rate in his operative series of approximately 50%.

The next major breakthrough in the treatment of rectal tumours came with Ernest Miles. Miles initially used the perineal approach for removal of the growth as practised by other surgeons of his day. He did, however, become increasingly disillusioned with the extremely poor results obtained. Between 1899 and 1906, Miles performed 50 resections, either via the perineum or via a trans-sacral excision. The average mortality from recurrent disease in this series was 96% (Miles, 1920). These appalling results stimulated Miles to study the modes of spread of the disease. This he achieved by meticulous dissection of tumour specimens. From these careful studies, he concluded that rectal carcinoma spread via direct extension into perirectal tissues, via lymphatics, and via the venous system. He further concluded that the most important of these modes of spread was lymphatic, and of the various directions by which tumour could spread within lymphatics ie downward, laterally and upward, the latter was of more significance. On the basis of this early pathological staging, Miles developed the operation of abdominoperineal excision, which was designed to encompass the areas of spread of the disease (Miles, 1908). In his original paper, Miles stated that an abdominal anus was a necessity and that the whole pelvic colon required removal, because of proximal lymphatic spread along vessels. He also stressed the
importance of clearing the pelvic mesocolon and common iliac lymph nodes, and that the perineal dissection should be as far lateral as possible. This operation was a formidable undertaking at the turn of the century. The initial report included 12 patients, of whom nearly half died (41.0%) in the postoperative period. However, after a maximum follow up of 23 months, the remaining seven were alive and clinically free from recurrence.

The procedure received a mixed reception from Miles' colleagues, who were impressed with the freedom from recurrence, but were horrified by the operative mortality. This latter problem precluded widespread adoption of abdominoperineal excision until the introduction of the safer one stage and combined approaches (Gabriel, 1932; Lloyd-Davies, 1939).

In a subsequent series (Miles, 1920), the operative mortality rate of abdominoperineal excision had dropped to approximately 18%, but this was still much higher than the 8.5% mortality achieved by Lockhart-Mummery in his series of 200 cases of perineal excision (Lockhart-Mummery J P, 1926). In this latter series, an overall five year survival figure of 54% was recorded, however, this included many tumours which had extended above the peritoneal reflection. For these tumours Lockhart-Mummery agreed that abdominoperineal excision was a superior operation.

The initial pathological examinations of Miles were expanded by Lockhart-Mummery in conjunction with a pathologist at St Marks...
Hospital, Cuthbert Dukes. Lockhart-Mummery's surgical findings combined with Dukes' classification of rectal carcinoma published in 1932 (Dukes, 1932), to which we will return later. It is however interesting to note that in his 1925 paper Lockhart-Mummery published five year survival rates for an early A, B, C, classification, which subsequently became known as Dukes' classification.

Because of Miles' initial observations on lymphatic spread of rectal carcinoma, predominantly upwards, but also occurring downward and laterally, it was felt that an operation for a rectal lesion, could only be curative if it removed all these possible routes. Abdominoperineal excision fulfilled these requirements. Several authors, however, began to query Miles' original observations (Wood and Wilkie, 1933; Westhues, 1934; Gabriel et al, 1935; Coller et al, 1940). These authorities repeated Miles' examinations of rectal specimens, and concluded that whilst upward lymphatic spread was common, downward or lateral lymphatic spread rarely occurred. Cases of downward spread did occur, but only in those tumours which were very advanced, and associated with blocked proximal lymphatics. Gollgher et al (1951) reviewed 1500 excised rectal specimens and found distal lymphatic spread in only 2%.

Thus it appeared that it was not always necessary to sacrifice the anus to achieve a cure. This idea of sphincter preservation was, however, not new. In 1833 Reybard of France (Frankin, 1937) reported resection of a portion of the sigmoid flexure followed by
end to end anastomosis, for which he was castigated by his colleagues. Harrison Cripps performed an anterior resection with anastomosis in 1897 (Cripps, 1907). Kraske (1885), reported that he had performed an anastomosis during a sacral approach. The patient developed a faecal fistula, which was subsequently closed, thus restoring continuity. Mayo was credited as the first American to perform a sphincter conserving operation (Balfour, 1910).

Other surgeons approached the problem of conserving the anus in a different fashion. H W Maunsell (1892) described an operation to anastomose the sigmoid stump to the anus, by invaginating the sigmoid through the anus. This technique was also used and modified by R F Weir (1901). Others also adopted the technique with various modifications (Ball, 1903; Aldrich-Blake, 1903; Lockhart-Mummery J P, 1923; Rayner, 1935; Babcock, 1939; Bacon, 1945; Turnbull and Cuthbertson, 1961; Cutait and Figiollini, 1961).

However, it was Dixon and colleagues of the Mayo Clinic who were the first to adopt and popularise the technique of sphincter preservation, following resection of tumours of the upper rectum (Dixon 1939, 1949). From the 1940s to the mid 1970s, anterior resection became a widely accepted technique for growths of the upper, and mid rectum when technically feasible. Several studies confirmed that anterior excision resection was as safe, and had equivalent results to abdominoperineal excision (Waugh et al, 1955; Morgan, 1955; Mayo and Fly, 1958; Whittaker and Goligher, 1978;
Lockhart-Mummery, H E et al, 1976). The operation thus became accepted for growths of the upper third of the rectum, and gradually anterior resection was also adopted for lower growths in the middle third (Cullen and Mayo, 1963; Morgan, 1965; Vandertoll and Beahrs, 1965).

Unfortunately, in many patients, restorative resection was not technically feasible. Although possible to remove the tumour, in an obese patient, or one with a narrow pelvis, construction of an anastomosis from within the abdomen proved impossible. The alternatives were the "pull-through" methods previously described, although the long term results of these operations were far from satisfactory (Mann, 1972). In the same year, Parks (1972) introduced the abdomino-transanal technique, which allowed a direct anastomosis of proximal colon to the anal canal, which thus enabled much lower growths to be resected, and continuity restored. The major technical breakthrough which allowed low anterior resection to become widely adopted, however, was the circular stapling device. The use of this instrument was first published by Fain et al (1975). The device allowed very low tumours to be resected and continuity restored. This technique, with modified stapling devices, has become widespread (Goligher et al, 1979; Heald 1980), thus allowing many more patients to retain their anal sphincter.
The one fear that many surgeons at the time had was that the use of such techniques would lead to higher local recurrence rates, and thus worsen survival.

However, for mid and low rectal carcinoma, the majority of investigators who have addressed the question, have found no difference in recurrence rates or survival in patients treated by abdominoperineal excision or by sphincter saving resection for equivalent lesions (Nicholls et al, 1979; Jones and Thompson, 1982; Heald et al, 1982; Williams and Johnston, 1984; Lasson et al, 1984; Williams et al, 1985), although some authors disagree (Hurst et al, 1982).

At the opposite end of the spectrum, various methods have been proposed to avoid major resection for rectal tumours by utilising local methods of treatment. Byrne in 1889 first described local electrocoagulation of the tumour. This approach was adopted by Kolisch for palliative therapy of inoperable lesions (Strauss et al 1935). Strauss (1935) revived interest in the technique, and reported some long term survivors. Wittoesch and Jackman (1958) utilised this approach in "poor risk" individuals, obtaining a 46% five year survival rate. Others have also successfully used electrocoagulation (Madden and Kandalaft, 1967; Crile and Turnbull, 1972; Salvata et al, 1976; Hughes et al, 1982), with encouraging results.
Other local methods of dealing with early rectal lesions, include endocavitary irradiation (Papillon, 1975) and local excision (Deddish, 1974; Morson et al, 1977; Lock et al, 1978; Whiteway et al, 1985). In selected cases, in experienced hands, the results are impressive, however the number of cases suitable for these procedures is small.
As previously stated in this introduction, the early results of local perineal excision, and trans-sacral excision were poor, because of local recurrence.

However, by the late 1920s, almost half (46.6%) the cases presenting to St Marks Hospital were considered suitable for resection (Bussey et al, 1960). By 1957 this had risen to over 93%. Over this period, the operative mortality had dropped from 10 to 4% (Bussey et al, 1960), and the overall five year survival had risen from 20 to 40%, although for the latter period, five year survival was approximately 50% (Dukes, 1957). From 1958-1963 at St Marks Hospital, five year survival had risen to 57.4% (Morgan, 1965).

Similar figures were reported by Whittaker and Goligher (1976) who analysed 550 cases of rectal cancer treated from 1955 to 1968. The resectability rate of 90.5% compared with that of the St Marks' series, and the overall five year survival of 56% was also similar. However, these figures relate to specialist centres, with a special interest in the disease. In non-specialist centres the picture appears much worse. Slaney (1971) reviewed 5800 cases of rectal carcinoma from Birmingham. Only 52% underwent rectal excision, with a corrected five year survival of 48.6%. Overall, however, less than a third survived five years. Similar figures were reported from the Bristol area, where overall five year survival...
survival of patients with rectal cancer was only 23.5% (Walker, 1971).

More recent series report five year survival after rectal excision from 44 to 74 per cent (Strauss et al, 1978; Nicholls et al, 1979; Jones and Thompson, 1982; McDermott et al, 1982; Williams and Johnston, 1984; Williams et al, 1985). However, it must be pointed out that these figures apply to patients who have undergone apparently "curative" resections, the overall five year survival figures remain around 50%.

There are two main reasons for these depressing statistics. Firstly, many patients present late. We know from extensive series of Dukes (1960), H E Lockhart-Mummery (1976), Whittaker and Goligher (1976), that if caught early, rectal carcinoma is curable. Unfortunately, very few patients present with surgically curable disease. More than 50% of patients are found to have evidence of spread, either at operation, or on examination of the specimen (Dukes, 1960; H E Lockhart-Mummery, 1976; Whittaker and Goligher, 1976). Such spread to lymphatics or to the liver mitigates against cure by rectal excision alone. In most series, Dukes' A tumours, comprise less than 20% of the total (Dukes, 1960; H E Lockhart-Mummery, 1976; Whittaker and Goligher, 1976; Phillips et al, 1984; Williams and Johnston, 1984).

The second reason for failure of surgical treatment, is recurrence of the disease after apparently "curative" excision. Such
recurrence occurs either locally, within the pelvis, or by distant metastasis, primarily to the liver. We must examine the reasons for this failure of "curative" surgery to eradicate the disease more closely.
The Reasons for Failure after "Curative" Excision

There are two main areas of failure following surgery in patients with rectal cancer, namely local pelvic recurrence, and distant metastasis, predominantly to the liver. Frequently the two co-exist.

Local Recurrence

Local recurrence accounted for many early failures of the perineal approach to rectal tumours in the last century. However, even today with advances in surgical technique, locoregional recurrence remains a major finding in patients dying of colorectal carcinoma. A study conducted in Malmo (Berge et al, 1973) on 267 patients who died of colorectal cancer, found 53% to have local recurrence. A similar post mortem study (F W Taylor, 1962) detected local recurrence in 24 (72%) of 33 patients who had undergone apparently "curative" resection. In only 25% of these patients was metastasis in the liver deemed to be the primary cause of death.

Gilbert (1982) reviewed 650 necropsies of patients who died of colorectal cancer. Of these, 45 who underwent an apparently "curative" operation subsequently died of recurrent disease. Local recurrence was found in 67% of these cases.

Gunderson and Sosin (1974) reported the findings of a series of 75 patients who underwent a programme of "second-look" operations
following apparently "curative" rectal excision. Their study reported that where recurrent tumour was present, locoregional recurrences had occurred in 92%. In only 8% were isolated liver metastases present without recurrent pelvic disease. Similar findings were reported by Pilipshen et al (1984).

Local recurrence of rectal cancer would therefore appear to be one major contributing factor to failure after "curative" excision. The questions to be answered are which patients are at risk of developing such recurrence, and can they be identified in the perioperative period?

The modes of spread of cancer of the rectum are well known, since the work of Cripps (1907) and Miles (1908), namely by penetration of successive layers of the bowel wall and beyond, via lymphatics, and via the bloodstream to distant organs. Peritoneal spread is rare as the majority of the rectum lies outside the peritoneal cavity.

Dukes (1932) was the first to categorise the patterns of spread to form the basis of his staging system, which with subsequent modifications is still the most important classification to date. This staging system was originally confined to rectal cancer, but subsequently has encompassed colonic cancer. The initial Dukes' staging has been modified and refined by several authors. Gabriel, Dukes and Bussey (1935) extended the classification by sub-dividing lymphatic spread into I and II depending on the level of nodal
Involvement. By 1949 Kirklin et al recommended a major modification by categorising 'B' tumours into B1, where the tumour has extended into, but not penetrated, the muscularis propria, and B2 where tumour had reached the muscular wall. This system gained wide acceptance in the United States.

A further modification, which further confused pathological staging was introduced by Astler and Coller (1954). This system modified that of Kirklin by subdividing the C category into C1, where tumour had not fully penetrated the muscularis propria, but lymph node metastases were present, and C2 where the muscular layer was breached and spread to nodes was present. These modifications in the original Dukes' stage have led to confusion, and difficulty in comparing reported series (Goligher, 1976).

There are other defects in Dukes' original system, the major being that no allowance is made for direct local spread. This omission is somewhat surprising, as since in 1958, Dukes and Bussey emphasised the importance of local spread on prognosis. In this study, local extension outside the rectal wall, was divided into slight, where spread had commenced to invade the extrarectal tissues, moderate, where spread was well established into the mesentery, and extensive, where tumour was deeply invasive with possible involvement of neighbouring organs. Corrected five year survival in patients with slight spread was 89.5%, and 57.0% in those with extensive spread.
Gunderson and Sosin (1974) also highlighted local extension as an important factor in prognosis and in subsequent local recurrence. Where tumour had obviously extended to neighbouring organs at laparotomy the local recurrence rate at "second-look" was 100%. If neighbouring organs were not involved at laparotomy, but there was macroscopic evidence of gross invasion, local recurrence occurred in 91.3%. However if the tumour extension was detected only by histological examination of the specimen, local recurrence occurred in 75%. The further conclusion from this study was that loco-regional failures increased when lesions extended completely through the bowel wall, and that the poor prognosis of these patients was related in part to the degree of extra-rectal extension.

Similar findings were reported by Moossa et al (1975), who reviewed 152 patients who underwent abdomino-perineal excision. Local recurrence occurred in 20.4%. One factor significantly related to local recurrence was direct spread into perirectal fat.

Godwin and Brown (1975) analysed a large number of prognostic variables in over 11,000 cases of colorectal cancer. The difference in survival by degree of extramural extension was highly significant. Patients with limited extra rectal extension had a 50% improvement in survival compared to those with more extensive spread. The authors concluded that local extent of disease was the most important prognostic variable.
Wood et al (1981) prospectively studied 404 patients with colorectal cancer. The presence of local tumour invasion was recorded at laparotomy and confirmed histologically. The patients were staged pathologically, using Dukes' classification. This study showed a highly significant decrease in survival rate for patients with extramural spread and proposed a modified staging system which took into account extramural spread. This system has, however, not been widely adopted.

Woods' system was utilised by Habib et al (1983) in a retrospective study of 301 colorectal tumours. The findings of this investigation supported those of Wood et al (1983). What was of particular interest, however, was the finding that patients without lymphatic involvement (Dukes 'B'), but with extramural spread, had a worse prognosis than those with nodal metastases, but without extramural spread (Dukes 'C'). This apparent anomaly in staging was also reported in the study of Wood et al (1981), previously alluded to.

Thus the main conclusion to be drawn from the studies outlined above is that local tumour spread is an extremely important prognostic factor in colorectal cancer, perhaps of more significance than lymphatic involvement. However lymphatic spread and direct extension often go hand in hand. Dukes and Bussey (1958) noted a close correlation between nodal spread, extent of local invasion, histology and venous invasion. They warned that dividing all cases into groups with and without nodal spread exaggerated the
importance of lymphatic spread, and disguises the fact that patients
with early lymphatic spread have a relatively good prognosis.

The effect of nodal metastases on local recurrence is unclear. 
Gunderson and Sosin (1974) found no difference in recurrence rates 
in patients with and without nodal deposits. The local recurrence 
was far more closely related to extramural spread. Thus early 
lymphatic spread appears to have little effect on local recurrence. 
If all involved nodes are resected, and tumour is confined to the 
wall, local recurrence is unlikely and the overall prognosis good.

There is little doubt however that the more nodes involved, the 
worse the overall prognosis (Dukes and Bussey, 1958; Spratt and 
Spjut, 1967; Copeland, et al 1968), and that if involved nodes and 
lymphatics are left behind in the pelvis, local recurrence is almost 
certain.

Patients with extensive local spread are thus unlikely to be cured 
by surgery alone, and are at high risk of developing local 
recurrence. It is important, therefore, to try and identify such 
patients as they are one group who require either more radical 
surgery or adjuvant therapy. The identification of this group in the 
perioperative period forms the basis of a major part of this thesis.

One important point that must not be overlooked, however, is that 
at laparotomy, a tumour which is adherent to, and appears to be 
invading surrounding structures, may merely be involved in
extensive inflammatory adhesions. It is impossible to identify these tumours macroscopically at operation. In a series of patients operated on for apparent extensive extramural tumour invasion, reported by Bonfanti et al (1982), of 52 structures apparently invaded by tumour, only 23 (44%) demonstrated microscopic invasion, the rest showed only inflammatory adhesions. Others (Jensen et al, 1970 Davies and Ellis 1975) have reported the incidence of inflammatory fixation varying from 26 to 38%.

Davis and Ellis (1975) found no difference in survival between patients with inflammatory adhesions as opposed to neoplastic extramural infiltration, who underwent extensive surgery. However, others (Bonfanti et al 1982; Jensen et al 1970) have found a significant improvement in survival in patients with inflammatory fixation compared with those exhibiting neoplastic fixation.

Many of the studies previously alluded to, combine both colonic and rectal tumours. To define the extent of extramural infiltration, and inflammatory fixation in rectal carcinoma alone, and the effect on survival, a retrospective study of 625 patients who underwent rectal excision for proven adenocarcinoma was undertaken. This study forms the basis of Chapter 2 of the thesis.

Liver Metastases

Unfortunately by the time many patients present, metastasis to the liver has already occurred in from 15-25% of cases (Bengmark and
Although there are occasional long term survivors, the majority of patients with untreated liver secondaries die within two years (Joffe et al, 1968; Oxley and Ellis, 1969; Wood et al, 1976). Many overt liver metastases are not detected until laparotomy for resection of the primary tumour. Ideally these patients should be identified prior to surgical intervention.

The degree of liver involvement is of prognostic significance. Several studies have demonstrated that patients with solitary liver metastases or those with only a few metastases confined to one lobe, have a significantly longer median survival compared with those patients who have extensive hepatic involvement (Jaffe et al, 1968; Nielsen et al, 1971; Wood et al, 1976; 1984).

As yet, for patients with extensive liver involvement, treatment aimed at cure has proved largely ineffective (I Taylor, 1985). If extensive liver involvement could be identified preoperatively, elderly and infirm patients could possibly be spared an unnecessary laparotomy. There is, however, evidence to suggest that removal of the primary tumour in such patients may prolong survival. Oxley and Ellis (1968) reported one year survival of 32% in patients in whom resection was performed, compared with the 15% where the primary was left. Against this argument, is the problem of operative morbidity and mortality. Elderly patients with disseminated disease may succumb in the early postoperative period. There are alternative modes of palliation for the primary tumour,
local radiotherapy or local fulguration can provide symptomatic relief, and may thus be preferable.

Conversely, patients with limited involvement of the liver may be suitable for resection of their metastases. The results of surgical treatment of liver metastases are inconclusive, however where there is only limited hepatic involvement, reported survival figures are encouraging. Reviewing the literature in 1970, Foster reported two and five year survival rates following hepatic resection for liver metastases of 47 and 21% respectively. Wilson and Adson (1976) reported similar results in their series of patients who had limited liver involvement. Their results where extensive involvement was present were, not surprisingly, poor. In a recent extensive review of the subject, I Taylor (1985) reported that the collective operative mortality of major resection for hepatic secondaries was approximately 5%, the one year survival 80%, and a three year survival of up to 55% in certain series. Thus surgical resection does have a role in the treatment of metastatic disease of the liver. The number of patients suitable is, however small, probably less than 10% of individuals with overt spread at the time of initial diagnosis. To optimise treatment in this group, however, such individuals require to be identified in the perioperative period.

It has been evident, for some time that the absence of overt liver metastases at laparotomy, does not necessarily imply that the liver is free of secondary spread. Goligher (1941) reported a series of 893 rectal cancers from St Marks Hospital. Liver metastases were
detected by the operating surgeon in 12%. Thirty one patients in the series subsequently died in the post operative period. None of these patients had obvious liver metastases at laparotomy, however on sectioning the liver at post-mortem, secondary deposits were found in 5(16%).

These findings have recently been supported by Finlay et al (1982). In a prospective study of 43 patients, with colorectal tumours, six had overt metastases found at laparotomy. The remaining patients, who apparently underwent a "curative" operation were closely followed up with serial liver function tests, and serial ultrasonic, isotopic and computed tomographic examination of the liver. Eleven of these patients (29%) subsequently developed proven liver metastases. Finlay et al termed these "occult" metastases and suggested that these secondaries were present, but undetected at the time of surgery. These data agreed with the experimental work of Mooney et al (1982) who detected such "occult" metastases in 30% of a comparable group of patients using a technique of injecting radio labelled microspheres into the portal vein.

Thus another group of patients who have residual disease after "curative" surgery of the primary lesion, have now been identified. If, as is suggested, these occult or micrometastases are present at the time of initial presentation, the aim should be to identify them at this stage. At such a point in their natural history, "micrometastases" may be more susceptible to adjuvant
chemotherapy, than when the patient subsequently develops widespread macroscopic disease.

Thus there are two main areas of failure following "curative" resection for rectal carcinoma. Surgery alone is inadequate in such cases, and this has therefore led to alternative avenues of therapy being explored.
Adjuvant Therapy of Colorectal Cancer

The fact that surgical treatment of rectal cancer has reached its zenith, has prompted clinicians to look for alternative therapies. To date the most widely adopted are radiotherapy and chemotherapy. Local radiotherapy and systemic or local chemotherapy have been extensively investigated in colorectal cancer. More recent adjuvant treatment, such as immunotherapy and photodynamic therapy are in their infancy, but may well have a useful role in the future.

The Use of Radiotherapy in Rectal Carcinoma

Radiotherapy has been utilised for treatment of rectal carcinoma almost since its invention. In the early days, radiotherapy was largely confined to the palliative treatment of inoperable tumours. J P Lockhart-Mummery (1934) records usage of the technique, and stated that radium treatment could be a possible alternative to excision, be useful as adjuvant, or provide palliation in inoperable cases. He recommended the use of radon seeds, which caused less problems than needles. Reasonable results were claimed, however dosage rates were extremely difficult to judge and local recurrence rates were high.

External beam radiotherapy using the early 250 Kv machines proved a complete failure (I G Williams, 1960). In 1936, a supervoltage machine was installed at St Bartholomew's Hospital which allowed
a higher dosage to be given via an external beam. From 1939-1955, 220 patients were treated by radiotherapy. Of these 83% were considered to have advanced disease. Twenty nine percent of these patients survived two years, but only 5% survived five years (I G Williams, 1960).

There is little doubt, however, that local radiotherapy has a role in treating inoperable rectal tumours. Relief of distressing symptoms can be achieved, and moreover, radiotherapy may convert an apparently inoperable tumour to an operable one (James and Schofield, 1985; Mendenhall et al, 1987). At the other extreme, as previously stated, for very early lesions, ie less than five cm diameter and confined to the rectal wall, endocavitary irradiation can provide a cure in a high percentage of patients (Papillon, 1975; Co-ordinating Committee on Cancer Research, 1982). Such cases, however, represent only a small percentage of patients who may benefit from X-ray treatment and it is the role of radiotherapy as an adjunct to surgical treatment which must be assessed.

**Adjuvant Radiotherapy in Rectal Carcinoma**

The rationale for local pelvic radiotherapy in operable rectal carcinoma came from the recognition of local tumour recurrence in the pelvis as a major contributor to subsequent morbidity and mortality (Lofgren et al, 1957; Morson et al, 1963; Gunderson and Sosin, 1974; Phillips et al 1984, a,b; Pillipshen et al, 1984). The first indication that adjuvant radiotherapy may prove beneficial
The results of these studies provided a more rational basis for the investigation of adjuvant radiotherapy in rectal carcinoma.

Since the initial report from the Memorial Hospital in New York, numerous trials of preoperative radiotherapy have been undertaken, with dosages ranging from 500 to 6000 cGy, some randomised, others not (Leaming et al, 1961; Stearns et al, 1974; Higgins et al, 1975; 1981; Rider et al, 1977; Medical Research Council, 1982; Kligerman et al, 1972; Boulis Wassif, 1982; Stevens et al, 1976). A second study conducted by the Memorial Hospital, (Leaming, 1961) failed to confirm the findings of their first study, and no benefit seemed to accrue in the irradiated group. The Veterans Administration Surgical Adjuvant Group (VASAG), in an initial randomised trial of 2000 cGy given preoperatively, also failed to demonstrate any overall benefit in survival (Higgins et al, 1975), however there was a significant survival advantage in those patients who underwent abdominoperineal excision. This has led VASAG to undertake a second trial (Higgins et al, 1981), as yet incomplete, and which is administering 3150 cGy to patients undergoing abdominoperineal excision.
The Toronto Group (Rider et al, 1977) used low dose radiotherapy (500 cGy). Again no difference in overall survival was apparent, but where lymph nodes were involved, a significant advantage in the treated group was apparent.

Several further trials, all of which have utilised a higher dosage of radiotherapy (Kligerman et al, 1972; Boullis Wassif, 1982; Stevens et al, 1976) have demonstrated more positive results. In the Yale trial (Kligerman et al, 1972), the numbers of patients were probably too small for meaningful analysis, however, five year survival was better in the treated group compared with the untreated group (41% versus 25% respectively). An interesting observation in this trial was that fewer patients in the irradiated group had involved lymph nodes, the suggestion being that radiotherapy had "downgraded" the tumour. A similar finding was reported by Boullis Wassif (1982). This later trial administered 3450 cGy prior to surgery, and although there was no improvement in overall survival of the treated group, pelvic recurrence rates were significantly reduced.

An unrandomised trial (Stevens et al 1976) of very high dose radiotherapy (5000–6000 cGy), demonstrated a definite improvement in survival when compared with historical controls. However of great interest was the fact that of the 44 patients who underwent a "curative" resection and had received radiotherapy, none developed pelvic recurrence.
In this country, several radiotherapy trials have been completed, or are in progress. The Medical Research Council undertook a trial of preoperative radiotherapy, administering either 500 or 200 cGy. (Medical Research Council Working Party 1982, 1984). This trial failed to demonstrate any improvement in overall survival of the treated group. However one factor to emerge from this study was an improvement, albeit non-significant, in survival of patients with fixed rectal tumours who received radiotherapy. This finding proved enough of a stimulus for the Medical Research Council to instigate a further trial of radiotherapy, specifically in patients with fixed or partially fixed tumours. This trial is underway at the present time.

At a meeting of the United Kingdom Co-ordinating Committee on Cancer Research, at the headquarters of the Medical Research Council in London, (1985) interim reports of several peri-operative radiotherapy trials were discussed. The North West Region radiotherapy trial, is administering 4000 cGy immediately prior to operation for fixed rectal tumours. After a median follow-up of 11 months this trial had already discovered a highly significant benefit in lowering pelvic recurrence in irradiated patients. This highly significant reduction has remained on longer term follow-up (D Jones et al, 1988). The Imperial Cancer Research Fund trial which began in 1983, which is employing a dose of 1500 cGy, is still underway. The trial has demonstrated an improvement in local recurrence rates, but this has not quite reached statistical significance. It would appear, therefore, that radiotherapy can
improve pelvic recurrence rates, but only if high doses are administered (at least 4000 cGy) close to the time of surgery. The major problem lies in selecting those patients who require such therapy, as in those who do not require it, unnecessary morbidity may result.

There is no evidence to suggest that low dose radiotherapy increases operative morbidity or mortality. The trials listed above using 3000 cGy or less have shown no increase in either morbidity or mortality. One particular worry of many surgeons is anastomotic dehiscence of an anterior resection after radiotherapy, however this does not appear to be a problem with low dosages. The Medical Research Council trial (MRC Working Party 1982, 1984) contained the largest number of restorative resections. Anastomotic leakage occurred in 30% of patients who underwent surgery alone, 8% in those who received 500 cGy, and 16% in those who received 200 cGy. Deaths related to radiation are rare, however Stevens (1976) reported two deaths in 58 patients who received 5000 cGy or more. The conclusions to be drawn from these studies on perioperative radiotherapy therefore appear to be that local irradiation can reduce the risk of local recurrence, but only if high doses are employed. Unfortunately with high dosage, the chance of complications is increased. Thus if high dose radiotherapy is to be used, it is essential that only those at risk of local recurrence be subjected to treatment. Who those patients are, and how they can be identified forms a major part of this thesis.
Local pelvic irradiation alone will not affect distant metastases; nor ideally should it be employed in patients with disseminated disease at presentation. For those patients with spread beyond the pelvis, one possible form of treatment is chemotherapy.

**Adjuvant Chemotherapy of Colorectal Cancer**

At the present time, the major alternative adjuvant therapy to radiotherapy in rectal carcinoma is chemotherapy. The majority of chemotherapeutic trials have, however, included colonic as well as rectal cancer.

One of the first chemotherapeutic agents used was nitrogen mustard (Mrazek et al, 1959). The results of this early trial demonstrated no benefit on survival. Another early drug evaluated was Thio TEPA (Triethylenethiophosphoramide, TPSA). In a trial undertaken in the United States this drug was administered to patients undergoing "curative" resection (Holden and Dixon, 1962). Due to complications of treatment the dosage employed was reduced after the first year of the trial. Almost 700 patients were entered, but again, disappointingly, there was no overall benefit in survival for the treated group. However, the 10 year follow up of the trial did show a slight advantage in survival for women treated with the initial higher dose (Dixon et al, 1971). A similar study by the Veteran Administration Hospitals Surgical Adjuvant Group (VASAG) failed to demonstrate any survival benefit (Dwight et al, 1969).
The most useful drugs for colorectal cancer appear to be the fluorinated pyrimidines, 5-fluorodeoxyuridine (5FUDR) and 5-fluorouracil (5FU), which were introduced in 1957. VASAG administered 5FUDR in 735 patients in a controlled randomised trial (Dwight et al, 1973). Unfortunately many patients in the study suffered from severe toxicity. At five years, no significant benefit in survival was apparent.

Following this study, VASAG and other groups commenced trials using 5FU in several treatment regimes (Higgins et al, 1978; Rousselot et al, 1972; Grage et al, 1979). Studies reported by Higgins (1978) and Rousselot (1972) did show improved survival in the treatment group, but this failed to reach significance on long term follow up. The Central Oncology Group trial (Grage, 1979), did however demonstrate a significant survival advantage in patients with rectal lesions who received 5FU. Patients with Dukes' C tumours of colon and rectum appeared to benefit also. The most impressive results of adjuvant 5FU were published by Li and Ross (1976) who demonstrated a significant survival advantage in patients who received the drug. This study is open to criticism, however, as it was unrandomised and used historical controls.

One major advance in the use of 5FU has been the study of I Taylor et al on portal perfusion in the perioperative period with 5FU introduced directly into the portal vein via the obliterated umbilical vein (I Taylor et al, 1985). This important study randomised 244 patients into surgery alone or surgery plus local
infusion of 5FU for seven days via a catheter placed in the portal vein. Those entered were patients undergoing "curative" resections, in whom there was no evidence of liver metastases detected either preoperatively or at operation. The results of the study were impressive, with a highly significant reduction in the development of subsequent liver metastases, and prolongation of survival in the group receiving intraportal 5FU. Patients with Dukes 'B' tumours of the colon appeared to derive most benefit. There was, however, no survival advantage for patients with rectal tumours when taken in isolation. One word of caution about this study is necessary, however. The patients who received 5FU also received heparin, and it is possible that this may have a role in the prevention of metastasis.

A further extremely important study has emerged recently from the Gastrointestinal Study Group (1985). In this investigation, 227 patients who underwent "curative" excision of a rectal cancer were randomised to surgery alone, surgery plus postoperative radiotherapy (4000-4800 cGy), surgery plus postoperative chemotherapy (5FU and MethylCCNU) or surgery, plus chemotherapy, plus radiotherapy. The results after five years were impressive. A significant improvement in both survival and disease-free interval was achieved in the group receiving combined chemotherapy and radiotherapy. This led the authors to conclude that post operative radiotherapy and systemic combination chemotherapy should be routinely administered to patients who undergo apparently "curative" resection.
There is no doubt, however, that chemotherapy is hazardous with associated major morbidity and toxicity. The average response rate to 5FU probably the best drug for colorectal tumours, is only 20% (Carter, 1976). Thus if the above policy were instituted, possibly 80% of patients would receive a worthless, potentially harmful drug. In the future, therefore, it is essential to carefully select "high risk" patients for chemotherapy, in order that those patients with early lesions do not receive unnecessary toxicity. Furthermore in an ideal situation, an assessment of individual tumour chemosensitivity is necessary, to avoid treating resistant tumours. This thesis addresses both of these problems.
Staging Systems for Rectal Carcinoma

The preceding discussion on the modes of treatment of rectal carcinoma has highlighted the fact, that if we are to improve overall survival, a careful assessment of each patient is essential. Moreover, before future trials into different forms of treatment, be they surgical, or adjuvant, are undertaken such assessment of patients will be essential if meaningful results are to be obtained.

The staging of rectal carcinoma has, to date, been largely pathological, based on the initial classification of Dukes (1932), with subsequent modifications (Dukes and Bussey, 1958; Astler and Coller, 1954; Kirklin et al 1949). This nomenclature has led to confusion, due to the fact that all the systems use an A, B and C classification, but as alluded to previously, all represent different stages in different systems. To try and overcome this discrepancy, the TNM system was introduced (Beart et al, 1978), however this has failed to gain wide acceptance, and most pathologists adhere to the original or modified Dukes' classification.

There is no doubt that these pathological classifications provide an excellent system for determining prognosis, however there are flaws. The recent work of Finlay et al (1982) on "occult" hepatic metastases had demonstrated that pathological examination of the specimen alone can be misleading. Furthermore, as previously stated, certain features of the primary tumour, such as extensive local spread may override conventional pathological staging criteria.
On an individual patient basis, therefore, if treatment is to be
tailored to the extent of that person's disease, both in initial
surgical management, and in deciding on any adjuvant therapy,
some form of clinical assessment is essential. In particular, for
selection of patients for adjuvant treatment, assessment in the
immediate perioperative period appears to be important both with
regard to spread, and the "aggressiveness" of the tumour. In the
few trials of adjuvant therapy which have demonstrated potential
benefit, both radiotherapy (Medical Research Council, 1983) and
chemotherapy (I Taylor et al, 1985) or a combination of the two
(Gastrointestinal Tumour Study Group, 1985), have been
administered in the perioperative period. Cuthbert Dukes (1932)
recognised the importance of a perioperative staging system. He
stated in his original article that: "If it were possible to decide
the category of the case before the operation, it would be very
important and useful information".

There have been attempts to classify colorectal tumours on a
clinicopathological basis (Davis and Newland, 1983; Chapuis et al,
1985; Devesa et al, 1984), however these systems, largely modified
and refined the pathological stage of the tumour, by adding clinical
data obtained at operation, or by basic preoperative investigations.
These systems do not go far enough, and incorporate colonic, as
well as rectal tumours.
As previously discussed, the major problems preventing successful treatment are local pelvic spread either direct or to lymphatic and distant metastasis, primarily to the liver. At the inception of this thesis, at the General Infirmary, Leeds, as in the majority of centres, patients with a rectal carcinoma were assessed both clinically and by preoperative investigation. Clinical assessment consisted of a general physical examination, with particular reference to abdominal palpation, and digital rectal examination. A rigid sigmoidoscopy was performed, the height of the lesion from the anal verge measured and biopsy taken for histological assessment. Blood was taken for liver function tests and an abdominal ultrasound performed, both to detect hepatic metastases. It was also routine practice to perform an intravenous pyelogram to exclude ureteric involvement, and confirm the presence and functioning of both kidneys, and chest x-ray to exclude pulmonary metastases. Upon these findings, the treatment options were considered. Let us examine this conventional assessment more closely, and commence with the problem of identifying local spread of the disease.
The Identification of Local Spread of Rectal Tumours

The advantage that a rectal cancer has over a colonic lesion, is that if situated in the lower two-thirds of the rectum, it can be palpated. Thus the assessment of local tumour spread has up until now been achieved by digital palpation of the tumour and assessment of local fixation, which implies tumour extension beyond the rectal wall. Harrison Cripps used careful digital examination to assess suitability for operation at the end of the last century, and since then, digital examination has become an established method of tumour assessment. Examination by the surgeon's finger is at best a subjective procedure and rather a crude method of assessing local tumour fixation. The majority of surgeons would recognise three grades of mobility, mobile, by which is meant the tumour was freely mobile, implying no extrarectal spread, tethered, whereby the tumour felt adherent, but not totally fixed, which implies a moderate degree of local spread, or totally fixed, which implies extensive extramural spread.

York-Mason (1976) has proposed a clinical staging system of the primary tumour, based on careful digital rectal examination. He has proposed a four stage system, (C S I-IV). Stage I (C S I) tumours are freely mobile growths which have not involved the muscularis propria, C S II tumours involve the muscle layer, CS III tumours are tethered, and C S IV are fixed. The rationale behind this system was to select patients for a more conservative surgical approach. Correlating the clinical stages to the pathological
findings, he found that lymph node involvement occurred in less than 10% of patients with C S I and II tumours, and from 10-90% in C S III and IV growths. Thus "early" growths could be selected on this basis for less radical surgery. Similar techniques of assessing size and mobility, in conjunction with histological assessment of a biopsy have been used by other authors in assessing rectal tumours suitable for local excision (Lock et al, 1978; Whiteway et al, 1985).

Nicholls et al (1982), tested the ability of clinicians to classify rectal tumours by digital palpation. In this study, the local extent of tumour spread in 70 patients with a palpable rectal cancer, was assessed by a variety of clinicians, and their findings compared with pathological examination of the specimen. The study highlighted considerable variation amongst examiners. In assessing tumours confined to the rectal wall, accuracy varied from 44 to 80%, and in those with moderate spread from 33-40% and with extensive spread from 36-45% respectively. Of note in the latter group were three patients in whom extensive spread was diagnosed by all clinicians but who were found to have only dense fibrosis on pathological examination. One further conclusion was that no useful information on nodal status could be obtained by the clinical examinations undertaken in the study.

Digital examination alone, therefore, can be criticised. York-Mason (1976) freely admits that accurate definition of a tumour digitally requires considerable experience. Furthermore only low tumours
can be fully assessed by the examining finger, particularly in the conscious state. Even if the lower border of a tumour is palpable, unless the upper part can be reached, incomplete information may result in the wrong treatment option, particularly for benign tumours such as a villous adenoma, or for early carcinomas.

Many surgeons argue, that the majority of rectal cancers can be reached by the examining finger. However, an epidemiological study in America (Berg and Howell, 1974) has revealed a surprising fact. It would appear that low rectal cancer is becoming less common. Berg and Howells' data revealed that only 30% of rectal carcinomas extended to within 6 cm of the anal verge and thus within easy reach of the clinician's finger.

At the opposite end of the spectrum, a very low tumour which involves the upper anal canal may not be palpable in the conscious state due to severe pain, and thus an examination under anaesthetic may be required.

One further criticism of digital examination was noted by Nicholls et al., (1982). They freely admit that, even if the tumour is easily and fully palpable, but found to be fixed, there is no way of detecting clinically whether this be due to extramural tumour spread, or a dense fibrotic reaction.

The palpation of involved lymph nodes also presents a problem. Although presumed nodal masses may be felt outside the rectum,
whether this enlargement is due to lymphatic metastasis or merely reactive hyperplasia cannot be determined on purely clinical assessment.

The question therefore arises whether any alternative methods for assessing the degree of local tumour spread exist. In this thesis I propose to explore two avenues, firstly biochemical, and secondly by use of pelvic computerised tomography.

**Biochemical Assessment**

In 1965 Gold and Freedman isolated a human tumour antigen from colonic carcinomas, not found in normal colonic tissue from the same patient. Foetal tissues obtained from gut, liver and pancreas were found to contain small amounts of the same antigen. Because of this distribution, the term carcinoembryonic antigen (CEA) was adopted. CEA is a glycoprotein with a molecular weight of approximately 180,000. Initially CEA was promoted as a specific antigen for colonic tumours which could be used to screen the population merely by taking a blood sample. It soon became evident, however, that CEA was not specific to colorectal carcinoma. Elevated levels of CEA have been found in a wide range of malignant disorders, and also benign and inflammatory conditions (Lawrence et al, 1972; Reynoso et al, 1972; Booth et al 1973; Zamcheck, 1975; Costanza et al, 1974; Hansen et al, 1974).
Certain authors have, however, found that preoperative serum levels of CEA may correlate with subsequent tumour recurrence and survival. Lo Gerfo and Herter (1975) studied 150 patients with colorectal carcinoma. After a follow-up period of from 24-36 months, following "curative" resection, they reported that, of those patients two had died of disease or who had recurrence, 57% had an elevated CEA preoperatively. In only 32% of patients free from disease was the serum CEA level elevated prior to operation. Moreover this relationship appeared unrelated to pathological staging of the resected specimen. Of 25 patients with a Dukes' 'A' lesion, five developed recurrence, and of these three (60%) had elevated CEA levels preoperatively. Of those free from recurrence, only one (5%) had an elevated CEA. Similar differential patterns of CEA were found in 'B' and 'C' cases.

Wanebo et al (1978) reported a higher recurrence rate in patients who had raised CEA levels preoperatively. Herrera et al (1976) reported that 83% of their series of patients with recurrence proved to have had an elevated CEA, compared with 35% who were disease free. The authors also related the preoperative CEA levels to the stage of the tumour, and concluded, albeit on flimsy evidence, that preoperative CEA levels provided a superior method of staging then pathological examination. Others, however, have failed to find CEA of significant value in prognosis of staging (Mackay et al, 1974, Lewi et al, 1984). However, in one of these studies (Lewi et al 1984), preoperative CEA levels did give an accurate prediction of the extent of disease. Seventy per cent of patients with 'D'
lesions were found to have an elevated serum CEA prior to surgery, compared with 25% of those with 'B' or 'C' tumours.

One study of considerable interest (Staab et al, 1981) has indicated that elevated CEA levels appear to correlate with locally advanced colorectal carcinoma. This would further support the theory that CEA merely reflects tumour load. It has, however, been suggested (Ward et al 1977) that the ability of CEA to predict operative findings is considerably enhanced by concurrent measurement of certain acute phase reactant proteins (APRP) in the serum. In Ward's study such a combination was able to discriminate preoperatively between metastatic and non-metastatic colorectal tumours. Elevated levels of alpha-1-antitrypsin and alpha-1-acid glycoprotein were found to correlate with metastatic disease.

Acute phase reactant proteins (APRPs) are mainly glycoproteins that alter their plasma concentration in response to stimuli produced by many forms of tissue injury, inflammation, connective tissue disorders and cancer. They have been defined (Koj, 1974) as "trauma-inducible, liver produced, glycoproteins", (Koj, 1974) however more recent evidence suggests that leucocytes can produce APRP locally (Gahmberg and Anderson, 1978). Typical APRP include alpha-1-acid glycoprotein (AGP), C-reactive protein (CRP), alpha-1-antitrypsin (AAP), alpha-1-antichymotrypsin (ACT), and haptoglobin. The acute phase reactants have proved of value in monitoring a variety of gastrointestinal carcinomas, and perhaps of more relevance to this thesis is the finding that serum levels of
such proteins may correlate with local tumour invasion. De Mello et al (1983) measured serum CEA and APRP in 100 patients with gastric carcinoma and 100 with colorectal tumours. In this study a CRP level of greater than 200mg/L identified 50% of patients with extensive local spread of their gastric tumour thus rendering them inoperable or suitable only for palliative treatment. Also of note, in this study, was the confirmation of the ability of combinations of CEA and APRP to identify patients with metastatic colorectal carcinoma. Utilising logistic discrimination, the serum levels of CEA and APRP defined a highly significant difference between metastatic and non-metastatic tumours. This is a point we will return to later when considering the question of liver metastases.

The potential for serum APRP to detect local tumour invasion has been reported in bladder cancer by Hollinshead et al (1977) who observed that serum levels of AGP and prealbumin provided an index, related directly to the degree of local invasion. Bastable et al (1978) measured serum levels of CRP, AGP and ACT and reported that by choosing appropriate levels, benign disease and superficial carcinomas of the bladder could be distinguished from invasive tumours.

This association between a progressive rise of APRP and a rapid evolution of tumours suggest that levels of these proteins are controlled by production of signals at the host tumour interface. It has been suggested that the strength of the signal is related to the depth of penetration of the tissue (Cooper and Stone, 1979). APRP
are also produced in quantity in response to inflammation (Koj, 1974). It is possible, therefore, that the highly elevated levels of APRP associated with an inflammatory response could prove of use in identifying the reaction around a rectal tumour fixed by acute inflammatory adhesions.

It would thus appear that measurement of CEA and APRP in the serum of patients with a rectal tumour is a potentially useful tool in assessing local tumour invasion. These compounds may also have a role in the detection of metastatic spread, and this possibility is discussed later in the section on Identification of liver metastases. Therefore in the latter part of Chapter 3 of this thesis, a study is outlined which explores the possibility of utilising serum measurements as an aid to defining the presence and type of local tumour fixation.

The second avenue explored to identify local spread of rectal tumours, is the use of pelvic computerised tomography.
Computerised Tomography of the Pelvis

Of all the recent advances in imaging techniques over recent years perhaps computerised tomographic (CT) scanning is the most significant.

The CT scanner allows a three dimensional picture of the body to be built up. Not surprisingly, after examining the normal subject, (Redman, 1977) interest turned to the diagnosis of disease, including colonic and rectal tumours, (Ellert and Kree, 1980; Mayes and Zornoza, 1980). Thoeni et al (1981) examined 39 patients, with rectal or rectosigmoid carcinomas, in an effort to determine the presence or absence of extrarectal spread. Extrarectal fat can be demonstrated well by CT scanning and thus it was proposed that tumour infiltration should be relatively easy to identify. The examination also included scanning the remainder of the abdomen for metastases. Using the CT scanner, Thoeni et al proposed a staging system, to include both local and distant spread. Using this classification, they were able to stage correctly 92% of their tumours, and accurately identify advanced local disease.

A K Dixon et al (1981) undertook a similar study in 52 patients with a rectal carcinoma, specifically to determine the ability of CT to detect extrarectal spread. The CT findings were compared with pathological examination of the specimen. The results of this investigation revealed that CT appeared satisfactory in excluding extramural spread where none was present, but could only detect
extrarectal spread when this was more than 2 cm from the rectal wall. Moderate degrees of tumour infiltration were not identified by the scans. With regard to identification of involved nodes, the CT scans were able to identify only seven of 18 (39%) involved nodes successfully.

In this later study, and in a follow up study by Nicholls et al (1982), the findings of CT were compared with those obtained from digital examination. Computerised tomography proved significantly superior to clinical examination in detecting extensive tumour spread, but could not improve over digital examination where lesser degrees of infiltration were present.

Computerised tomography may also have a role in the staging of early rectal carcinoma, suitable for local excision or other modes of local therapy. Hamlin et al (1981) successfully utilised the technique in five patients with early rectal tumours. Based on the CT findings, the patients were selected for surgery or endocavitary irradiation.

Koehler et al (1984) also reported the efficacy of CT scans in selecting patients for therapy, particularly with respect to the local status of the tumour. These authors studied 23 patients and reported that in only three (13%) did the CT scan not influence the choice of treatment. In almost half the patients (48%) the results of the pre-operative CT, contributed to, or were directly responsible for, a change in treatment, from that decided on
clinical grounds. They conclude that CT scanning should be performed in all patients with invasive rectal tumours.

There is, therefore, mounting evidence to support the use of CT scanning pre-operatively in the staging of local tumour spread. Therefore the first part of Chapter 3 outlines a study designed to assess the potential contribution of this technique to overall peri-operative assessment. The use of CT scanning in the detection of hepatic spread is discussed later in this chapter.

Since the inception of the study, early reports of alternative imaging modalities, such as transrectal ultrasound, (Beynon et al, 1986) and nuclear magnetic resonance (Butch et al, 1986), to assess the local extent of rectal neoplasms have appeared. Although extremely promising, these newer techniques have not been fully evaluated.
The Identification of Hepatic Metastases:

As alluded to previously in this introduction, spread of rectal carcinoma to the liver presents major problems in patient management. Many patients are found to have overt liver spread at operation. Although in the majority of these patients, a major abdominal approach will be required for symptomatic relief of the primary, occasionally in the elderly or infirm patient, if the clinician was aware of metastatic spread prior to surgery, some consideration could be given to purely local, palliative treatment.

The other major group of patients are those with an apparently normal liver at laparotomy, who subsequently develop metastases. It has been suggested, as mentioned earlier, that these metastases are present, but are too small to be detected by the surgeon at laparotomy, (Golligher, 1941; Finlay and McArdle, 1982). The work of I Taylor et al (1985) has demonstrated a reduction in the development of metastases in such patients, whose livers appear normal at laparotomy, who are treated with chemotherapy in the immediate post-operative period. Whether this success is due to the relatively small size of these "occult" metastases, which allows greater drug penetration, or whether the chemotherapy destroys cells shed into the circulation, and which may subsequently implant in the liver, or for other reasons, is not known. For these two groups of patients therefore, those with overt hepatic spread at presentation, for whom pre-operative knowledge of the situation can influence surgical approach, and those with "occult" spread,
who may require adjuvant chemotherapy in the peri-operative period, accurate identification of liver spread is required. How can this be achieved?

In a small number of patients with advanced liver involvement, clinical examination may reveal an enlarged metastatic liver. This is, however, relatively rare. One must therefore turn to investigations which to date, comprise, biochemical measurement of liver function, in conjunction with some form of hepatic imaging.
Biochemical Assessment:

Shortly after the development of routine measurement of liver function, in particular alkaline phosphatase, Gutman et al (1940) reported elevated levels of this latter enzyme in 30 patients with a variety of liver metastases.

The ability of biochemical assessment in the pre-operative period of liver function, in predicting metastases is variable. Castagna et al (1972) were only able to detect abnormalities of liver function in 58% of patients with proven metastases. Other groups (Baden et al, 1971; McCarthy et al, 1970), have reached similar conclusions. The range of predictive accuracy of liver metastases from colorectal cancer, in these studies ranged from 65-77%.

Measurement of liver function biochemically can lead to both a high false positive and false negative rate. Temple et al (1983), reported a false positive result in 76.5%, and a false negative result in 34.8% of 40 patients in whom alkaline phosphatase was measured pre-operatively. The positive predictive accuracy of this study was 65.2%, the negative predictive accuracy only 23.5%. It would therefore appear that pre-operative biochemical assessment by measurement of liver function is too inaccurate for routine peri-operative assessment of patients with overt metastases in the majority of surgical departments.

Neither do pre-operative liver function tests appear, in isolation, able to predict which patients have "occult" spread and who will
subsequently develop metastases. Tartter et al (1984) studied pre-operative alkaline phosphatase in almost 300 patients with normal livers at laparotomy. 38% of these patients had an elevated level of alkaline phosphatase pre-operatively, however there was no significant association with the subsequent development of hepatic metastases. Twenty six per cent of patients with normal levels prior to operation developed metastases subsequently, compared with 17%, whose alkaline phosphatase was raised.

Mooney et al (1980) in a similar study identified 23 risk factors for the development of liver metastases. The three most significant factors were pre-operative weight loss, a Dukes' 'C' tumour, and an elevated alkaline phosphatase pre-operatively. Combinations of these three factors correctly predicted the subsequent development of liver metastases in 72% of those who developed spread within 15 months of operation. Thus the exact role of assessment of liver function in assessing patients with rectal tumours remains unclear. In this investigation, routine liver function tests were performed in all patients entered into the study, in an attempt to define their role in peri-operative assessment.
The Role of CEA and APRP in the Identification of Liver Metastases:

It has been accepted since the early days of measurement of CEA in colorectal cancer that highly elevated serum levels are indicative of widespread metastatic disease (Dhar et al, 1972; Herrera et al, 1976; Staab et al, 1981). Szymenera et al (1982) reported that almost 60% of patients with advanced liver metastases had a pre-operative CEA level of greater than 20ng/ml. In a subsequent study (Szymenera et al 1985), this percentage rose to 95%. Similar results were reported by Lewi et al (1984) who reported raised CEA levels preoperatively in 70% of patients with disseminated disease and Wanebo et al (1978b), who found that 65% of patients with liver metastases had raised levels of CEA pre-operatively. Wanebo et al stated in their paper (1978b), that CEA levels tended to be higher in patients with liver or other distant metastases, compared with those in whom there was extensive local spread. It was also implied in this study that patients with elevated CEA levels pre-operatively, in whom no overt liver metastases were detected at laparotomy, and in particular those whose CEA remained elevated, were at greater risk of developing metastases subsequently. This suggests that CEA levels may be of use in detecting "occult" metastases.

Finlay and McArdle (1983), however failed to confirm the usefulness of CEA in the detection of "occult" spread. Of 13 patients with a "normal" liver at laparotomy, who subsequently...
developed metastases, and were thus deemed to have "occult" metastases at the time of surgery, only one had an elevated CEA. In a further seven, CEA levels did rise before death of the patient.

Ward et al (1977) suggested that the addition of pre-operative levels of certain APRP may refine the ability of CEA to predict metastatic from non-metastatic colorectal carcinoma. Ward et al reported that pre-operative levels of alpha-1-antitrypsin (AAT) and alpha-1-acid glycoprotein (AGP) were highest when the primary tumour was complicated by metastases. Using logistic discriminant analysis, Ward et al were able to predict metastases in 19 of 22 (86%) patients.

Cooper and Stone (1979) have, however, demonstrated that AAT levels show marked genetic variation, and therefore in a further study (De Mello et al, 1983), other acute phase reactants were estimated pre-operatively. Using a discriminant function, which included CEA, gamma-glutamyl transferase, phosphohexose isomerase alpha-1-antichymotrypsin and sex of the patient, De Mello et al were able to identify 16 of 18 (89%) patients with metastases. Only six of 82 (7%) patients were unclassified. Thus it would appear that combination of CEA and APRP can predict metastasis from colorectal tumours, with some degree of accuracy. As detailed in Chapter 3, these compounds were measured pre-operatively, in order to help define local spread. Therefore the ability of these compounds to predict liver metastases has also been addressed in the subsequent chapter.
Imaging Investigations in the Identification of Hepatic Metastases:

There are three main imaging techniques which have been used for the detection of liver metastases, ultrasound, isotopic scanning, and more recently computerised tomography. There are now several studies which have compared the relative value of these three modalities (Snow et al 1979; Taylor et al 1981, Smith et al 1982; Alderson et al 1983, Temple et al 1983).

Three of these studies (Snow et al 1979; Smith et al 1982; Alderson et al 1983), assessed all three techniques, and a mean derived from these publications suggests a sensitivity of ultrasound of 73%, isotopic scanning 85% and CT scanning 88%. Taylor (1981) compared ultrasonic examination with isotope scanning and reported a similar sensitivity for detection of overt metastases, of 93% for isotopic examination and 87% for ultrasound. Taylor continued that as ultrasound is widely available, this should be the screening modality used to detect liver metastases pre-operatively. He did, however, recognise that ultrasound examination was extremely operator dependent, and had a failure rate of at least 10%.

In a more recent study (Temple et al 1983), isotopic scanning was compared with CT scanning in 40 patients. Again the overall diagnostic accuracy of the two modalities was similar, being 81% and 85% respectively. One advantage of CT over isotope scanning in this study was that CT was more accurate in predicting the "tumour load" in the liver.
All these comparative studies can, however, be criticised, on the grounds that the "gold standard" for detection of metastases was the presence or absence of overt spread at laparotomy. As previously alluded to, however, this is a very inaccurate assessment. Finlay and McArdle (1982) have reported that 29% of patients with "normal" livers subsequently develop metastases. This study suggested that pre-operative CT scanning may help to identify such "occult spread", and was superior to both ultrasound and isotope scanning.

A study by Ostfeld and Meyer (1981) revealed similar results. Twenty one percent of patients with normal isotope liver scans, who died in the immediate post-operative period were found at post-mortem to have liver metastases, the majority less than 2 cm in diameter.

Of all three modalities, however, isotope scanning appears least attractive. Although of equal sensitivity when compared with ultrasound and CT scanning, it has a high false positive rate, 26% in one series (Snow et al 1977) and is subject to a high degree of interobserver variation (Gjorup et al 1985).

Moreover, for detection of "occult" metastases, many of which are below 1 cm, which is beyond the resolution of standard scintigraphy, isotope scanning does not appear likely to be effective. Ultrasound has a slight advantage in detecting colorectal liver metastases as these appear more echogenic and are thus more
likely to be detected (Debongnie et al, 1981). However, CT scanning, which appears able to detect intrahepatic lesions of from 1-1.5cm (Philips et al, 1975) would appear a more suitable method for detecting "occult spread".

Ultrasound examination, at the commencement of this thesis was the standard imaging method used for scanning patients with rectal tumours for hepatic metastases. In Chapter 4, therefore, a study is outlined, which compares this standard method, with CT scanning of the liver, in an attempt to define which modality is superior for the perioperative assessment. Since the inception of this study, alternative imaging methods have been suggested, including measurement of altered blood flow (Leveson et al, 1985) and intra-operative ultrasound examination, (Gozzetti et al, 1986). However, these as yet are unproven.

The combination of biochemical measurement of liver function, measurement of CEA and APRP and imaging techniques is evaluated in the discussion in Chapter 4.
PART II

Assessment of Biological Parameters of Rectal Tumours

The previous section of this introduction has outlined the importance of the spread of rectal carcinoma and outlined the methods by which it can be detected.

In the second part of this overview, the assessment of certain biological aspects of the tumour, its relation to spread of the disease, and implications for therapy are addressed.

Firstly, the question of histological assessment of a rectal carcinoma is discussed.
At the Leeds General Infirmary, in common with the majority of clinicians dealing with rectal tumours, it was routine practice to perform a preoperative biopsy via the sigmoidoscope. This was performed firstly, to confirm the diagnosis of neoplasia, and secondly to assess the degree of tumour differentiation.

It was Broders (1925) who first developed a grading system for rectal carcinomas. This system was based on the proportion of undifferentiated cells and mitotic figures within a tumour.

In 1939 Grinnell proposed a modified grading system based on the observation that the propensity for malignant cells to stream out and mingle with normal cells had prognostic value. The grading also took into account the degree of glandular arrangement, the polarity of the cells, and the position of nuclei relative to the basement membrane.

Dukes (1940) proposed his own histological grading system. Dukes' grading comprised five groups, I to V. At one extreme, Grade I tumours closely resembled adenomas, but with evidence of infiltration. At the opposite end of the spectrum, grade V or colloid tumours, were highly anaplastic. Dukes applied this system to nearly a thousand consecutive rectal tumours. He extended this grading to colonic carcinomas, and discovered that those tumours are generally more favourably differentiated, a finding which
echoed that of Grinnell (1939). Dukes subsequently redefined his system, and incorporated some of the ideas of Grinnell (1939). Dukes' modified grading now comprised low, average and high grades of malignancy, and a separate category of colloid carcinoma.

Dukes and Bussey (1958) subsequently applied this modified histological grading system to 2,097 rectal cancers from St Mark's Hospital. Five year survival for patients with low, average and high grades of malignancy were 77.3%, 60.6% and 28.9% respectively. Thus as a prognostic indicator, degree of differentiation appeared important. The value of such a grading system was confirmed by other groups, (Dunning et al, 1951; Copeland et al, 1988).

The grade of a tumour is important when comparing the published results of treatment, but more recently has assumed an important role in selection of patients for both surgical and adjuvant treatment.

With regard to the selection of surgical approach, the grade of the pre-operative biopsy, is frequently used to determine which operation is performed, particularly in the choice of procedure for early carcinomas. Lock et al (1978) states that local excision should not be performed where the pre-operative biopsy demonstrates a high grade of malignancy, a position adopted earlier by Morson et al (1977). A similar policy was advocated by Whiteway et al, (1985) who also addressed the selection of tumours
for local treatment. This group did however discuss a potential problem with reliance on grading a single pre-operative biopsy, a subject I will return to shortly.

Many factors must be taken into account when deciding whether a rectal tumour, which requires a major resection, should be removed by a sphincter saving approach or via an abdominoperineal excision. Histological grading of the pre-operative biopsy is one of these factors. Some authors (Golligher, 1984) advocate that a cancer of the mid and low rectum, which prove to be poorly differentiated on pre-operative biopsy, is not suitable for a sphincter saving procedure. The major reason for this stance, is inadequacy of the distal resection margin, if an anterior resection is attempted. Poorly differentiated tumours are more likely to have spread more than 1cm distally (Williams et al, 1983) and thus would seem to require a greater distal margin of excision. The problem of the extent of the distal resection margin has become more complex since the introduction of the circular stapling gun, which allows a greater number of low tumours to be considered for anterior resection. Thus histological grading of a pre-operative tumour sample carries considerable weight in the choice of surgical approach for the majority of clinicians.

The degree of differentiation may also play a role in the selection of adjuvant treatment. Poorly differentiated tumours are more likely to be associated with extrarectal spread, (Dukes and Bussey, 1958; Wood et al, 1981) and a high rate of local recurrence
Poorly differentiated lesions one could argue are "high risk" tumours and should thus be considered for adjuvant therapy.

Histological assessment of pre-operative biopsies therefore assumes an important role in a potential peri-operative assessment system, in particular, with regard to a choice of surgical procedure. There are, however, considerable problems with histological assessment of differentiation, which have recently come to light.

There is at present a general lack of agreement on specific, reproducible criteria for grading rectal carcinomas which then becomes a subjective assessment by different pathologists. In addition, it is known that varying histological grades can be found in different areas of the same tumour, (Grinnell, 1939; Qualheim and Gall, 1953). Furthermore, it is questionable whether a pre-operative biopsy actually reflects the differentiation of the main tumour bulk.

Accepting these limitations led Qualheim and Gall (1953) to question whether histological grading of colon carcinoma was a valid procedure. They reported that only 28% of tumours showed a uniform grading throughout, and concluded that most biopsy samples would not be representative of the main tumour. It has further been suggested that the more poorly differentiated cells may lie at the deepest part of the tumour, well away from the biopsy forceps.
which can remove only a superficial portion of tumour, (Whiteway et al, 1985).

One further problem with relying on a histological grade to determine treatment, is that of observer variation. Grading of a rectal tumour is as yet not objective. Blenkinsopp et al (1981) conducted a large survey of pathological reporting, of large bowel cancers, which involved pathologists at 22 hospitals. The proportion of tumours placed into the three categories varied widely, well differentiated 3-93%, moderately differentiated 8-82%, and poorly differentiated 5-30%, thus demonstrating very different grading criteria.

Thomas et al (1983) have further illustrated the problem. In this study 100 rectal tumours, with corresponding pre-operative biopsies were assessed "blind" by several experienced pathologists. The intra-observer agreement between the pre-operative biopsy and the main tumour varied from 56-69%. Furthermore, poorly differentiated tumours, which would appear to be the most important group with regard to treatment, could only be correctly predicted from the pre-operative biopsy by one experienced gastro-intestinal pathologist, in only 52%. Thus almost half the poorly differentiated tumours, which would appear to be the most important group with regard to treatment, could be correctly predicted from the pre-operative biopsy. The situation proved even worse when inter-observer variation in reporting poorly differentiated tumours was considered. In only 33% of biopsies was
agreement between observers reached that the tumour was poorly differentiated. The taking of multiple pre-operative biopsies in the study failed to improve the results.

It would appear therefore, that to have treatment decisions on such a variable factor as histological assessment of a pre-operative sample is irrational and should not be included in a peri-operative assessment. A less subjective assessment of cellular differentiation is required, and the recently introduced flow cytometer, may provide a more objective solution.

Flow cytometry is a relatively new method for determining the nuclear DNA content, and thus establishing the ploidy status of a cell, (Sugarbaker et al, 1979). Various cytogenetic investigations have indicated that karyotypic abnormalities are present in malignant tumours (Atkin, 1962; Gripenberg et al, 1977; Sandberg, 1981). Such karyotypic changes in tumours appear to reflect genetic instability and a higher mutation rate, (Ohno, 1971). Initially, attempts to determine the prognostic value of tumour ploidy were made with the use of the Feulgen stain and chromosome counts, (Atkin, 1962; Bohm and Sandritter, 1975; Nasiell et al, 1978). In these early studies Atkin (1964, 1972) concluded that tumours of the uterine cervix and breast, with a predominantly diploid cell population, ie a normal amount of chromosomal material, had a better prognosis than those containing mostly cells with an abnormal amount, so called aneuploid, or non
diploid cells. These findings were later confirmed in other

The recent development of flow cytometry, a more rapid technique
for cellular DNA analysis has allowed a number of tumours to be
analysed for ploidy status. Several studies have, using this
technique, confirmed abnormalities of DNA distribution in various
human tumours, (Bichel et al, 1977; Vindelov, 1977; Tribukait and

Flow cytometric assessment of cellular DNA content is based on
rapid measurements of fluorescence of specific dyes which bind
stoichiometrically to double-stranded DNA. The fluorescence is
then measured by passing the cells singly through a laser beam
with an appropriate wavelength. The fluorescence detected is
directly proportional to the amount of DNA in each cell. By
computer analysis, a DNA histogram is built up which can be
compared with that of a standard diploid population of cells.
Further details of the technique are outlined in Chapter 5.

Flow cytometry has been applied to colorectal carcinomas. Woolley
et al (1982) measured the cellular DNA content of 33 colonic
carcinomas and compared these findings with the clinical outcome
of the respective patients. Twenty tumours were deemed to be
diploid, the remaining thirteen aneuploid. Of the 20 with diploid
tumours, 65% of patients were alive and free from disease at five
years. However, of those patients whose tumours were aneuploid, only 7.5% survived five years, a highly significant difference.

Further validation of the prognostic value of ploidy in colorectal cancer came from Armitage et al (1985). This group examined paraffin embedded material from 134 patients with colorectal tumours, the survival details of whom were known. Fifty four percent of the tumours were DNA aneuploid. Five year survival for those patients with aneuploid tumours was 19% compared with 43% for patients with diploid cancers, again a highly significant difference. One further interesting factor revealed in this study, was that ploidy status appeared to be independent of both Dukes' stage of the tumour, and degree of differentiation. Armitage et al concluded that cellular DNA content can be regarded as a measure of tumour aggressiveness, and appears to be an independent indicator of survival in colorectal cancer.

Similar conclusions have been reached by other workers in this field. Banner et al (1985) examined 56 invasive colonic carcinomas. This study confirmed that ploidy appears independent of histologically determined degree of differentiation, but differed from the findings of Armitage et al (1985) in that aneuploidy appeared to correlate with a more advanced Dukes' stage of the tumours. Furthermore depth of invasion and nodal status were strongly associated with ploidy status. Banner et al concluded that ploidy status should replace subjective histological criteria in staging colorectal tumours.
Kokal et al (1986) in a similar study, to that of Armitage et al (1985), examined paraffin embedded specimens from 77 colorectal tumours. Dukes' D tumours were, however, excluded. This study concurred with that of Banner et al (1985) in that aneuploidy was associated with an advanced Dukes' stage of the disease. Twenty five of the 29 (86%), Dukes' C1 and C2 tumours were aneuploid, 25 of the 48 (52%), A and B tumours were aneuploid. Almost half the aneuploid tumours developed recurrent diseases during the course of the study, whereas no patient with a diploid growth had developed recurrence over a similar period of time. Using a logistic regression analysis, Kokal et al concluded that ploidy of a colorectal tumour, was the single most important prognostic factor. This conclusion has been questioned by Goh et al (1987) who, whilst agreeing with the prognostic importance of ploidy, using similar regression analysis, found standard pathological staging to be of greater significance.

There is agreement generally, however, that determination of tumour ploidy status is an objective measure, superior to standard subjective histological examination of colorectal cancer, and provides a good prognostic guide, with regard to survival and recurrence. This appears the case for rectal, as well as colonic carcinomas, (Forslund et al, 1984). It would therefore appear advantageous to include such an assessment in a peri-operative assessment system of rectal tumours. Furthermore, by identifying a group at high risk of recurrence, ie, aneuploid tumours, flow cytometric assessment seems a more logical basis for selection of
treatment, both operative and adjuvant. Should, therefore, flow cytometric analysis replace histological examination of pre-operative biopsies?

One word of caution is necessary at this stage, however. Colorectal tumours are known to have a heterogeneous cell population, and this has been demonstrated flow cytometrically with regard to ploidy status in a large percentage of tumours, (Peterson et al, 1980; Rognum et al, 1982; Tribukait et al, 1983; Quirke et al, 1985). Thus the same problem arises with flow cytometry, as with histological examination of pre-operative biopsies:— does the ploidy status of the biopsy reflect that of the main tumour. An attempt to answer this question is made in Chapter 5. This chapter addresses the accuracy of pre-operative biopsies, examined both histologically, and by flow cytometry, in predicting the status of the main tumour assessed in a similar manner, and attempts to define whether routine flow cytometry should replace subjective examination of a rectal neoplasm, in peri-operative assessment.

Let us now turn to examine more closely another biological parameter, a potential mechanism, by which rectal carcinoma may spread and metastasize, the role of proteolytic enzymes.
The Role of Proteolytic Enzymes in the Invasive Potential of Rectal Tumours

The importance of local spread of tumour within the pelvis in the prognosis of rectal carcinoma, leads to the obvious question of the mechanism by which this is achieved. In 1925, Fischer, described the ability of explants of tumour tissue, in organ culture, to release factors which were capable of degrading extracellular protein. The extracellular matrix in a dense lattice work of collagen and elastin embedded in a viscoelastic ground substance composed of proteoglycans and glycoproteins. For any tumour to invade, the cells must first breach the basement membrane, the structural backbone of which is type IV collagen (Llotta, 1986). The basement membrane is a continuous structure, impermeable to large proteins, (Vrako, 1974). This membrane only becomes permeable to cell movement during tissue healing and remodelling, inflammation and neoplasia. Thus for malignant cells to invade normal tissues, this barrier and the surrounding ground substance, the majority of which is collagen, must be dismantled. It was the discovery, by Gross and Lapierre (1962) of a specific enzyme, which degraded collagen, and was thus termed collagenase, which led the way to an understanding of the possible role of such an enzyme, in tumour invasion. Human collagenases comprise a variety of zinc and calcium dependent enzymes, which operate over a wide pH range, (5.2 to 9.5) at physiological temperatures. The characteristic action on the collagen molecule, is to split it into 3/4 and 1/4 fragments, (Woolley et al, 1980). Collagenase is found
under normal conditions in the colon and rectum, and appears to play an important role in the healing of colonic anastomoses, (Sturzacker and Hawley, 1975; Hawley, 1976; Young and Wheeler, 1984).

Dresden et al (1972) examined several human neoplasms, including colorectal tumours, and were able to isolate collagenase from them. The enzyme produced by these tumours appeared similar in every respect to the enzyme found in normal tissues. Woolley (1982) has also demonstrated collagenase in several human tumours, including gastro-intestinal, by using a specific antibody to immunolocalise the enzyme. Collagenase activity appears to increase in colonic tumours. A study by Tighe et al (1981) measured collagenolytic activity in 16 colonic tumours. Compared with normal colon, taken from the same patient, in 11 tumours, significantly higher collagenase activity was detected.

What is as yet unclear, is whether the tumour cells themselves are able to synthesise collagenase, or whether the tumour cells can stimulate host cells to produce the proteolytic enzyme. Woolley (1982) has suggested that both mechanisms may operate. Bauer et al (1979) have demonstrated that tumour cells can transform stromal cells in vitro, causing them to produce proteolytic enzymes. Several studies which have demonstrated that such enzyme activity is maximal at the infiltrating edge of a tumour. (Ljotta et al, 1977; Woolley, 1982; Shamberger and Rudolf, 1987.)
Collagenase, however, is not the only enzyme responsible for collagen degradation, other proteolytic enzymes are known to be involved. Collagenase achieves the initial cleavage of the collagen molecule. This activity occurs extracellularly, at neutral pH. Further degradation is thought to be achieved by the cathepsins, a group of enzymes found within intracellular lysosomes. These enzymes are active only at a relatively acid pH, (Van den Hoof, 1983), not normally found in the extracellular matrix, unless undergoing some inflammatory process, however a relatively low pH is found intracellularly. Of the cathepsins, Cathepsin B, appears to be of most importance in collagen breakdown. Etherington (1976) has postulated that the collagen fragments produced outside the cell by collagenase activity, are phagocytosed and digested by intracellular cathepsin B. This is not the only possibility, however. Graf et al (1981) have proposed an alternative method of action. They have suggested that cathepsin B acts outside the cell, not directly on the collagen molecule, but by transforming latent collagenase, the proenzyme of collagenase, in which form it is produced by the cell, to active collagenase.

Increased activity of cathepsin B has been discovered in a number of malignant tumours (Poole et al, 1978; Graf et al, 1981; Recklies et al, 1980), and similarly, most activity appears in tissue obtained from the region behind the invasive edge (Recklies et al, 1980). The role of cathepsin B may, however, be more complex than purely digestion of collagen. Sylven (1968) has reported the detachment of cells from glass surfaces by a cathepsin B at
physiological pH, implying that the enzyme can act on cell surface components, reducing cell adhesiveness, and facilitating invasion and probably metastasis. Thus, there is a growing body of evidence to suggest that proteolytic enzymes have potentially, an important role in the invasiveness of certain tumours.

The above outline of potential mechanisms by which tumour cells breakdown the extracellular matrix is obviously an oversimplification. A cascade of proteolytic enzymes is probably involved (Liotta, 1986). However, this in itself is insufficient to account for all the facets of tumour invasion. Other factors, such as free locomotion, lack of response to growth restraints, high metabolic activity and changed cell surface characteristics are all likely to play a part.

The role of proteolytic enzymes in rectal tumours has not been specifically addressed. Chapter 6 of this thesis contains the results of a study into the possible role of these enzymes in the local invasion and metastasis of tumours of the rectum and rectosigmoid, and their relationship to the pathological stage of the tumour.
Assessment of Tumour Growth Rate

The third biological parameter of rectal carcinoma addressed in this thesis concerns the growth rate of the tumour and its assessment by the development of a tumour model. Tumours with a fast growth rate constitute a "high risk" category. Present methods of assessment of tumour growth can only be achieved retrospectively.

In Chapter 7, a study is outlined in which an attempt is made to develop an in vitro methodology which would allow an assessment of tumour growth rate to be made, and relate this to the stage and aggressiveness of the cancer in individual patients.

Histopathological assessment of tumour stage, provide a static picture of the cancer at one particular point in its natural history. A more accurate picture of tumour aggressiveness may be furnished by measurement of growth rate and cell turnover. Measurement of growth rate in vivo is difficult to achieve and therefore an accurate in vitro model is required.

The development of an in vitro model from individual patients would also have important implications for therapy, particularly chemotherapy.

Once systemic spread of rectal cancer has occurred, be it macroscopic, or occult, the only chance of eradication lies in some
form of systemic therapy. The use of chemotherapy for rectal tumours has already been discussed. Unfortunately, as previously stated, the response rate in colorectal cancer is poor, with only 20% of tumours responding to the most effective drug - 5 fluorouracil, (Carter, 1978). Obviously if tumour sensitivity could be predicted prior to treatment, only those tumours found to respond could be treated and unnecessary toxicity avoided.

The ability to predict in the laboratory, the sensitivity of a tumour to drug therapy has been the subject of much research effort, involving in vitro and in vivo tumour models.

A comprehensive review of all the experimental models used in this field is beyond the scope of this thesis. The main avenues of investigation, however, have been in vitro culture, as monolayers or cell aggregates, and in vivo, with experimentally induced tumours, and transplantable tumours grown as xenografts in immune suppressed animals. The main aim of these experimental techniques to date has been to assess chemotherapeutic sensitivity.

**In Vitro Methods:**

Attempts at culturing tumour cells in vitro met with little success until the introduction of 'Clonogenic' methods (Salmon, 1979). Prior to this, whole fragments of tumour were cultured and often failed to grow, with stromal elements predominating. The clonogenic method requires a single cell suspension, from which
stem cells are selectively cultured. Unfortunately, colorectal
tumours have proved extremely difficult to culture, and early
attempts were largely unsuccessful (Buick et al, 1980; Richmond
and Billington, 1981). However more recent studies have improved
on this, with up to two-thirds of tumours producing satisfactory
colonies, (Trotter et al, 1984).

However, with clonogenic assays of single cell suspensions, and with
monolayer cultures, there are theoretical objections. Primarily that
such techniques fail to reproduce the conditions found in a tumour
in vivo, and thus any results obtained are of doubtful relevance to
the clinical situation.

**Carcinogen-Induced Tumours:**

There are now animal models for colorectal cancer, Druckrey et al
(1967), discovered that administration of dimethylhydrazine to rats,
induced colonic carcinoma. All grades of tumour can be produced,
from polyps through to extensive carcinoma, however one major
disadvantage is the time taken for these to be induced,
approximately six months. It is, however, interesting to note that
several groups who have used this model have reported similar drug
sensitivities to those found in human tumours, (Sych et al, 1978;

A modification of this animal model was the introduction of
transplantable tumour cell lines, Corbett et al (1977) used the
mouse as a model to produce colorectal carcinoma. From these
tumours, cell lines were developed which could be reproduced and
used for screening a wide variety of drugs. The technique is
however difficult. Of over 80 tumours originally produced, only
four resulted in transplantable cell lines.

These tumours are inoculated subcutaneously, and one criticism is
that in this situation the tumour may behave in a different way.
However, these tumours were found to have a similar range of
sensitivity to chemotherapy as did human tumours. Other workers
using this model have reported similar results. (Tsuruo et al, 1980;
Ball and Double, 1975). There are now a variety of cell lines
available, produced from human tumours, which can be utilised in a
similar manner to screen potentially useful chemotherapeutic
agents. (Agrez et al, 1983). Many of these cell lines bear little
relationship to a tumour in vivo, although more recently produced
cell lines do appear to retain some characteristics of the parent
tumour. (Whitehead et al, 1987).

**Xenografts:**

There are many criticisms which can be levelled at the use of in
vitro methods for determination of drug sensitivity. Ideally the
aim should be to determine the sensitivity of each individual
tumour to a "panel" of drugs. One potential method is use of a
xenograft. In this technique a fragment of a patient's tumour is
implanted into an experimental animal in a site which allows
assessment. Chemotherapy can then be administered, and the response of the xenograft measured. To achieve success, however, the recipient animal must be rendered immune-deficient. This can be achieved either by breeding congenitally athymic animals, such as mice, or by rendering an animal immunodeficient by thymectomy and irradiation.

Although such methodology retains many similarities to tumour growth in the human, one immediate problem arises. The host tumour interface is crucial in determining tumour progression. By using immune compromised animals, this interface becomes highly artificial. This apart, however, the xenograft model does provide the closest approximation to the clinical situation. Furthermore, human tumours maintained as xenografts appear to retain the characteristics of the parent tumour. (Houghton and Taylor, 1978; Steel et al, 1983), and show similar sensitivities to chemotherapeutic agents used in clinical trials (Giovanella et al 1983; Steel et al, 1983). The production of xenografts is, however, a costly and time consuming procedure. The reported success rate for achieving xenografts from colorectal tumours is approximately 50% (Steel et al 1983) and the results of chemosensitivity testing may take several months. There is as yet no ideal tumour model for colorectal cancer. A system which reproduces the conditions found inside a tumour growing in the body, but which has the advantages of in vitro methodology, namely reproduceability, cheapness and ease of manipulation but which provides quick results, is required. One tumour model which
perhaps comes the closest to this ideal is the multicellular tumour spheroid (MTS).

**Multicellular Tumour Spheroids:**

Multicellular tumour spheroids (MTS) are spherical aggregates of malignant cells which have the characteristics of in vivo tumours at an early avascular stage of tumour growth. Experimentally induced aggregates of animal cells have been widely used in biological research for many years. In early studies in amphibian embryos Holtfreter (1944) described a method for the generation of stable, spherical aggregates of embryonic cells. These aggregates were produced by culturing embryonic explants or single cells on agar as a substratum to avoid cell attachment to the bottom of the culture dishes. Subsequently the dishes were agitated to reduce cell-substratum interaction. Moscona (1952) published a study on the reaggregation of single cells from chick embryos on plasma clots. This technique was used to assess the capacity of embryonic and malignant cells (Moscona, 1957a, b) for reaggregation, proliferation and differentiation. In particular, the invasive potential of melanoma cells was demonstrated in vitro, (Moscona, 1957b). Adapting this technique Dabrowska-Piakowska (1959) demonstrated the ability of aggregates of C3H mammary tumour to invade muscle fragments in vitro. Moreover there appeared to be a remarkable structural similarity between the tumour cell aggregates and the original tumour tissue. Moscona (1961) further elaborated the technique for induction and growth of multicellular
aggregates. Using the modified methodology, Halpern et al (1966) were able to demonstrate that malignant cells were much more likely to produce aggregates than normal cells. Similar findings were reported by McAllister et al (1967) who demonstrated that virus induced hamster tumours would grow as aggregates in soft agar, whereas normal hamster cells would not. This loss of "anchorage-dependence" appears to be a feature of malignant cells, indeed the ability for colonial growth in soft agar is taken by some authorities as one criterion for malignancy (Bruland et al, 1985).

There are pronounced similarities between the primary tumours and the respective colonies produced. (McAllister et al 1967). Furthermore, these similarities persist if the aggregates are implanted as xenografts and allowed to form tumours. One further point reported by McAllister et al (1967) was that the almost spherical aggregates consisted of a central necrotic area with a variable rim of viable cells, with interspersed degenerate forms.

Studies on the response of these cellular aggregates to radiation therapy were initiated by Sutherland and his co-workers (Sutherland et al 1970, 1971; Inch et al, 1970). The cell aggregates investigated were obtained by culturing V79-I71 hamster lung cells in liquid media in "spinner-flasks". Because of their almost spherical shape, the aggregates were termed "multi-cell spheroids" or multicellular spheroids. Since these cellular structures resembled the modular structures observed in C3H mouse mammary tumours, (Sutherland et al, 1970; Sutherland and Durand, 1972) or in several
types of human tumour, (Thomlinson and Gray, 1955; Bennington, 1959) and since the survival curves were similar in radiation experiments to solid tumours, (Sutherland et al, 1970, 1971), multicellular spheroids were considered a suitable in vitro model for mimicking basic biological properties of cancer cells in vivo.

Spheroids exhibit a histological structure similar to that of solid tumours. This is not only true for the distribution of viable and necrotic areas, but apparently also true for the extracellular matrix. Similar matrix components, such as glycosaminoglycans can be identified in tumour spheroids, and corresponding original tumours, (Angello and Hosick 1982; Grover et al, 1983; Nederman et al, 1984).

The capacity to synthesise extracellular matrix, however, is lost when the cells are from monolayers. This confirms the general findings that the degree of structural and functional differentiations in the primary tumour may be retained in spheroid culture, a feature not seen in monolayer culture. It is often difficult for even experienced pathologists to differentiate morphologically from a primary tumour and spheroids derived from it.

A further characteristic common to both tumour and spheroid is the existence of large spatial variations in cellular proliferation, i.e., of proliferation gradients. Solid tumours contain proliferating areas close to microvessels, in areas well supplied with oxygen and nutrients, (Rajewski, 1965; Burns and Tarnock, 1970; Brammer et
Away from these vessels, cell cycle times are increased, as a consequence of diffusion-limited nutrient supply and restricted removal of toxic waste, (Born et al, 1976). These cells may become quiescent, arrested in Go-phase. Such proliferation gradients, not found in monolayer culture, have been well documented in multicellular spheroids. Several groups have demonstrated that proliferating cells in spheroids are localised within superficial layers; with a thickness of between 50-100 um, (Sutherland et al, 1971; Dertinger and Lucke-Huhle, 1975; Durand, 1976; Haji-Karim and Carlsson, 1978; Yuhas and Li, 1978).

With increasing depth into the spheroid, cell cycle times are prolonged and pass into a non-proliferating state. Bauer et al (1982) have reported that these cells in Go-phase match those found in vivo much more closely than those in monolayer culture.

Thus it appears that cellular heterogeneity, which is a general property of solid tumours occurs in multicellular spheroids. This is not the case in monolayer culture.

As a consequence of the proliferation gradients, the volume growth kinetics of spheroids are thus similar to that of solid tumours. In general, three phases of spheroid growth are seen. The first phase, during which all the spheroid cells are proliferating, is characterised by exponential growth, up to spheroid diameters of 50-200um, (Durand and Sutherland, 1975; Sutherland and Durand, 1976; Landry et al, 1982). During the second phase, cell-cycle
distribution starts to change towards an increasing accumulation of non-proliferating cells in central regions of the spheroids. (Durand, 1976; Haji-Karlm and Carlsson, 1978).

In addition mitotic cells are sequestered from peripheral parts of the spheroid into the culture medium, (Landry et al, 1982). Both these mechanisms result in a progressive reduction in the proliferating function of cells, (Durand, 1976), which results in a linear increase in spheroid diameter with time during the phase (Inch et al, 1970; Folkman and Hochberg, 1973; Sutherland and Durand, 1976; Yuhas et al, 1977, 1978; Yuhas and Tarleton, 1978, Yuhas and LI, 1978).

In the third phase of growth, lack of oxygen and nutrients at the centre of the spheroid, combined with accumulation of waste leads to a pronounced slowing of growth. Eventually growth stops and the spheroids attain a maximal diameter of from 1-4 mm depending on the cell type and method of culture, (Folkman and Hochberg, 1973; Haji-Karlm and Carlsson, 1978). Spheroids which have reached this diameter can be maintained in culture for several weeks, with virtually no increase in volume despite replenishment of culture medium. It is of interest to note that a similar situation occurs if spheroids are grown as xenografts in a host animal, unless vascularisation occurs, (Folkman, 1974). If the spheroid does become vascularised, then exponential growth occurs, (Folkman, 1971, 1976). Saturation of growth is a general feature
of tumour growth in vivo, however the host usually succumbs before this occurs, (Folkman, 1976).

The behaviour of spheroids gives useful insight into the possible evolution of tumours in vivo. Solid tumours may start as a single cell, or group of cells which have lost physiological control of proliferation, and form small avascular aggregates. These structures may remain dormant unless vascularisation occurs. A similar situation may arise with metastases, where clumps of cells break from the main tumour, lodge in a distant organ and undergo a similar process.

Nodular structures, similar to spheroids can be identified in the solid tumours, even in advanced states of growth in both the experimental animal and human tumours, (Inch et al, 1970. Sutherland et al, 1970; Folkman and Hochberg, 1973; Thomlinson and Gray, 1955; Bennington, 1969). These tumours could therefore be described as an assemblage of spheroids which are supplied by vessels at their surface. Thus tumour spheroids appear an ideal model for studying the early avascular growth of tumours in vivo. Spheroids can be considered as in vitro models of tumour microregions, or early metastases, which reflect the particular environmental conditions of cancer cells located at different distances from the nutritive vessels. Spheroids therefore, combine the attractions of in vitro methodology with many of the characteristics of in vivo tumour behaviour and thus approximate to an ideal tumour model.
In view of the attractions of the model, spheroids have been utilised widely for investigation of tumour biology. The model provides an ideal situation for investigation of cell to cell contact in tumours. (Landry and Freyer, 1984; Glaser, 1982; Lieberman et al 1982) and for studies on proliferation and differentiation (Landry and Freyer, 1984).

The early studies of Moscona (1957) and Dabrowska-Plakowska (1959) on invasion of normal tissues have been expanded (Schleich et al 1974; 1981) and also the mechanisms of tumour metastasis explored, (MacDonald and Sordat, 1980; Umbrecht and Erbe, 1979). Other studies have investigated host versus tumour reactions, (Sutherland et al, 1977, MacDonald and Howell, 1978; MacDonald and Sordat, 1980), including infiltration of the spheroid by lymphocytes.

Spheroids provide an excellent model for the study of the tumour micro-milieu, in particular to the metabolic aspects of tumour growth, (Sutherland et al, 1986; Muller-Klieser and Sutherland, 1985).

The potential use of multicellular tumour spheroids in selection of patients with rectal tumours for adjuvant therapy is also of particular relevance to this thesis. Although not specifically addressed in this study, the majority of work on adjuvant forms of therapy has been on ionising radiation. The majority of these studies have used cell lines, (Sutherland et al, 1970; Durand and
Sutherland, 1976; Dertinger and Lucke-Huhle, 1975). More recently, however, chemotherapeutic sensitivity evaluations, either using spheroids in culture, or from xenografts have been reported, (Sutherland and Durand, 1976; Yuhas and Li, 1978; Twentyman, 1980; 1982; Kwok and Twentyman, 1985; Jones et al 1982). Again many of these studies utilised cell lines derived from animals or humans. It would appear, however, that the response to chemotherapeutic agents in vitro, closely follows that when the same spheroid is tested in an experimental animal, (Yuhas et al, 1978). This obviously is an advantage, as results of chemosensitivity can be achieved more easily and much more expeditiously by using in vitro methodology.

There have been few studies which have specifically addressed the formation of, and evaluation of colorectal tumour spheroids, and those which have, have used cell lines derived from human tumours, (Lees et al, 1981; Sutherland et al 1986). In Chapter 7, therefore, a study is outlined, which investigates the growth of spheroids from primary colorectal carcinoma, their relationship to tumour 'aggressiveness' and the ability to define high risk groups. The use of the model in the assessment of chemotherapeutic agents for adjuvant treatment of the disease is also evaluated. As outlined above, the spheroid model possesses many characteristics of in vivo tumour behaviour, in particular the situation found in early metastatic lesions, such as the "occult" liver metastases, as previously described. Such a model could potentially identify biologically aggressive tumours which may require adjuvant therapy.
In addition to surgical excision. Furthermore if one could identify an effective chemotherapeutic agent, in the immediate perioperative period, in a patient whose macroscopic disease is resectable, then a case can be made for adjuvant treatment with that particular agent. The spheroid model, which can theoretically provide such information within one to two weeks may provide an ideal solution.

**Specific Aims of this Thesis**

The aim of this thesis is to assess individual patients with rectal tumours much more carefully than hitherto, firstly by identifying spread of the disease, and secondly by investigating aspects of the biological behaviour of the tumour. Thus patients at high risk can hopefully be identified and a more rational basis for treatment selection established.

The thesis naturally divides into two sections therefore. In Section I, the more accurate identification of tumour spread, both local and to the liver is addressed. In Section II, the biological parameters of individual tumours, histological grading, proteolytic enzymes and in vitro culture are explored.

In Chapter 8, both sections are brought together, as an overall perioperative assessment of rectal neoplasia, and the implications for basing treatment on a more individualistic approach discussed.
SECTION I

The Assessment of Local and Distant Spread
of Rectal Neoplasia
CHAPTER 2

The effect of local spread of rectal carcinoma on local recurrence and survival
Introduction

The fact outlined earlier in the introduction that mortality from carcinoma of the rectum has remained virtually static for the past 40 years, (Dukes, 1957; H E Lockhart-Mummery et al, 1976; Eisenberg et al, 1967; Whittaker and Goligher, 1976; Ohman 1982), has led to attempts to define certain high risk groups. One such group on which attention has focused consists of patients with colorectal tumours which at operation seem fixed or partially fixed. These patients have a reduced chance of survival compared with those who have mobile growths (Habib et al, 1983).

In a proportion of cases, however, the tumour is not fixed by direct malignant infiltration, but by an intense inflammatory response (Fig. 2.1.). The effect that inflammation has on survival, is however, unclear (Davies and Ellis, 1975; Bonfanti et al, 1982), but is is likely that in patients with tumours which exhibit this characteristic, adjuvant therapy is unnecessary and possibly harmful.

Previous studies have addressed colorectal tumours as a whole and therefore this initial clinical study was undertaken to clarify the effect that fixation of a rectal carcinoma, either by inflammation or malignant extension, has on recurrence and survival after apparently "curative" rectal excision.
PATIENTS AND METHODS

Six hundred and twenty-five patients who underwent rectal excision for adenocarcinoma of the rectum between 1957 and 1973, and in whom records were available for the analysis, were studied.

Of these 225 patients (38%) had a fixed, or partially fixed tumour, defined by the surgeon at laparotomy. Forty-six patients (20.4%) in this group had evidence of distal intra-abdominal spread, or were deemed inoperable, and were excluded, six (2.6%) were lost to follow-up and cause of death could not be established in the remaining 4 (1.8%). Histological examination of the specimens consisted of taking a minimum of two blocks through the full thickness of the excised tumour, including the lateral resection margins. Multiple sections were cut from these and examined by pathologists experienced in gastro-intestinal disease.

Of the 169 excised specimens of the cases included in the study, 124 (73.4%) had evidence of direct malignant spread to adjacent structures, but in 45 (26.6%) inflammatory adhesions only were present.

The recurrence rates and overall survival of this group were compared with an equivalent group of 180 patients, selected from the remaining 100 individuals with mobile tumours. The two groups were matched for age, sex and Dukes' stage (Table 2.1). The degree of differentiation and height of the lesion above the anal
margin were similar (Table 2.2). Although more individuals with fixed tumour had poorly differentiated lesions, this difference was not statistically significant. By definition, there were no Dukes' A lesions in the group fixed by malignant spread.

All the patients in the mobile group were considered at laparotomy to have undergone a 'curative' resection, this being defined by the surgeon as excision of all macroscopic tumour. Twenty-two patients (13%) in the fixed group were deemed to have undergone only a 'palliative' resection and were thus excluded from the overall survival data. Eight patients (4.5%) in the mobile group and 20 patients (11.8%) in the fixed group, who died within 28 days of operation were also excluded.

Accurate survival data were usually available from the patients' records, since most patients attended at least once annually following operation. Information not available from the patients' records was obtained from the patient's relatives, or from the General Practitioner.

The development of local and distant recurrence was defined according to previous criteria (William and Johnston, 1984). Data on recurrences were obtained from post-mortem reports, available in 32% of patients who died or from unequivocal clinical, histological or radiological evidence.
Differences between the groups were analysed using the $X^2$ test, with Yates correction for small numbers where appropriate. Five and 10 year corrected survival rates were calculated by life table analysis. Difference in survival were analysed using the method described by Colton (Colton, 1974 pp 237-250).
RESULTS

The operations performed are listed in Table 2.3. Sixty per cent of patients with mobile tumours underwent abdominoperineal excision (APER), as opposed to 74.5% of those whose tumour was fixed (p<0.01). The operative mortality rates were 4.5% for patients with mobile tumours, and 10.6% following curative resection of a fixed lesion (p<0.01), 18.8% of patients who underwent palliative resection died in the post operative period.

SURVIVAL (Figures 2.2 - 2.4)

Overall corrected five and 10 year survival was 66.9 and 62.3% respectively for patients with mobile tumours, 39.8 and 33.9% for those with fixed tumours (p<0.01).

In patients with direct malignant infiltration five and 10 year survival rates decreased to 28.5 and 22.9% respectively, whereas if inflammatory adhesions were present, the corresponding rates were 64.6 and 56.1% respectively (p<0.01). Survival rates of patients with tumours fixed by inflammation did not significantly differ from those of patients with mobile growths.

When the influence of lymphatic involvement was examined (Fig. 2.4) 62.9% of patients with mobile 'C' lesions survived five years compared with 43.5% with fixed 'B' tumour (p<0.01). Although this difference persisted at 10 years, the survival rates being 52.3 and
39.4\% \text{ respectively, this failed to reach statistical significance (p=0.14).}
LOCAL RECURRENT

41.3% of patients with fixed tumour due to direct spread, who underwent a "curative" operation, subsequently developed local recurrence, as opposed to 15.1% of patients with no extramural infiltration (p<0.01). The incidence of distant metastases was 43.4 and 30.2% respectively (p<0.05). Of patients who had evidence of inflammatory fixation 20% developed local and 30% distant recurrence, not a statistically significant difference from patients with mobile lesions.
DISCUSSION

This study was retrospective and thus relied entirely on the quality of previous records. It is therefore fortunate that meticulous records and accurate data were available on all patients included. As with any retrospective study, however, the groups were not entirely comparable. In an attempt to overcome this problem, patients with fixed and mobile tumours were matched for other known prognostic variable, such as age, sex and Dukes' stage. A higher proportion of patients with fixed tumours did, however, have poorly differentiated lesions. This difference was not statistically significant in itself and is unlikely to account for the difference in survival and recurrence rates between the groups.

A greater percentage of patients with fixed lesions underwent APER. Since this operation is considered to be more radical than its alternatives, differences in survival and recurrence are unlikely to be biased by this discrepancy.

The problem of inter-surgeon variation has been highlighted by Phillips (Phillips et al, 1984) who described widely differing rates of local recurrence differences. One study (Davies and Ellis, 1975) reported no difference in survival between patients who had neoplastic adhesion and those with inflammatory fixation. This is contrary to the findings in this study and also to those of Jensen et al (1970) who reported five year survival rates of 13.5 and 50%
for patients with neoplastic and inflammatory fixation respectively, and Bonfanti et al (1982) who quoted 32 and 75% respectively.

The true incidence of recurrence will be underestimated by a retrospective investigation. Patients may die without clinical evidence of recurrent disease and not all undergo a post mortem examination. Local recurrence, particularly after APER, is difficult to detect clinically. A higher proportion of patients in this study underwent APER and therefore any underestimate of local recurrence would be biased in favour of this group. Despite this bias, the results indicate a significantly higher rate of local recurrence in patients with extramural infiltration, and this finding lends further weight to the argument.

Patients with local malignant involvement subsequently developed distant metastases more frequently than those with no spread, and this may be explainable on the greater degree of venous invasion seen in these tumours (Dukes and Bussey, 1941). Again the incidence of distant recurrence is probably an underestimate, a proportion of patients may have had "occult" metastases (Finlay and McArdle, 1982) not detected preoperatively or at laparotomy, but this applies equally to both groups.

Although Dukes recognised the importance of extramural spread (Dukes and Bussey, 1958), he did not include it in his classification. The newer staging system suggested by Wood (Wood et al 1981) which would take account of the prognostic significance of local
spread, but this is again a pathological classification and as such has the limitation that the information is only available after treatment has been initiated. Several trials of adjuvant chemotherapy and radiotherapy in rectal cancer are underway at present. The recent results of the Medical Research Council (MRC) trial of low dose preoperative radiotherapy (5 Gy or 20 Gy) in operable rectal cancer (Second Report of an MRC Working Party 1984) failed to confirm the benefits on survival demonstrated by the Toronto trial (Ride et al, 1977), and the National Veterans Administration Trial (Roswit et al, 1975), but did suggest that patients with tumour fixed or partially fixed on pre-operative assessment, may derive some beneficial effect from preoperative irradiation with multiple fractions. These results have prompted a further trial to evaluate the effect of a higher dose of radiotherapy (40 Gy) in patients who on clinical examination have fixed or partially fixed rectal cancers. Clinical assessment of fixation of rectal tumours is only accurate if performed by experienced clinicians (Nicholls et al 1982). Only tumours in the lower half of the rectum are easily palpable, and there is the epidemiological evidence from America to suggest that distribution of cancer within the rectum is changing with only 12% palpable on digital examination (Berg and Howell 1974). Furthermore, if palpable even the most experienced surgeon cannot differentiate between malignant and inflammatory fixation. This study would suggest that a quarter of clinically fixed tumours are tethered by inflammation and fibrosis, and some of these "fixed" lesions are Dukes 'A' carcinomas. These patients do not require preoperative
radiotherapy which may lead to unwarranted morbidity particularly with the higher dosage which is being used in present trials.

This study confirms the poor prognostic significance of fixation of rectal tumour, but only when this is due to contiguous malignant spread.

Identification of these high risk patients who may benefit from adjuvant therapy requires a more accurate method of preoperative assessment than clinical examination as previously outlined. Furthermore, clearer definition of patient groups for the future planning of clinical trials of adjuvant therapy is a necessity, if erroneous results are to be avoided. The following Chapter evaluates a more extensive assessment of extra rectal tumour spread, in an effort to identify more accurately these high risk patients. The studies outlined in the next chapter compare standard clinical assessment of local tumour spread, with assessment by sophisticated imaging and biochemical methods.
<table>
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<tr>
<th>Age Range</th>
<th>Mobile (n=180)</th>
<th>Fixed (overall) (n=169)</th>
<th>Fixed by malignancy (n=124)</th>
<th>Fixed by inflammation (n=45)</th>
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<td>30-39</td>
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<td>6 (3.6)</td>
<td>5 (4.0)</td>
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<td>40-49</td>
<td>19 (10.0)</td>
<td>14 (8.3)</td>
<td>12 (9.7)</td>
<td>2 (4.4)</td>
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<td>50-59</td>
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<td>10 (22.3)</td>
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<td>60-69</td>
<td>72 (40.0)</td>
<td>68 (40.2)</td>
<td>49 (39.5)</td>
<td>19 (42.3)</td>
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<td>70-79</td>
<td>40 (22.0)</td>
<td>40 (23.7)</td>
<td>28 (22.5)</td>
<td>12 (26.6)</td>
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<th>Fixed by inflammation (n=45)</th>
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<td>97 (53.8)</td>
<td>94 (55.6)</td>
<td>68 (54.8)</td>
<td>28 (57.7)</td>
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<td>F</td>
<td>83 (46.2)</td>
<td>75 (44.4)</td>
<td>56 (45.2)</td>
<td>19 (42.3)</td>
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<th>Fixed by inflammation (n=45)</th>
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<td>59 (34.9)</td>
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<td>80 (47.3)</td>
<td>61 (49.2)</td>
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<tr>
<td>C2</td>
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<td>23 (13.7)</td>
<td>21 (16.9)</td>
<td>2 (4.5)</td>
</tr>
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</table>
### Site of lesion above anal margin and degree of differentiation

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<thead>
<tr>
<th>Degree of differentiation</th>
<th>Mobile</th>
<th>Fixed</th>
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<tbody>
<tr>
<td>Well</td>
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<td>23 (13.7)</td>
</tr>
<tr>
<td>Moderate</td>
<td>44 (24.4)</td>
<td>35 (20.7)</td>
</tr>
<tr>
<td>Poor</td>
<td>102 (56.8)</td>
<td>111 (65.6)</td>
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<tr>
<td>Site of lesion</td>
<td></td>
<td></td>
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<tr>
<td>Upper third (13-19 cm)</td>
<td>23 (12.8)</td>
<td>19 (11.2)</td>
</tr>
<tr>
<td>Middle third (7-12.5 cm)</td>
<td>87 (48.3)</td>
<td>78 (46.2)</td>
</tr>
<tr>
<td>Lower third (0-6.5 cm)</td>
<td>70 (38.9)</td>
<td>72 (42.8)</td>
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Page 112
<table>
<thead>
<tr>
<th>Operation</th>
<th>Mobile</th>
<th>Fixed</th>
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<tr>
<td>Abdominoperineal excision</td>
<td>108 (60.0)</td>
<td>126 (74.5)</td>
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<td>Anterior resection</td>
<td>64 (35.5)</td>
<td>34 (20.1)</td>
</tr>
<tr>
<td>Abdomino-anal pullthrough</td>
<td>8 (4.5)</td>
<td>9 (5.4)</td>
</tr>
</tbody>
</table>
Figure 2.1. Histological section of rectal tumour (arrowed) surrounded by dense inflammatory response and fibrosis.
FIGURE 2.2
Corrected 10 year survival for patients with mobile and fixed tumours.
FIGURE 2.3
Corrected 10 year survival for patients with mobile tumours, those fixed by malignant infiltration and tumours fixed by inflammatory adhesions.
FIGURE 2.4
Corrected 10 year survival for patients with Dukes' B and C lesions: mobile and fixed by malignant infiltration.
CHAPTER 3

The Identification of Local Intrapelvic Spread of Rectal Tumours
INTRODUCTION

As outlined in Chapter 2, local tumour extension into perirectal tissues is an important prognostic indicator, and the presence, or absence of such spread, may well define the requirement for adjuvant treatment.

The standard method of assessing local tumour spread is digital rectal examination. If performed by an experienced operator, low rectal tumours can be accurately assessed (York Mason, 1976; Dixon et al, 1981). It may be difficult to assess a rectal tumour digitally, if it is high in the rectum, or if the patient is obese. An unexpected finding in an American study (Berg and Howell, 1974) was that in the United States, low rectal cancer appeared to be less common than 20 years ago. Indeed in this series, only 30% of rectal carcinomas were below 8 cm, and thus within easy reach of the examining finger.

The introduction of computerised tomography allows the pelvis and rectum to be examined in a fashion previously unavailable. Several studies have now reported on the accuracy of detecting rectal and sigmoid tumours by pelvic CT, and moreover CT allows more accurate assessment of extrarectal tumour spread (Redman, 1977; Thoeni et al, 1981; Hamlin et al, 1981; Koehler et al, 1984). In this section of the study, therefore, clinical assessment of extrarectal spread, performed by two experienced clinicians, was compared with assessment by pelvic CT scanning. The role of
serum measurements of oncofetal antigens as a screening procedure for colorectal cancer has not fulfilled initial expectation and although raised levels of carcinoembryonic antigen (CEA) are found in the serum of a proportion of patients with colorectal cancer (Dhar et al, 1972) this is too non-specific for use as an early diagnostic tool. Raised levels of CEA are, however, found in patients with metastatic and locally invasive disease (Staab et al, 1981), and may thus prove of use as a preoperative measure of tumour spread.

Acute phase reactant proteins (APRP) are mainly glycoproteins produced primarily by the liver as a non-specific response to a variety of stimuli, including acute and chronic inflammation and cancer (Koj, 1974). Several authors have reported on the use of CEA and APRP in staging gastrointestinal tumours (Staab et al, 1981; Ward et al, 1977; de Mello et al, 1983), and in view of this, serum levels of CEA and two acute phase reactant proteins, alpha-1-acid glycoprotein (AGP) and C-reactive protein (CRP) were measured preoperatively, initially in a group of 99 patients with colorectal tumours, to evaluate the accuracy of the technique and subsequently in 76 patients who comprised the peri-operative assessment study group. Thus this part of the study was undertaken to reevaluate the role of these serum markers in the extended peri-operative assessment, particularly in refining the distinction between malignant and inflammatory fixation.
PATIENTS AND METHODS

a) **Comparison of Clinical Assessment and Pelvic CT Scanning in Assessment of Local Spread**

The patients included in the studies outlined in this and the following chapters comprised 76 consecutive individuals who presented to the Leeds General Infirmary with a rectal neoplasm at or below 12 cm from the anal verge, as measured by rigid sigmoidoscopy.

Any patient considered unfit for surgical intervention was excluded, as was any patient whose tumour had complicated ulcerative colitis or familial adenomatous polyposis.

Of the 76 patients 43 (56.6%) were male and 33 (43.4%) female.

The median age of the patients was 69 years, with a range of 45–87 years.
Clinical Examination and Assessment of Local Spread

A full clinical examination of each patient was undertaken independently by two experienced clinicians, myself and one consultant surgeon.

Digital rectal examination was performed with the patient in the standard left lateral position. It was noted whether the tumour was palpable and for this the tumour had to be sufficiently accessible for the assessment of fixity. If considered fixed, each clinician had to specify whether this was:

1) **minimal** extrarectal spread, defined as tethering of the tumour;

2) **moderate** if there was established extrarectal spread, or

3) **extensive** where extrarectal structures were invaded.

For the latter the structure involved had to be specified. The clinician was also asked to specify whether such fixation, in his opinion, was due to neoplastic infiltration, or peritumoral inflammation. Palpable extrarectal lymph nodes were sought.
Each clinician was also asked to assess the extent of circumferential spread of the tumour by specifying the quadrants of rectum involved.

As digital rectal examination of an anaesthetised patient often allows a more extensive assessment of a tumour, particularly for very low lesions involving the upper anal canal, which are often painful and difficult to assess adequately, each patient was reassessed by the same two clinicians under anaesthetic, with the patient in lithotomy position. If this altered the original assessment, this was recorded.

**Imaging Techniques**

**Computerised Tomography (CT Scan)**

CT scans of the pelvis were performed on each patient to assess pelvic spread, and the presence or absence of involved lymph nodes.

CT scans were obtained using a rotating fan beam machine with a scan time of 4.8s (Phillips T310). Each patient took 800 ml 4% gastrografin orally 40 minutes before the scan. For examination of the rectum, the patient lay prone and contiguous slices of 6 mm were taken from the level of the anal canal, up to the mid part of the sacro-iliac joints, and more cranially if initial review suggested the lesion had not
been fully encompassed. Local tumour spread was defined as present or absent (Fig. 3.1). If present spread was classified as slight, moderate or extensive. Spread was categorised as slight if the tumour had breached the muscular wall of the rectum and extended just into perirectal fat (Fig. 3.2), moderate when there was established spread to extrarectal tissues (Fig. 3.3) and extensive where other pelvic organs or bony structures were involved (Fig. 3.4).

The CT scans were performed throughout by one consultant radiologist experienced in the technique. The scans were assessed "blind", with no knowledge of the clinical findings.

Laparotomy Assessment

Every patient in the study underwent laparotomy, during which the local status of the tumour was classified as fixed or mobile.

Evidence of intra-abdominal dissemination was also sought, and if suspected confirmed by biopsy. The laparotomy findings and operative details are recorded in Tables 3.1-3.2. Thirty two tumours (42%) were in the mid rectum and 44 (58%) in the lower rectum. Thirty seven tumours (49%) were classified as mobile, and 39 (51%) were classified as fixed or partially fixed.
Ten patients (13%) had overt liver metastases.

Twenty two patients (29%) underwent abdominoperineal excision, 48 (63%) low anterior resection, and two patients had a Hartmann's procedure.

In 48 patients (63%) the operating surgeon deemed the resection to be "curative", however in the remaining 28 (37%) residual tumour was left behind, either as liver metastases (n=10), residual pelvic tumour (n=18) or a combination of both.

**PATHOLOGICAL EXAMINATION OF RESECTED SPECIMEN:**

The resected specimen was prepared in the manner originally described by Dukes (1940) in which it was opened along the anti-mesenteric border and pinned out on a cork board to prevent shrinkage. Four unfixed blocks were taken from the tumour. Two thirds of the material was sent for DNA analysis using flow cytometry (See Chapter 5), and the other one third was used to determine histological grade. Further details of the histological assessment are given in Chapter 5.

After fixation of the man specimen by immersion in 10% formalin for 48 hours, Dukes' stage was determined in the customary way (Dukes, 1932) (Table 3.3). Dukes' 'D' tumours were defined as those lesions incompletely excised,
either due to metastases or where the surgeon felt tumour was left behind in the peritoneum.

The lateral margins of the specimen were specifically examined for the presence or absence of extramural infiltration, or the presence of an inflammatory reaction. On the basis of these findings the patients were categorised as to having mobile or fixed growths, the latter was then subdivided into, fixed by malignant infiltration (FM) or fixed by an inflammatory reaction (FI). If fixed by malignancy, the degree of spread was categorised into slight, moderate or extensive. (Table 3.4.)

Slight spread was defined as extension just into perirectal fat, moderate spread, defined as established spread into perirectal tissue, and extensive spread, defined as involvement of adjacent structures or organs.

After approximately 18 months of the study, the pathological examination of the lateral excisional margin was extended. A separate study was commenced (Quirke et al, 1986), which was designed to examine the entire lateral excisional margin of the tumour. Prior to this lateral spread was determined from a single slice taken through the macroscopically determined area of maximum lateral spread. The study was designed to investigate the relationship between microscopic lateral spread and local tumour recurrence. In 34 of the
patients in the staging study the extended technique was applied to the excised specimens.

The technique is fully described elsewhere, (Quirke et al 1986) and consists of standard fixation on a cork board, with serial transverse sectioning of the entire tumour and mesorectum at 5–10 mm intervals. The slices were then cut at 8 mm intervals and the entire lateral excisional margins of the tumour were examined for microscopic tumour deposits. All specimens in the study were, however, initially assessed according to the initial pathological examination as outlined above. This enabled a comparison to be made between the two techniques, and the effect on pre-operative staging assessed.

The protocol thus allowed a comparison to be made between the accuracy of initial clinical, and extended assessment in predicting the operative and pathological findings. In addition, it was possible to determine how the additional information obtained on extended staging might later affect the perioperative management.
PATIENTS AND METHODS

b) **Comparison of Clinical Assessment and Preoperative CEA and APRP in Assessing Local Spread**

**Initial Study Group (n = 99)**

In order to validate the accuracy of preoperative measurements of CEA and APRP in colorectal cancer, an initial study was undertaken in a group of 99 consecutive individuals who presented between 1982 and 1984 with a diagnosis of a colonic or rectal tumour made on sigmoidoscopy or barium enema.

All patients were assessed clinically by two experienced clinicians and particular attention was paid to abdominal findings, and rectal findings where relevant.

All patients subsequently underwent laparotomy and the degree of fixation was assessed. The presence or absence of macroscopic hepatic metastases was documented. The excised specimens were subjected to a detailed examination by two experienced pathologists. A minimum of four blocks was taken from each tumour and the lateral margins were specifically examined for the presence or absence of microscopic extramural infiltration of the presence of an inflammatory reaction. On the basis of these findings,
patients were categorised as having mobile and fixed growths, the latter being divided into those fixed by malignancy and those fixed by inflammation.

Preoperative Staging Group (n = 76)

The patient's details, method of preoperative examination, laparotomy findings and details of pathological examination are documented earlier in this Chapter.

In an attempt to refine the discrimination between mobile and fixed tumours, in the initial study group, the patient's age, sex, weight loss (<or>5kg), site in the colon (rectum or other), Dukes' stage, preoperative haemoglobin, white cell count, percentage lymphocyte count and alkaline phosphatase were recorded and together with the preoperative serum values of CEA and APRP were used to develop a statistical model, using polychomatous logistic regression (vide infra), designed to differentiate between mobile tumours (M), those with malignant extension (FM) and those with an inflammatory reaction (FI). The model developed was subsequently tested against the preoperative serum levels of CEA and APRP of the 76 patients of the preoperative staging study group.
Analytical Methods

Blood samples from both groups were collected prior to operation by venepuncture, allowed to clot at room temperature, centrifuged at 3000 rpm and the serum stored at -25°C for subsequent analysis.

Determination of CEA

CEA was determined by a direct radiolmmunoassay using a proprietary kit, Phadebas CEA Prist kits, supplied by Pharmacia Diagnostics AB (Uppsala, Sweden). This kit uses paper discs as the solid phase. During the first incubation, anti-CEA antibodies, covalently coupled to the paper discs react with the patient sample. After washing a fixed amount of $^{125}$I labelled immunosorbent purified anti CEA antibodies is added. During a second incubation, the added antibodies form a specific complex with the CEA molecules which are found, to the antibodies on the paper discs. The radioactivity of this complex is then measured in a scintillation counter. The amount of radioactivity found is proportional to the amount of CEA in the sample. A more detailed methodology is given in Appendix 3.5.
**Measurement of APRP**

The method utilised was that described by Mancini et al (1965), and was a direct immunoassay to specific antiserum, supplied by Behringwerke, Marburg/Lahn (FRG). A full methodology is given in Appendix 3.5. In order to establish variation in levels of APRP, in 10 patients daily measurements for three days were obtained. (Our normal laboratory values are AGP < 1.4g/L and CRP < 10mg/L).
Statistical Methods

Statistical analysis in both studies included in this chapter was by \( X^2 \) test, with Yates correction when appropriate, Fishers Exact Test, or by Mann-Whitney U test for unpaired data.

For the purpose of the study, sensitivity was defined as the proportion of tumours that were correctly predicted as fixed, specificity was the proportion predicted as mobile and diagnostic accuracy the proportion of fixed and mobile that were correctly predicted. Results are expressed throughout as median and range.

The statistical model utilised to differentiate between types of fixity was developed using polychomatous logistic regression (Marshall and Chisholm 1985).

The 12 variables measured were entered into a trichomatous model (ie designed to distinguish between M, FM and FI groups) and statistical tests (likelihood ratio) for the inclusion of each variable in the model was calculated. The logistic regression analysis was performed on the Leeds University ANDAHL 470 computer using commercially available software.
RESULTS

a) Clinical Examination v. Pelvic CT Scanning

Clinical Examination

Assessment of Local Tumour Spread

In the conscious state, 53 (70%) tumours were palpable and could be fully assessed on digital examination. Under general anaesthetic a further 8 (10.5%) became palpable and were assessed. Thus 15 of 76 (20%) patients could not be assessed digitally by either surgeon.

Of the 61 palpable tumours, 32 (52%) were fixed at operation, and on pathological examination 14 (23%) had extensive extrarectal tumour spread, 6 (10%) had moderate spread, 5 (8%) slight spread. In 7 (11%) specimens, the fixation was due to an inflammatory reaction.

Clinical assessment correctly identified 13 of the 14 (93%) patients with extensive extramural infiltration but only identified two of the six (33%) patients with moderate, and two of the five (40%) with slight extrarectal involvement.
Of the seven specimens which exhibited inflammatory fixation, clinical examination wrongly classified five of these (71%) as having extramural tumour spread.

In those tumours clinically assessed as fixed (n = 9) both observers agreed on the presence of fixation except in one case (5%), although there was disagreement about the extent of fixation in four (21%) cases.

Twenty nine (48%) of the 61 palpable tumours demonstrated no extrarectal spread, however in two cases (7%) clinical examination classified them as fixed.

Thus, of the 32 fixed tumours which were palpable, clinical assessment correctly classified 19 (59%).

Overall therefore, of the 61 palpable tumours, digital examination agreed with the pathological findings in 46 cases (75%). However, if non-palpable tumours are included in the analysis, then digital examination of rectal tumours, in this study, correctly assessed only 61% of cases.

**Palpation of Extrarectal Lymph Nodes**

In no patient in this study could either surgeon palpate extrarectal lymph nodes with any degree of confidence.
Assessment on Imaging

Assessment of Extrarectal Tumour Spread by Computerised Tomography (CT)

(Table 3.5.)

Using pelvic CT scanning, all 76 patients with rectal lesions were assessed (Cf. clinical examination $X^2 = 9.42$, $p<0.005$).

Of the 29 patients with extrarectal tumour spread, CT of the pelvis correctly identified 27 (93%). This difference is significant in comparison with clinical examination which only correctly identified 17 (59%) $X^2 = 9.42$, $p<0.005$).

CT scanning identified all patients with extensive and moderate spread but failed to detect slight extramural spread in two patients.

CT correctly predicted inflammatory fixation in eight patients (80%) and identified 33 of the 37 (89%) mobile tumours.

Thus of the 39 tumours found to be fixed at operation, CT was correct in 35 (90%) compared with 19 (49%) on clinical examination ($X^2 = 15.41$, $p<0.005$).

Overall CT scanning correctly classified 68 (89%) of the 76 rectal neoplasms in this study, with regard to presence or
absence of extramural tumour invasion, compared with clinical examination which was correct in 46 (61%) \((X^2 = 17.00, p<0.005)\). 

The sensitivity, specificity and overall diagnostic accuracy of CT scanning of the pelvis in this study was thus 93%, 87% and 89% respectively and for clinical examination 59%, 62% and 61% respectively \((p<0.005)\).

**Detection of Involved Lymph Nodes**

**by Pelvic Computerised Tomography**

Twenty nine patients (38%) had evidence of lymph node deposits on pathological examination of the specimen. CT scanning identified 12 (41%) of these patients with nodal metastases. There was one false positive.

Although CT scanning identified less than 50% of patients with involved nodes, this proved significantly better than clinical evaluation, which failed to identify any \((X^2 = 15.3, p<0.0005)\).
RESULTS

b) Clinical Examination v Serum CEA and APRP

Pathology

The pathological details of the 76 perioperative assessment study group are described previously. Of the 99 patients in the initial evaluation group, 67 (67%) had a rectal lesion. One patient who underwent laparotomy was deemed to have a tumour which was technically inoperable because of extensive local fixation; biopsy confirmed that fixity was due to malignancy. Four patients in this group proved on microscopic examination of the specimen, to have had benign villous adenomas. Dukes' staging of the initial group is detailed in Table 3.6.

Of the 95 patients with adenocarcinoma 32 (34%) were classified as fixed, 16 (17%) by malignancy (FM) and 16 (17%) by an inflammatory response.
Serum Measurements

Initial Group

The preoperative serum measurements of CEA and APRP are listed in Appendix 3.2 and graphically in figures 3.7-3.9. Analysis of the preoperative levels of AGP revealed that all four patients with benign lesions and 58 (92%) of those with mobile lesions had a value of 1.4g/L or less. Median preoperative AGP concentrations were significantly higher in the serum of patients with both FM and FI tumours (Median FM, 1.54/L (Range 0.96 - 2.90), median FI, 1.94 g/L (range 1.34-2.79) compared to those with mobile growths (median M, 0.99 g/L (range 0.63 - 1.89)) (M v FM, U = 121, z = -4.7916, p<0.0001, M v FI, U = 20, z = -5.9575, p<0.0001).

There was no significant difference between those patients with FM tumours and those with FI growths (U = 72 p = NS).

A similar pattern was demonstrated with CRP. Again levels were significantly elevated in both tumours fixed by malignancy and by inflammation compared to mobile growth (M v FI, U = 162, z = -5.8990, p<0.0001. M v FM U = 162, z = -4.3280, p<0.0001). However, there was a significant difference between FM and FI tumours with higher levels found in those patients with an inflammatory reaction (U =
Median values (+ range) were: M, 6 (0-30.0); FI, 54.5 (18.0 - 181.0) and FM, 15.0 (4.0 - 79.0) respectively.

The presence of liver metastases appeared to make little difference to the results of assessing fixity (figures 3.7 - 3.9). Excluding the 16 patients with hepatic involvement, again preoperative serum AGP and CRP were significantly higher in those patients with fixed tumour. The results excluding the 16 with metastases were:-

**AGP:**
- M v FI : \( U = 13, z = -5.7891, p<0.0001 \)
- FI v FM : \( U = 20, p<0.01 \)
- M v FM : \( U = 70, z = -3.2366, p = 0.0012 \)

**CRP:**
- M v FI : \( U = 26, z = -5.8267, p<0.0001 \)
- FI v FM : \( U = 24, p<0.05 \)
- M v FM : \( U = 43, z = -3.7598, P = 0.0002 \)

The only major difference was that levels of AGP in patients with inflammatory fixation had now become significantly higher than those with malignant fixation.

Daily variation of serum concentrations did occur. In the 10 patients studied longitudinally, the mean daily variation in AGP was 0.1 g/L, and CRP 7.8 mg/L. Maximal variation was found in the upper ranges of both proteins, but in none of these patients did the variation alter their subsequent...
classification in the predictive index. Overall coefficient of variation was <10%.

Turning to the preoperative serum CEA, significantly raised levels were found in those patients with malignant extension compared with both mobile tumours and those fixed by inflammation (M v FM : U = 118, z = -4.8319, \(p<0.0001\). FI v FM : U = 27, \(p<0.01\)). There was no difference between mobile growths and those with an inflammatory reaction (M v FI : U = 421, z = -1.3300, \(P = 0.1834\)).

Highest values of CEA were found in patients with widespread or metastatic disease (Fig 3.9.), however after excluding the 16 patients with liver metastases, preoperative CEA levels were still significantly higher in those growths which were locally invasive (M v FM : U = 0), z = 4.5692, \(p<0.0001\). FI v FM : U = 23, \(p<0.05\)).

As a predictive index of fixity, arbitrary cut off points, which gave the best discrimination for AGP 1.4 g/L or CRP >15 mg/L were taken. Patients with preoperative serum levels greater than these values were predicted as having fixed tumours, irrespective of type. Correlation with histological findings revealed a sensitivity for AGP of 81%, and for CRP of 84%, and a specificity of 90% and 91% respectively; thus in this initial group of 99 patients with a prevalence of 32% of tumours that were fixed, preoperative serum levels of AGP
and CRP gave an overall diagnostic accuracy in determining fixity of 87% and 89% respectively.

The highest values of both proteins were, however, found in those patients with inflammatory fixation; all 16 patients with inflammatory fixation (100%) had a preoperative serum CRP >15 mg/L and all but one (94%) had a value of AGP >1.4g/L. There was, however, too great an overlap with the group with malignant infiltration for APRP levels alone to discriminate between types of fixation. CEA values, however, were a good discriminant of fixity. All 16 patients with inflammatory fixation had a preoperative CEA of 45ng/ml or less. Only four of those with malignant extension were below this level. Thus combination of high preoperative levels of either AGP or CRP, in association with a low CEA (<45ng/ml) was highly suggestive of inflammatory fixation. Conversely high levels of APRP with high CEA levels was indicative of local infiltration or hepatic metastases. Overall of 32 colorectal tumours fixed histologically, a combination of preoperative measurements of CEA, AGP and CRP correctly identified 27 (84%), 16 of 16 FI tumours (100%) and 11 of 16 (69%) FM tumours.
Fixity Analysis of Logistic Regression

The variables utilised in the model are listed in Appendix 3.3. Entering these variables into the model revealed that all can be omitted without substantial loss, that is once the acute phase reactants and CEA were in the model, there was no improvement by including any additional variables. A further reduction in the model was achieved by testing for "indistinguishability" (Marshall and Chisholm 1985) of CRP, AGP and CEA between the three groups. This revealed that CRP and AGP were "indistinguishable" with regard to the FI and FM groups, whilst CEA is indistinguishable between the M and FI groups. The form of the model for the probabilities (P) of FI, FM and M was written as follows:

\[
\begin{align*}
    P(\text{FI}) &= \frac{\exp(-5.02)}{d} \\
    P(\text{FM}) &= \frac{\exp(-6.00 + 0.021 \times \text{CEA})}{d} \\
    P(\text{M}) &= \frac{\exp(-0.09 \times \text{CRP} - 1.47 \times \text{AGP})}{d} \\
    \text{Where } d &= \exp(-5.02 - \exp(-6.00 - 0.021 \times \text{CEA})) + \exp(-0.09 \times \text{CRP} - 1.47 \times \text{AGP})
\end{align*}
\]

The fact that CEA appears only in the numerator of P(FM) implies the indistinguishability of the groups FI and M where CEA is absent. Similarly the inclusion of CRP and AGP in the numerator of P(M) implies indistinguishability of FI and FM with regard to these proteins. Applying this model to the data from the initial group of 99 patients, and assigning each
patient to the group with the largest probability, the model
correctly assigned 77% of the FI group, 90% of the FM group
and 87% of the mobile group. (Table 3.7.) These
probabilities appear very good on the surface, however, it
must be stressed that they were based on re-classifying the
data used to develop the model. Therefore in order to
evaluate the formula, it was subsequently applied to the data
from the perioperative staging group.

**Perioperative Staging Group (n = 76)**

The data from this group of patients are detailed in Appendix
3.2 and graphically in figures 3.10 - 3.12. A similar pattern
emerged to that of the initial study of 99 colorectal tumours.
Preoperative AGP and CRP were both significantly higher in
the FI and FM groups compared to the mobile group, median
(+ range) values (g/L) for AGP (g/L) were: M : 0.94 (0.60 -
1.60), FI : 1.67 (1.03 - 2.79), FM : 1.42 (0.91 - 2.19) and
CRP (mg/L): M : 4.0 (0-134.0), FI : 18.5 (5.0 - 153.0), FM :
13 (0 - 79.0). (AGP : M v FI, U = 17, z = -4.3691, p<0.0001
; M v FM, U = 123, z = -5.3438 p<0.0001 CRP : M v FI, U =
19, z = -4.3286 p<0.0001, M v FM, U = 120, z = 5.3882,
p<0.0001). Again there was no significant difference between
FI and FM groups with respect to AGP and CRP (U = 87, z -
1.8345, p = 0.606, and U = 119, z = -0.8364, p = 0.4025,
respectively).
Excluding patients with liver metastases again did not alter the results with regard to local tumour extension. Median values (+ range) excluding metastases are detailed in Appendix 3.2.

\[\text{AGP : M v FI, } U = 17, z = 4.2871, p<0.0001, \text{ M v FM, } U = 82, z = 4.6233, p<0.0001. \text{ CRP : M v FI, } U = 17, z = -4.2997, p<0.0001, \text{ M v FM, } U = 61, z = 5.1409, p<0.0001).\]

Preoperative CEA values were significantly raised in patients with FM tumours, compared to both M and FI groups. Median values (+ range) for CEA (ng/ml) were: M : 3.4 (2.5 - 54.9, FI : 4.2 (2.8 - 19.3) and FM : 24.6 (2.8 - 250.0). (CEA : M v FM, U = 135, z = -5.1916, p<0.0001, FI v FM, U = 39, z = -3.4102, p = 0.0007). Again no significant difference was apparent between mobile and inflammatory groups. (U = 128, z = -1.4862, p = 0.1372).

Excluding patients with liver metastases, serum CEA values were still significantly raised in the FM group. (FI v FM U = 38, z = -2.8327, p = 0.0046, M v FM U = 80, z = -4.8056, p<0.0001).

Applying the fixity criteria derived above from the initial study to this group, revealed the sensitivity of AGP to be 59%, of CRP, 58% and specificity of AGP 95%, CRP 100%. Thus in this perioperative study group, the overall diagnostic
accuracy was 76% and 78% respectively. Highest values of APRP were once more found in patients with an inflammatory reaction, with AGP and CRP correctly predicting 80% and 70% respectively.

As in the initial study group, high levels of CEA were indicative of a locally extensive tumour, or hepatic metastases. The differentiation between the two may be resolved by the results of the CT scan and this is addressed in the discussion at the end of this and the following Chapter.

Thus applying the overall fixity criteria previously defined from the earlier group, preoperative levels of CEA and APRP correctly defined 24 of the 37 (65%) mobile tumours, 9 of 10 (90%) FI tumours and 15 of 29 (52%) FM growths. Compared with initial clinical assessments, these results are not significantly better overall.

In the FM group there was no significant difference between AGP and CRP levels depending on the extent of fixation, however CEA levels were higher in those tumours with extensive spread, compared to those with moderate or slight spread. (U = 54, p<0.05). Furthermore, the previously defined fixity criteria correctly identified nine (60%) of tumours with extensive fixation, compared with only two (14%) of those with moderate or slight extrarectal spread. However, CEA and APRP measurements did significantly
improve the diagnosis of inflammatory fixation. Overall clinical examination only identified two (20%) patients correctly (cf serum measurements p=0.0026, Fishers Exact Test).

However, applying the regression model, previously described, derived from the initial 99 patients, prospectively to the 76 patients of the perioperative assessment group, 36 (97%) of patients with mobile tumours, 17 (59%) of those with malignant extension, but only three (30%) of tumours with inflammatory fixation were correctly assigned. (Table 3.8.)
DISCUSSION

It is clear, both from the study detailed in Chapter 2 and from other groups (Wood et al, 1981; Habib et al, 1983) that the degree of fixity of a rectal cancer is a most important prognostic factor but only when due to malignant extension and not inflammation (Jensen et al, 1970; Bonfanti et al, 1982). Perhaps it is of more relevance than Dukes' stage (Wood et al, 1981). It is for this reason that patients with fixed lesions have been selected for inclusion in trials of adjuvant pre-operative radiotherapy.

The MRC trial treating fixed rectal tumours with pre-operative radiotherapy is underway at the present time, and although as yet, too few patients have been entered to reach statistical significance, there is a trend towards an improvement in local recurrence rates in those patients receiving radiotherapy. (UKCCCR meeting 1986.) This finding is supported by interim results of the North West Region trial into immediate post operative radiotherapy. (Jones et al, 1988). There is, however, uniform agreement that pre-operative radiotherapy can cause tumour regression, and convert an inoperable lesion to an operable one (Second Report of MRC Working Party, 1984, Tepper et al, 1988). Even if radiotherapy proves of no benefit, patients with extrarectal tumour spread require some form of adjuvant therapy if we are to improve the prognosis. If chemotherapy is used, it would appear necessary to commence this during operation (I Taylor, 1981). Thus in such circumstances it
would be necessary to know whether a tumour fixed at laparotomy is fixed by tumour or inflammatory adhesions. Similarly this information may well influence operative procedure, whether the patient should have a SSR or an APER.

In those tumours which are palpable, digital examination is reasonably accurate in detection of extensive extrarectal spread, however, it cannot assess high rectal tumours, nor can it accurately detect lesser degrees of spread. Digital examination is also poor at differentiating neoplastic from inflammatory fixation.

In this study group, 70% of tumours were palpable in the conscious state. This figure was increased slightly by examination of patients under general anaesthesia. This figure of 70% is quite high in comparison with the study of Berg and Howell (1974) which reported that only 30% of rectal cancers extended to within six centimetres of the anal verge, and were thus easily assessed by the examining finger. These authors also found a decreasing incidence of low rectal tumours compared with 20 years ago.

York-Mason (1976) published a detailed clinical staging system for rectal tumours, based on careful digital palpation of the lesion. He, however, readily admits that this system is only suitable for low tumours which can be fully assessed.

Digital examination of low rectal lesions is accurate when performed by experienced clinicians. However, inter-observer
variation does occur and this was highlighted in a study by Nicholls et al (1981).

The detection of extrarectal involved lymph nodes on clinical examination proved difficult in this study. In no case could either surgeon confidently predict the presence of involved nodes.

Pelvic CT proved an extremely accurate method of detecting extramural tumour spread. Furthermore, in the majority of cases, differentiation was made between malignant and inflammatory fixation. Measurement of serum carcinoembryonic antigen and serum acute phase proteins appears to refine the diagnostic capabilities of CT examination and is discussed further in Chapter 8.

The correlation between pelvic CT findings and pathological examination of the specimen in this study was excellent, with CT capable of identifying even minor degrees of extramural infiltration in the majority of cases. The definition of extensive spread ie spread to the lateral excisional margins and/or involvement of contiguous organs was based on the standard pathological technique available in the pathology department on commencing this study. However, a further study undertaken by the author in conjunction with the pathology department of the Leeds General Infirmary has defined a group of patients with microscopic lateral spread, not previously detected by routine pathological studies (Quirke et al, 1986). This study on microscopic lateral spread was commenced
after the perioperative assessment study outlined in this thesis had commenced. It does, however, have major implications, as approximately double the number of patients considered on routine pathological examination to have spread to the lateral excisional margins (one of the criteria for extensive spread) have microscopic spread on examination of the specimen (Fig. 3.5. - 3.6.). These patients have a very high risk of local recurrence and obviously would require adjuvant treatment. This could theoretically nullify the usefulness of pelvic CT in pre-operative staging as it is unlikely to detect such microscopic lateral extension, and alter the classification of extensive extramural spread.

However, the latter 34 patients (45%) in the perioperative assessment study were included in the lateral spread study and of these 10 (29%) had microscopic lateral spread; in the modified pathological examination. Five of these patients (50%) fulfilled the original pathological criteria for FM tumours and all were classified as extensive spread on pelvic CT. Of the other five, all were shown on pelvic CT as demonstrating extramural spread, three as moderate and two slight, which corresponded with the original routine pathological examination for lateral spread. The implication of this finding is that pelvic CT can identify extramural extension but may underestimate the lateral extent of microscopic spread. Therefore extrapolating these findings to the study as a whole and to avoid missing patients with microscopically extensive spread, patients with any degree of extramural extension demonstrated on pre-operative pelvic CT scanning, theoretically warrants inclusion
in the decision on adjuvant therapy. This information should also be considered with regard to operative procedure. These implications are discussed further in Chapter 8.

Pelvic CT was significantly better at detecting involved lymph nodes than clinical examination, however it managed to identify less than 50%. Such lack of sensitivity thus precludes the routine use of CT scanning techniques for treatment of small tumours, as nodal metastases may well be missed.

The findings of this study on the use of pre-operative pelvic CT scanning validates the results of previous studies (Thoeni et al; 1981; Koehler et al; 1984; Dixon et al, 1981). As with these studies, extramural spread of tumour could be accurately detected but depth of spread within the bowel wall itself could not be accurately defined. If, however, as recommended, the criterion for adjuvant therapy is presence or absence of extrarectal spread, then accuracy of the technique within the bowel wall is not important.

More recently, alternative imaging techniques to detect extramural spread have been reported. Pelvic scanning by nuclear magnetic resonance (Butch et al, 1986) appears to give similar results to CT, however, the cost and relative inaccessibility of the technique mitigates against routine use in assessment of rectal tumours.

Transrectal ultrasound (Dragsted and Gammelgaard, 1983; Beynon et al, 1986) is a new, inexpensive technique which appears a
relatively accurate way of detecting intrapelvic spread of rectal tumours. The quoted accuracy is comparable to that of CT found in this study, with regard to both detection of lateral spread and nodal metastases. The technique is, however, not widely available and is still under evaluation. However, it appears promising and may well have a role in perioperative assessment in the future. The main advantage of the technique appears, however, to be its ability to define accurately tumour spread within the rectal wall (Beynon et al, 1986). As it would appear that it is spread outside the wall which is prognostically important, the value of this technique from a therapeutic viewpoint remains unproven.

Turning to serum measurements, several workers have suggested that serum movements of CEA (Staab et al, 1981; Wanebo et al, 1978; Steel et al, 1982) and APRP (Ward et al, 1977; de Mello, et al, 1983) may be useful in the preoperative assessment of gastrointestinal cancer. Preoperative levels of CEA can help determine prognosis (Staab et al, 1981; Wanebo et al, 1978) although Steel et al (1982) found this only to be true for colonic carcinoma, and not rectal cancer. Staab et al (1981) reported that raised CEA levels, greater than 5ng/ml, in patients with extramural spread were associated with a poor prognosis. In the majority of patients in this study, with extramural spread, CEA levels were markedly elevated, particularly where that spread was extensive.

Acute phase reactant proteins were defined by Koj (1974) as 'trauma-inducible liver produced glycoproteins'. Recently, however,
Gahmberg and Anderson (1978) have suggested that AGP may be produced locally by leucocytes, which proliferate in association with inflammation and cancer, rather than by stimulation of liver synthesis alone. This would certainly agree with the results of this study which has demonstrated maximal levels of AGP in the serum of patients with an inflammatory reaction around the tumour. The alternative explanation is that the rise in APRP, which is observed in malignant disease, occurs in response to an as yet unknown signal from the host–tumour interface, and it is suggested that the strength of the signal is related to depth of invasion (Bastable et al, 1979).

Ward et al (1977) suggested that a combination of APRP and CEA could distinguish metastatic from non-metastatic malignant tumours preoperatively, and may be of use in monitoring the progress of large bowel cancer. Certainly CRP does show a marked rise in patients with disseminated malignancy, and often heralds death, usually within two to three months of such a rise (Milano et al, 1978). One major criticism of Ward's study, however, is that the proteins measured were alpha-1-antitrypsin (AAT) and haptoglobin which demonstrate considerable genetic variation in their response to the reaction produced by a malignant tumour. AGP and CRP do not demonstrate such a phenotypic variation (Cooper and Stone, 1979).

Other workers have utilised a combination of APRP and CEA to stage a variety of tumours. De Mello et al (1983) estimated CEA
and APRP in 100 patients with gastric cancer. A CRP value >20 mg/L identified 50% of patients with technically inoperable tumours. Another correlation between APRP and the invasive propensity of bladder tumours was reported by Hollinshead et al (1977) who used a polyacrylamide gel technique to detect changes in plasma AGP and prealbumin, the index of which correlated with the stage and extent of the tumour. Similarly Bastable et al (1979) reported that serum levels of alpha-1-antichymotrypsin (ACT), AGP and CRP were able to differentiate between early and invasive forms of bladder tumour.

The laboratory techniques for estimation of these compounds are well established and accurate. It should be emphasised, however, that normal values differ from laboratory to laboratory, and the values quoted in this study are not universal. One potential source of error is that the normal range was estimated from young volunteers, who were of a different age range to the patients studied. This, however, should not alter the predictive value of the results. Levels of APRP do fluctuate from day to day, but variation in this study was minimal.

Whilst appreciating that numbers of patients in the individual groups in each part of the study are relatively small, pre-operative values of AGP or CRP appeared able to identify the majority of patients with fixed tumours, particularly those with inflammatory fixation. The sensitivity and specificity of these serum proteins in the initial 99 patients were good, but on applying the predictive
criteria to the pre-operative staging patients, the estimations became much less sensitive in detecting fixity, although the majority of patients with extensive, fixation were detected. Specificity was, however, maintained, with virtually all the mobile growths being predicted.

The use of discriminant analysis in this field is not new. Ward et al (1977) used a stepwise approach to set up a discriminant function using logistic discrimination for separating patients with large bowel cancer into those who remained tumour free for at least one year after surgery, and those who developed metastases. Polychomatous logistic regression (Marshall & Chisholm, 1985) has advantages over standard regression analysis. In this study a polychomatous logistic model was derived from the data of the initial 99 patients. Perhaps, not surprisingly, when the model derived was reapplied to the data, the predictive value was excellent. However, on applying the formula to the pre-operative study group, the predictive value was inferior, although again, patients with mobile growths were identified accurately. The addition of other clinical information to the model did not improve its predictive value.

Elevated levels of CEA indicated either the presence of a locally invasive tumour, gross hepatic metastases, which are discussed in the next Chapter, or a combination of the two. The pre-operative CT scan may help differentiate between the two and this is discussed further in the following Chapter.
Thus in the overall assessment of local spread of a rectal tumour, pre-operative computerised tomography appears significantly superior to both clinical examination, and pre-operative serum measurements of CEA and APRP. Measurement of the latter compounds are of most use in assessment of local fixation, particularly differentiating between malignant and inflammatory fixation. This distinction is extremely important, with regard to both prognosis, as outlined in the previous chapter, and also the necessity for adjuvant radiotherapy.

Serum CEA and APRP levels measured pre-operatively, would it seem from the data in this study appear more useful in the evaluation of distant spread. This point is addressed in the following Chapter.
### TABLE 3.1.

**Findings at Laparotomy**

(Perioperative Assessment Group)

(\(n = 76\)) (%)

<table>
<thead>
<tr>
<th>Site of Lesion</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid - Rectal (8 - 12 cm)</td>
<td>32 (42)</td>
<td></td>
</tr>
<tr>
<td>Low - Rectal (0 - 7cm)</td>
<td>44 (58)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumour Fixity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile</td>
<td>37 (49)</td>
<td></td>
</tr>
<tr>
<td>Fixed/partially fixed</td>
<td>39 (51)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liver Metastases</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>10 (13)</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>66 (87)</td>
<td></td>
</tr>
</tbody>
</table>
### Operative Procedures
*(Perioperative Assessment Group)*

(\( n = 76 \)) (%)

<table>
<thead>
<tr>
<th>Type of Reaction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominoperineal excision</td>
<td>22 (29)</td>
</tr>
<tr>
<td>Low anterior resection</td>
<td>48 (63)</td>
</tr>
<tr>
<td>Hartmanns procedure</td>
<td>6 (8)</td>
</tr>
</tbody>
</table>

**"Curative" v "Palliative"**

| "Curative"                          | 48 (63) |
| "Palliative"                         | 28 (37) |
| - Liver metastases                   | 10      |
| - Residual "tumour" in pelvis        | 18      |
| - Combination of the above           | 4       |
### TABLE 3.3.

**DUKES' STAGE OF TUMOURS**

(Perioperative Assessment Group)

\[
\begin{array}{lcr}
& \text{Villous Adenoma} & 11 \ (14) \\
\hline
\text{DUKES} & A & 6 \ (8) \\
 & B & 25 \ (33) \\
 & C1 & 13 \ (17) \\
 & C2 & 6 \ (8) \\
 & D & 15 \ (20) \\
\end{array}
\]
<table>
<thead>
<tr>
<th>Pathological Assessment of Tumour Extension</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(Perioperative Assessment Group)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>(n = 76)</em></td>
<td>(49)</td>
<td>(%)</td>
</tr>
<tr>
<td>No evidence of extrarectal spread</td>
<td>- 37</td>
<td>(49)</td>
</tr>
<tr>
<td>Extrarectal Spread Present</td>
<td>- 29</td>
<td>(38)</td>
</tr>
<tr>
<td>slight</td>
<td>- 7</td>
<td></td>
</tr>
<tr>
<td>moderate</td>
<td>- 7</td>
<td></td>
</tr>
<tr>
<td>extensive</td>
<td>- 15</td>
<td></td>
</tr>
<tr>
<td>Dense inflammatory reaction</td>
<td>- 10</td>
<td>(13)</td>
</tr>
</tbody>
</table>

Page 160
Comparison of digital rectal examination and pelvic CT in assessment of rectal tumours - numbers (%) agreeing with pathological examination of the specimen.

<table>
<thead>
<tr>
<th></th>
<th>Rectal Examination</th>
<th>Pelvic CT scan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile (n=37)</td>
<td>27 (73)</td>
<td>33 (89)</td>
</tr>
<tr>
<td>FI (n=10)</td>
<td>2 (20)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>FM-overall (n=29)</td>
<td>17 (59)</td>
<td>27 (93)</td>
</tr>
<tr>
<td>- extensive (n=15)</td>
<td>13 (87)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>- moderate (n=7)</td>
<td>2 (29)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>- slight (n=7)</td>
<td>2 (29)</td>
<td>5 (71)</td>
</tr>
</tbody>
</table>

$X^2 = 17.00$

$p < 0.005$
**TABLE 3.6.**

**Dukes' Stage of Initial Patient Group**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villous Adenoma</td>
<td>4 (4)</td>
</tr>
<tr>
<td>A</td>
<td>13 (13)</td>
</tr>
<tr>
<td>B</td>
<td>39 (40)</td>
</tr>
<tr>
<td>C₁</td>
<td>20 (20)</td>
</tr>
<tr>
<td>C₂</td>
<td>6 (6)</td>
</tr>
<tr>
<td>D</td>
<td>17 (17)</td>
</tr>
</tbody>
</table>

n = 99

(%)
Table 3.7

Table of Probabilities Derived by Logistic Regression in Initial Group

(n = 99)

a) Table of Counts

<table>
<thead>
<tr>
<th>Assigned Class</th>
<th>FI</th>
<th>FM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual</td>
<td>FI</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Class</td>
<td>FM</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

b) Table of Probabilities

<table>
<thead>
<tr>
<th>Assigned Class</th>
<th>FI</th>
<th>FM</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actual</td>
<td>FI</td>
<td>0.7692</td>
<td>0.0000</td>
</tr>
<tr>
<td>Class</td>
<td>FM</td>
<td>0.1538</td>
<td>0.9000</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.0769</td>
<td>0.1000</td>
</tr>
</tbody>
</table>
TABLE 3.8.

Polychotomous Logistic Regression
Assignment of Perioperative Assessment
Group from Derived Model

(n = 76)

<table>
<thead>
<tr>
<th>Assigned Class</th>
<th>M</th>
<th>FI</th>
<th>FM</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>37</td>
<td>1</td>
<td>0</td>
<td>38</td>
</tr>
<tr>
<td>FI</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>FM</td>
<td>7</td>
<td>4</td>
<td>17</td>
<td>28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Actual Class</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>45</td>
<td>8</td>
<td>23</td>
<td>76</td>
</tr>
</tbody>
</table>

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FIGURE 3.1

Pelvic CT scan demonstrating absence of extrarectal tumour spread.

This lesion proved to be a villous adenoma on histological examination.
FIGURE 3.2

Pelvic CT scan demonstrating slight extrarectal spread of tumour (arrowed).
FIGURE 3.3

Pelvic CT scan demonstrating moderate extrarectal tumour spread.

The tumour is extending well into perirectal fat (arrowed).
FIGURE 3.4

Pelvic CT scan demonstrating extensive extrarectal tumour spread.

The tumour is invading the sacrum (arrowed).
FIGURE 3.5.

Whole block section through a rectal tumour stained with H&E demonstrating contiguous spread to lateral excisional margin (arrowed).
FIGURE 3.6.

Whole block section through a rectal tumour stained with H&E demonstrating apparent clearance around tumour. However, a discontinuous microscopic tumour deposit (arrowed) was found at the lateral excisional margin.
FIGURE 3.7

Initial group (n = 99).

Preoperative serum levels of AGP (g/L)
Initial group (n = 99).

Preoperative serum values of CRP (mg/L)
FIGURE 3.3

Initial group (n = 99).

Preoperative serum values of CEA (ng/ml)
FIGURE 3.10

Study group (n = 76).

Preoperative serum values of AGP (g/L)
FIGURE 3.11

Study group (n = 76).

Preoperative serum values of CRP (mg/L)
FIGURE 3.12

Study group (n = 76).

Preoperative serum values of CEA (ng/ml)
CHAPTER 4

The Identification of Hepatic Spread of Rectal Tumours
Introduction

The primary site of distant, blood-borne spread of rectal carcinoma is to the liver. As outlined in the introduction to this thesis, involvement of the liver is a major factor in the continuing high mortality from this disease. There are two groups of patients in whom identification of liver metastases in the perioperative period may well affect prognosis, those with "overt" metastases at the time of presentation, and those with so called "occult" hepatic involvement.

The detection of overt hepatic metastases prior to operative treatment may well affect management, particularly in the elderly, where a potentially hazardous laparotomy could be avoided, and more appropriate local therapy instituted. Even at laparotomy, however, despite an apparently normal macroscopic appearance, the liver may contain small metastases. Goligher (1941), demonstrated that 16% of apparently normal livers assessed at laparotomy contained undetected metastases. These so called "occult" metastases have recently been highlighted by Finlay et al (1982). The pre-operative detection of hepatic secondaries remains a conundrum. Biochemical tests of liver function may give some guidance. Alkaline phosphatase is reported as being raised in approximately one third of patients with liver deposits (Cederqvist and Nielsen 1972), however this is too inaccurate for perioperative assessment. Techniques for imaging the liver are becoming more refined, and include ultrasound, isotope scanning and arteriography.
It remains unclear, however, which, if any, is the most reliable (Temple et al, 1983; McCarthy et al, 1970; Castagna et al, 1972).

It is standard practice of many colorectal surgeons, in their routine pre-operative assessment to perform an ultrasound examination of the liver, a service which is readily available and relatively inexpensive. Computerised tomography, though less available, would appear a more accurate method of assessing the liver, particularly for small hepatic metastases, and may be capable of identifying "occult" spread (Finlay and McArdle, 1982). As demonstrated in the previous Chapter, serum levels of CEA in particular, are raised in patients with liver metastases.

Thus in the second part of this section of the study, a comparison was made between what is regarded by most surgeons as routine preoperative investigations ie a clinical abdominal examination combined with liver function tests, and ultrasonic examination of the liver, with an abdominal CT scan and measurement of preoperative serum CEA and APRP. The aim was to determine which, if any, was the most reliable method of assessing the liver for secondary spread.
Patients and Methods

Clinical Assessment

The patients included in this part of the study are those 76 individuals outlined in the previous Chapter, who comprised the perioperative assessment group.

All patients underwent a thorough abdominal examination by the same two clinicians who performed the clinical assessment of local tumour spread. In particular hepatomegaly was sought, and if found each clinician was asked to specify, if in his opinion, this was due to tumour spread or not.

Biochemical Assessment

Blood samples were taken from every patient in the study preoperatively. Serum levels of bilirubin, alkaline phosphatase and transaminases were measured in the routine biochemical laboratory of the Leeds General Infirmary.

Imaging Techniques

All patients underwent an ultrasound examination of the liver preoperatively, using an ATL real time sector scanner (Squibb Medical Systems). The patients were starved prior to the procedure.
The scans were performed and reported by one experienced radiologist, and were assessed "blind" without knowledge of the results of other investigations.

Hepatic metastases were defined as present or absent.

**Computerised Tomography**

The scans of the liver were obtained in all 76 patients concurrently with the pelvic CT described in the preceding chapter.

For examination of the liver, however contiguous slices of 12mm were taken in rapid sequence made after a bolus injection of 100 ml meglumine diatrizoate (Conray 280, May and Baker). As with the pelvic scans, the films were reported by one radiologist, experienced at interpreting CT scans of the liver, and without the knowledge of previous findings. Hepatic metastases were defined as present or absent (Fig. 4.1.).

**Serum Measurements of CEA and APRP**

Blood samples were obtained from all 76 patients, and analysed as outlined in the preceding chapter. The methods outlined fully in the appendices to Chapter 3. Full details are given in the previous Chapter.
Laparotomy Assessment

As previously stated, all patients underwent a full laparotomy. The details of operative findings are given in Table 3.1. The presence of overt hepatic metastases was sought by meticulous bimanual palpation of the liver. Any possible lesion was biopsied and confirmed by histological examination.
RESULTS

At laparotomy, 10 patients (13%) had proven overt liver metastases confirmed by histological examination of a biopsy. One patient was found to have lung secondaries on routine pre-operative chest X-ray.

Clinical Examination

In none of the 76 patients examined was a definite diagnosis of hepatomegaly due to overt liver metastases made by either clinician. In particular, none of the 10 patients subsequently proved to have liver metastases, was any abnormality suspected on abdominal examination.

Biochemical Assessment of Liver Function

The pre-operative serum levels of bilirubin, alkaline phosphatase and SGOT in the 10 patients with overt liver metastases, found at laparotomy are listed in Table 4.1.

As can be seen in only one of these patients was there any abnormality detected ie a raised bilirubin. In the remaining 66 patients, all the biochemical tests of liver function were within the normal laboratory values.
Ultrasound Examination of the Liver

Of the 10 patients (13%) with proven, overt liver metastases at laparotomy, pre-operative ultrasound examination identified five (50%), with three false positives and five false negatives.

Thus overall, ultrasound correctly assessed the liver for overt spread in 68 patients (89%), not significantly different to combination of clinical examination and liver function tests which although only predicting one positive patient correctly, predicted absence of liver metastases in 66 patients ($X^2 = 3.81, p<0.05$).

Computerised Tomography of the Liver

Computerised tomography of the liver identified eight (80%) of the patients with proven overt metastases, with two false negatives.

CT scanning correctly assessed the liver for overt spread in 74 (97%) patients, significantly better than clinical examination combined with liver function tests, and ultrasonic examination ($X^2 = 4.80, p<0.05$, $X^2=3.85, p<0.05$ respectively) (Table 4.2.).

Serum Measurement of CEA and APRP

The detailed results of pre-operative CEA and APRP in the 76 patients are given in the preceding chapter and in figures 3.10.-3.12.
In the 76 patients of the study group, as in the initial 99 patients with colorectal tumours detailed in the preceding chapter, the presence of liver metastases did not affect serum levels of either AGP or CRP.

Median (+ range) of pre-operative values for AGP (g/L) in the 66 patients without overt liver metastases (1.26, 0.60 - 2.79) were not significantly different to that of the 10 patients with metastases (1.23, 0.86 - 1.78) (z = 0.0615, p = 0.95, Mann Whitney U Test).

Similarly for CRP (mg/L) values did not differ significantly (without live r metastases: 7.5, 0 - 153; with metastases: 6.5, 0 - 50;) (z = -0.60, p = 0.55, Mann-Whitney U Test).

However, a different pattern emerges for CEA (ng/ml) levels. The pre-operative serum values of patients with liver secondaries (103.0, 20.1 - 250.0) were significantly higher than those values in patients without metastases (3.8, 2.5 - 203.1) (z = -4.51, p < 0.001, Mann Whitney U Test).

CEA levels were also markedly elevated in those patients with locally extensive disease as detailed in the previous Chapter and these individuals accounted for the highest levels found in patients without overt metastases (see figure 3.12, previous chapter). If the patients with extensive extrarectal spread, but no overt liver metastases at laparotomy, are excluded, then the ability of CEA to predict hepatic metastasis assumes greater importance.
Eighty per cent of patients with overt hepatic metastases in the study had a pre-operative serum CEA of 45 ng/ml or greater. One patient with a tumour confined to the rectal wall, and with no evidence of overt metastases, either on pre-operative scanning and at laparotomy, had a CEA value of 54.2 ng/ml. This patient unfortunately died post-operatively. At post mortem, multiple small (<0.5 cm) liver metastases were found within the liver substance. This suggests that elevation of CEA levels in the absence of obvious metastatic disease could identify certain patients with "occult" hepatic spread.

In both patients with liver metastases missed by CT scanning, CEA levels were above 45 mg/ml. Pelvic CT failed to show evidence of extra rectal spread. Thus by combining the results of CT and pre-operative CEA levels, all 10 patients with overt metastases were identified pre-operatively (cf clinical examination p<0.001, Fishers' Exact Test).
Discussion

The reliable detection of overt liver metastases prior to operation may alter the management of patients with rectal carcinoma. Furthermore there would be no indication for administration of adjuvant radiotherapy to the pelvis in such patients. Similarly patients with small mobile lesions, but who have hepatic metastases, might be more suitably treated by some form of local treatment as opposed to a major abdominal procedure with its concomitant greater morbidity. Combination of physical examination and liver function tests identified only one patient as having metastases, this due to a marginally elevated bilirubin in that case. These findings are somewhat different to those of Temple et al (1983) who found raised alkaline phosphatase in 65% of patients with liver metastases, although in this study, there was a false positive rate of 76%. Studies by Baden et al (1971) and McCarthy et al (1970) have also reported similar figures for alkaline phosphatase. It is difficult to explain the lack of derangement in biochemical tests of liver function in this study, although in a recent study (Schreve et al, 1984) alkaline phosphatase was raised in only 33% of patients with liver metastases. It cannot be due only to small liver metastases being present in the study undertaken for this thesis, as in five patients (50%), at least 75% of the liver substance has been replaced.

As in previous studies (Snow et al, 1977; Finlay et al, 1982; Schreve et al, 1984) CT scanning of the liver proved superior to
ultrasound in detection of overt metastases in the liver. However the "gold standard" for detection of metastases in this study was careful bimanual palpation of the liver, and as Colligher stated (1941), palpation of the liver underestimates the percentage of patients with liver secondaries. Thus it might be argued that the three false positives seen on ultrasound were in fact lesions missed at laparotomy. It is doubtful if this were the case as the three "metastases" noted on ultrasound were multiple and of large diameter, and should have been palpable. Certainly metastases of smaller diameter identified by CT were subsequently palpable. Also, in none of the three were CEA levels elevated pre-operatively. Furthermore these three patients are alive and well with no clinical signs of metastases, a mean of 24 months following resection.

CT scans appear to be accurate for detecting metastases more than 1.5 cm in diameter (Levitt et al, 1977). Both patients who were missed by the tomography had metastases which were smaller than this. With such a sensitive technique and the knowledge that the surgeon often underestimates the presence of metastases, it is surprising that CT scanning did not detect more lesions. There can be no doubt, however, that no matter how carefully hepatic assessment is performed, the surgeon will miss tiny metastases deep within the liver. These have been termed "occult" metastases for this reason, (Pinlay et al, 1982) and their presence is claimed to be responsible for the poor results in patients who have undergone "curative" resections. Despite a careful search for these lesions on
CT and ultrasound in this study, none were detected. This is perhaps not surprising as the only study which did detect them using these modalities (Finlay et al, 1982) could only be sure about their presence on subsequent serial scans at three monthly intervals. The radiologists interpreting the initial scan were encouraged to be definite even on occasions of doubt. This led to a false positive rate of 33% (I G Finlay - unpublished data) which is too high to be of clinical value.

Newer techniques, such as dynamic hepatic scintigraphy (Leveson et al, 1985) seemed initially encouraging to predicting presence of overt metastases and the subsequent development of metastases in patients clinically clear at laparotomy, however the initial optimism has been tempered by lack of both sensitivity and specificity on long term follow-up (P J Robinson - unpublished data).

Whether "occult" metastases can be detected pre-operatively is of doubtful significance. Such patients may benefit from Intra-portal chemotherapy (Taylor, 1981). If "occult" metastases are present, however, it would be wrong to deny patients with apparently normal livers of the potential benefit of chemotherapy, as part of the therapeutic effect may be the prevention of circulating malignant cells from seeding in the liver.

Perhaps of greater relevance at the present time is the accurate detection of macroscopic disease, as it impinges on clinical management. Three of the patients in the study with liver
metastases were suitable for a local procedure. Instead all underwent major resection. Two developed major complications, and all three died within 12 months of operation. Perhaps these patients would have been better served by a more conservative approach if the presence of major hepatic involvement was known prior to operation.

Conversely accurate detection of metastases pre-operatively might influence the selection of patients who have solitary lesions to be considered for hepatic resection (Fortner et al, 1978; Wilson and Adson, 1976; Adson and Van Heerden, 1980). This did have relevance in this study, one patient with an apparently solitary metastasis at laparotomy, proved on his pre-operative scan to have multiple small metastases elsewhere.

The role of pre-operative estimation of serum markers in the detection of liver metastases appears to be complementary to the CT findings. CEA levels pre-operatively appear to correlate with tumour load. An elevated CEA in this study indicated either extensive local tumour spread in the pelvis, metastases in the liver, or a combination of the two. Computerised tomography of the abdomen can help in identifying which mode of spread is present. A CEA level of greater than 45 ng/ml, in the absence of extrarectal spread on pelvic CT was highly suggestive of liver metastases. These findings confirm the results of the studies of Herrera et al (1976) and Lewi et al (1984) in suggesting that
highly elevated CEA levels pre-operatively suggest extensive disease.

The role of the APRP's measured in this study, is less clearly defined. Ward et al (1977) suggested that APRP measurement may more clearly define those patients with metastasis disease. The results of this study do not support these findings.

Similarly de Mello et al (1983) developed a discrimination function which used CEA and several acute phase reactants to define 89% of patients with Dukes' D colorectal cancer. The two acute phase reactants measured in this study, however, failed to add any additional information to that obtained by measurement of CEA alone.

CRP levels are known to rise, as a pre-terminal event in many tumours (Cooper and Turner, 1980), and elevated levels are found in a high percentage of inoperable gastric tumours (de Mello et al, 1983). In the small number of patients with liver metastases in the study, however, no evidence of such a link between CRP and liver spread was found.

Thus the overall results of this study suggest that combination of pre-operative computerised tomography of the liver, in conjunction with serum measurement of CEA provides the optimum chance of identifying overt hepatic metastases prior to laparotomy.
CT scanning does not appear able to detect "occult" spread, however an elevated CEA level combined with an abdominal CT scan which shows no evidence of extensive pelvic disease is highly suggestive of "occult" spread in the liver.

Thus at the present time a pre-operative CT scan of the liver combined with measurement of serum CEA appears the most accurate method of detecting hepatic spread of rectal carcinoma.
Table: 4.1.

Pre-operative liver function tests in patients with overt hepatic metastases at laparotomy (n=10). (Normal laboratory ranges given in parentheses).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Bilirubin (umol/l)</th>
<th>Alk Phos (KA units)</th>
<th>SGOT (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>7.0</td>
<td>6.4</td>
<td>18.0</td>
</tr>
<tr>
<td>DK</td>
<td>8.0</td>
<td>9.7</td>
<td>9.0</td>
</tr>
<tr>
<td>FM</td>
<td>12.0</td>
<td>5.9</td>
<td>17.0</td>
</tr>
<tr>
<td>MT</td>
<td>4.0</td>
<td>7.0</td>
<td>14.0</td>
</tr>
<tr>
<td>MW</td>
<td>11.0</td>
<td>10.3</td>
<td>14.0</td>
</tr>
<tr>
<td>FL</td>
<td>28.0*</td>
<td>7.1</td>
<td>9.0</td>
</tr>
<tr>
<td>MP</td>
<td>7.0</td>
<td>9.3</td>
<td>19.0</td>
</tr>
<tr>
<td>OS</td>
<td>11.0</td>
<td>12.7</td>
<td>25.0</td>
</tr>
<tr>
<td>JC</td>
<td>8.0</td>
<td>4.6</td>
<td>13.0</td>
</tr>
<tr>
<td>JMcD</td>
<td>8.0</td>
<td>11.1</td>
<td>27.0</td>
</tr>
</tbody>
</table>

* abnormal
Table 4.3.

Detection of hepatic metastases (n=10) by clinical examination and liver function tests (LFT), ultrasound (US) and computerised tomography (CT) (%).

<table>
<thead>
<tr>
<th>Clinical Exam.</th>
<th>US</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ LFT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correct</td>
<td>1 (10)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>False +ve</td>
<td>0</td>
<td>3 (30)</td>
</tr>
<tr>
<td>False -ve</td>
<td>9 (90)</td>
<td>5 (50)</td>
</tr>
</tbody>
</table>
FIGURE 4.5

Abdominal CT scan demonstrating obvious liver metastases (arrowed).
Section II

The assessment of Biological Parameters in Rectal Neoplasia

Implications for Prognosis and Treatment
CHAPTER 5

Histological assessment and flow cytometry of pre-operative biopsies from rectal tumours
INTRODUCTION

The first of the biological parameters addressed in this thesis, is histologically defined tumour differentiation.

The pathological assessment of colorectal adenocarcinoma has remained unchanged for the past 50 years. The staging system of Dukes (Dukes, 1932) remains, with modifications, the standard classification. This system is usually combined with subjective grading of the extent of tumour differentiation, (Stewart and Spies, 1929; Dukes, 1936; Grinnell, 1939) to determine the final prognosis. Tumours which are poorly differentiated on histological examination have a poor prognosis (Dukes, 1936) and patients with such tumours are known to constitute a high risk category.

Although differentiation undoubtedly correlates with survival (Dukes, 1936), the assessment of tumour differentiation remains subjective, and colorectal tumours are known to be heterogeneous in this respect (Qualheim and Gall, 1953). It remains routine practice, however, to biopsy a rectal tumour, not only to confirm the diagnosis, but also to assess the degree of differentiation. Some authors (Lock et al, 1978) base treatment decisions on the grading of the pre-operative biopsy, particularly when the tumour is poorly differentiated. However, the accuracy of such grading has been questioned (Thomas et al, 1983).
In the first part of this section of the study therefore, an attempt has been made to improve the accuracy of histological examination of pre-operative biopsies by increasing the number of samples taken. Thus the grading of one pre-operative sigmoidoscopic biopsy has been compared with four samples taken from different areas of the same tumour. Recently, however, a new method of assessing cellular differentiation, flow cytometry has been reported.

Flow cytometry is a relatively new technique which can measure cellular DNA content. If cells from a tumour are measured, flow cytometry can differentiate between cells containing a normal amount of DNA (diploid) from those containing abnormal amounts (non-diploid/aneuploid). It has recently been suggested that assessment of colorectal cancer by flow cytometry may add a new prognostic dimension (Wolley et al, 1982). Recent studies (Wolley et al, 1982; Armitage et al, 1985) have demonstrated that patients with a colorectal tumour containing a diploid cell population have a significant survival advantage over those whose tumours are non-diploid. Flow cytometry may thus provide a quantitative method of assessing tumour behaviour and biological aggressiveness which avoids the problem of subjective assessment. Several studies (Wolley et al, 1982; Armitage et al, 1985) have confirmed the ability of flow cytometry to identify high risk patients. If these patients are to be identified pre-operatively and treatment decision based on the information from a pre-operative biopsy then the ability of such a biopsy, assessed by flow cytometry in assessing the bulk of the tumour is crucial to management.
The second part of the study therefore is an investigation into the ability of flow cytometric analysis of pre-operative tumour biopsies to predict the cellular status of the resected tumour, and to compare this predictive accuracy with that achieved by conventional histological assessment.

If flow cytometry of pre-operative biopsies could provide a reproductive, quantitative and accurate prediction of the cellular status and therefore behaviour of the main tumour, it would provide a more rational basis for assessing the biological aggressiveness of the tumour, and thus allow a more rational basis for treatment.
Methods

Single versus Multiple Biopsies

All 76 patients in the perioperative assessment group, detailed previously, study underwent a pre-operative sigmoidoscopy in the outpatient department where one biopsy of the tumour was taken and submitted for routine histological assessment, all samples were examined by one experienced pathologist.

As previously described, all patients underwent an examination under anaesthetic, at which time four biopsies were taken via the sigmoidoscope. Where possible two deep biopsies were taken via a Tru-cut biopsy needle to determine if differentiation altered with depth of tumour penetration. These biopsies were also submitted for routine histological assessment of differentiation performed "blind" by the same experienced pathologist.

After resection of the tumour, the main specimen was submitted for detailed pathological assessment, and determination of overall tumour differentiation, this again performed by the same pathologist. A comparison was then made between differentiation determined by a single biopsy versus the resected tumour, and that obtained on multiple biopsies.
Flow Cytometric Analysis of Pre-operative Biopsies and Resected Tumour

Flow cytometric analysis of the pre-operative multiple biopsies and the resected tumour was performed in 36 (47.4%) of the patients in the study.

Two methods of flow cytometric analysis were used. Initially in the first 17 patients the cells were stained with propidium iodide (PI), which enables nuclear DNA quantitation only (Figs. 5.1. - 5.2.), but in the latter 19 patients the cells were stained with acridine orange and ethidium bromide (AO/EB) which stains the whole cell and allows quantitation of both DNA and RNA, and also estimation of cell death (Figs 5.3. - 5.4.).

Sampling

In all 36 patients, half of each of the four pre-operative biopsies was placed in Hanks balanced salt solution (HBSS) for subsequent flow cytometric analysis. The remainder was fixed in 10% neutral buffered formalin, embedded in paraffin wax and 4 μm sections cut and then stained with haematoxylin and eosin for subsequent histological examination.

After resection, a central slice of tumour was removed and four samples taken, two superficial and two deep (Fig. 5.5.). Each of these blocks were divided as shown and the central portion...
prepared for histopathological assessment as detailed above. The other two portions were placed in HBSS for flow cytometry.

**Histopathological Assessment**

The stained sections from the various samples were randomised, coded and graded, for the PI technique by one pathologist, but for the AO/EB technique by three independent pathologists. The latter was undertaken in an attempt to increase the level of agreement between the pre-operative biopsy and resected specimen and to minimise interobserver error. Established criteria were used (Grinnell 1939) and each observer estimated the proportion, expressed as a percentage, of well, moderate or poorly differentiated adenocarcinoma in the samples. The final grade was established by averaging the results from the three observers. If this produced equal proportions of two or more grades, then the least differentiated grade was allocated as the final grade. This method was utilised to minimise inter-observer error and to increase the chance of agreement with flow cytometric data which is quantitative. This method also takes into account the heterogeneous nature of rectal cancer and avoids the conventional pathological practice of grading simply on the worse differentiated area, for which there is no valid prognostic basis.
Tissue Preparation for flow cytometry

Tissue samples were stored in HBSS at 4°C until analysis which was conducted with 24 h of removal. After removal, tissue was minced by scalpel and suspended in 4 ml Hepes buffer, pH 7.3 containing 1mM Ca++ and 200 units collagenase (Sigma Chemical Company, Poole, Dorset). The suspension was incubated in a water bath for 0.5 - 1h at 37°C. Cells were then collected by centrifugation, suspended in 2ml PBS and incubated with 1 unit of papain (Sigma Type III) for 5 min at 37°C in 2ml PBS. Cells were again collected by centrifugation and resuspended in 2-4ml PBS (50%) and HBSS (50%) and stained with either propidium iodide (PI) or acridine orange/ethidium bromide (AO/EB).

Propidium iodide staining

The staining solution comprised 0.25% solution of Triton X100, 30 ug/ml propidium iodide and 100 pg/ml RNAse. Two ml of stain were added to 1ml of cell suspension and incubated for 30 min at 20°C before analysis. After staining the sample was placed in the flow cytometer, the flow started and allowed to equilibrate for three minutes before data acquisition began.

Acridine orange/ethidium bromide staining

An equal volume of PBS (50%) and HBSS (50%) containing five uM acridine orange (AO) and three uM ethidium bromide (EB) was
added to an aliquot of cells. After 27 min staining, the sample was placed in the flow cytometer and the sample flow started. Coefficients of variation ranged from 6-8%.

**Flow cytometry measurements**

A diagrammatic representation of the flow cytometer is depicted in Fig. 5.6. All measurements were performed in an Ortho Diagnostic Cytofluorograph systems 50H with a Lexel 95-4 W argon ion laser, routinely used at 250mw at the nm line. With the dichroic mirror and filter systems employed, the wavelengths measured were green fluorescence 53-565 nm and red fluorescence >640nm. Instrument calibration of the 2C channel number, C being the standard unit of measure, was performed using normal peripheral blood lymphocytes, isolated by layering on to lymphocyte separation medium (Flow Laboratories Ltd).

Date acquisition, storage, retrieval and analysis were performed with an ortho 2151 computer system. Data was transferred to computer disc immediately on completion of sample measurements. The software available allows statistical analysis of any cell subpopulation defined within a scattergram in relation to the entire population analysed.
Interpretation of flow cytometric results

The two techniques used in this study produce different stains. PI staining stains only the nuclear DNA within the cell, whereas AO/EB stains both nuclear DNA, cytoplasmic RNA, and also enables measurement of the number of dead and dying cells. Figure 5.3. depicts a diagrammatic representation of the format of data presentation of the two methods. Nuclear staining produces a DNA histogram; diploid tumours were defined as those containing one stemline only. DNA aneuploidy was defined as the presence of more than one G1/G0 peak and signifies the presence of an abnormal stemline with an increased DNA content. Figure 5.2. shows a diploid and an aneuploid DNA histograms from two rectal tumours. AO/EB staining produces a DNA/RNA scattergram (Figure 5.4.), the areas of which can be divided and the number of cells in each area quantitated (Dyson et al, 1985). Acridine orange stains the DNA to give green fluorescence, RNA to give red fluorescence, and ethidium bromide, which can only traverse cell membrane rendered permeable by degeneration, stains dead and dying cells.

The areas of the AO/EB scattergram are shown in Figure 5.4. Area 1 obtained from normal human lymphocytes contains non cycling diploid G0 cells. Area 2 from 2.5 to 4.5c contains mitotic cells in the S phase of the cycle and those in the G2 +M phase with double the normal DNA content (4c) together with G0 and G1 cells of abnormal ploidy. An example of a diploid and an aneuploid tumour is depicted in Figure 5.7. Area 3 (>4.5c) contains
hypertetraploid cells which with few exceptions are tumour cells. Area 4 contains dead and dying cells.

For the purposes of this study, diploid tumours were defined as those tumours which had <10% of the total cell population in area 3, whereas DNA aneuploid tumours had >10% of the cell population in this area of the scattergram. The number of cells in area 4 was expressed as a percentage of the total number of cells measured and were not included in the assessment of viable cells in area 1.3. Examples of diploid and DNA aneuploid scattergrams from rectal tumours are depicted in Figures 5.8-5.9, where the percentages of cells in each area has been quantitated and expressed as a percentage. Area 3 is subdivided, in an attempt to refine the technique further.

**Statistical Methods**

Differences between groups were compared using the $X^2$ test with Yates' correction, or when numbers were small, Fishers' Exact Test.
RESULTS

Histopathology

i) **Comparison of single versus multiple pre-operative biopsies**

*in the determination of tumour differentiation.*

Assessed by one pathologist, the grade of the single pre-operative biopsies compared with the differentiation of the main tumour specimens is shown in Table 5.1. In 17 patients (22%) the tumour was missed by the initial biopsy. Of the remaining 50 patients, the single initial biopsy agreed with the differentiation of the main resected tumour in only 35 (59%). The overall accuracy of a single pre-operative biopsy was thus 35/76 or 46%. Multiple biopsies were available in all 76 patients. The differentiation of these biopsies compared with the resected tumour is listed in Table 5.2. Agreement between differentiation of multiple biopsies and of the resected tumour occurred in 53 patients (70%). Although this was an improvement on a single biopsy, this failed to reach statistical significance ($X^2 = 1.16, p = 0.28$).

Deep biopsies were only available in 45 patients (59%). Assessed separately, differentiation did not differ from the superficial multiple biopsies.
Of the 19 poorly differentiated tumours in which single pre-operative biopsies were available, on experienced pathologist correctly identified six (32%) of the 31 well or moderately differentiated tumour, the same experienced pathologist, on single biopsy correctly identified 16 (52%). The sensitivity, specificity and diagnostic accuracy for identification of poorly differentiated lesions was thus 32%, 52% and 42% respectively.

Of the 24 poorly differentiated tumours, examination of multiple biopsies by the pathologist correctly identified 12 (50%) (cf. single biopsy $X^2 = 0.819, p = 0.36$), and of the 41 well or moderately differentiated tumours, the pathologist identified 30 (75%) correctly (cf. single biopsy $X^2 = 2.69, p = 0.10$). Thus the sensitivity, specificity and diagnostic accuracy for identification of poorly differentiated lesions on multiple biopsies by a single pathologist was 50%, 75% and 65% respectively, not significantly different to that obtained from a single biopsy.

ii) Comparison of histopathology and flow cytometric analysis of pre-operative biopsies.

Histological Grading

For the propidium iodide stained tumours ($n = 17$), one pathologist graded 11 (65%) correctly. For the acridine orange/ethidium bromide technique ($n = 19$), where multiple
biopsies were assessed independently by three pathologists, agreement was reached in only six (32%) (cf. one pathologist $X^2 = 2.73, p = 0.10$). Overall agreement in the 36 cases was thus 47%. Twenty-five of 36 resected tumours (69%) demonstrated heterogenicity, containing tumour samples of different grade.

Five of 12 (45%) poorly differentiated tumours were correctly identified pre-operatively, as were 12 of the 25 (48%) well or moderately differentiated lesions.

Flow Cytometric Results (Table 5.3.) (Figs. 5.10.-5.11.)

Of these 17 tumours assessed by propidium iodide staining 16 (94%) of the pre-operative biopsies agreed with the resected tumour. Agreement between pre-operative biopsy and resected specimen using the acridine orange/ethidium bromide technique, was 19/19 (100%). Overall agreement by flow cytometry was thus 35/36 (97%) (cf. histological grading, $p = 0.0001$, Fishers' Exact Test).

A possible objection to this comparison is that three histological grades (Well, moderate and poorly differentiated) were compared to two flow cytometric grades (diploid/non-diploid). To overcome this problem, the histological gradings were reduced to two, poorly and non-poorly differentiated tumours. The detection of a poorly differentiated tumour is
of particular prognostic importance, and may well influence management. Despite this, the overall accuracy of histological grading was only 47% compared with 97% achieved by flow cytometry (p=0.0001, Fishers Exact Test).

**Dead and Dying Cells**

The significance of the number of dead and dying cells within a rectal carcinoma, and the effect on prognosis is unknown. The number of such cells can easily be quantified on the DNA/RNA scattergrams produced by the AO/EB technique.

In the 19 tumours so assessed, agreement between pre-operative biopsy and resected specimen, in the percentage of dead and dying cells present was reached in 14 (73%) (Table 5.4.), significantly better than as histological assessment by three observers ($X^2 = 5.17$, $p = 0.02$), but not as accurate as assessment of the DNA aneuploid population.
DISCUSSION

This section of the study has further confirmed the findings of earlier workers (Qualheim and Gall, 1953; Thomas et al, 1983) in that the histological assessment of pre-operative biopsies from rectal cancer is unreliable. This has important implications.

Rectal tumours are heterogeneous in consistency and thus the assessment of one or two pre-operative biopsies is unlikely to be representative (Quirke et al, 1985; Qualheim and Gall, 1953). The taking of multiple biopsies, both superficial, and where possible deep, might therefore be expected to improve consistency, however although the single specialist pathologist did achieve slightly more consistency on multiple biopsies, this failed to reach statistical significance. Furthermore, when three independent specialist pathologists are involved in assessment, agreement appears even less likely, despite the use of a semi quantitative measure. This failure of multiple biopsies, to improve accuracy confirms the work of Thomas et al (1983), who found that intra and inter observer variation, made interpretation of pre-operative biopsies of rectal cancer extremely unreliable.

As there is good evidence to demonstrate that poorly differentiated lesions have a much poorer prognosis (Lockhart-Mummery et al, 1976), if the pathologist could identify this group accurately, then the surgeon would have a firmer basis on which to select therapy. The degree of differentiation of pre-operative biopsy samples is
used by some authorities to make decisions on operative management, particularly in the selection of patients for local excision or low anterior resection. Lock et al (1978) advise radical excision for poorly differentiated lesions, rather than local excision, even if other factors are favourable. Goligher (1984) suggests at least a 5 centimetre distal clearance for tumour which are poorly differentiated or pre-operative biopsy, and this would mean, in a proportion of patients, sacrifice of the anal sphincter. Unfortunately this study has demonstrated that poorly differentiated lesions cannot be accurately detected pre-operatively, even by multiple biopsy. Over half the tumours (69%) demonstrated differences in grade in one or more samples, confirming that heterogeneity of grade is a common occurrence in rectal adenocarcinoma. Such heterogeneity may well explain the poor agreement obtained between the grade observed in pre-operative biopsies and the subsequently resected tumour (Chapuis et al, 1982).

Measurement of DNA aneuploidy by flow cytometry is quantitative and reproducible. There is now a substantial body of evidence that aneuploidy is a marker of poor prognosis in colorectal cancer as a whole (Wolley et al, 1982; Armitage et al, 1985) and in rectal cancer when taken in isolation (Quirke et al, 1986).

In this study two methods of flow cytometric analysis were used. Initially nuclear staining with propidium iodide was utilised, which produces a DNA histogram, but subsequently cell staining with acridine orange and ethidium bromide was used. The latter
technique which provides DNA/RNA scattergrams is a more sensitive technique which allows the presence of small numbers of cells in any area to be recognised more readily and by the careful quantitation of the cell number above 4.5c, the subjectivity of assessing the presence or absence of an aneuploid G_0, G_1 peak on a DNA histogram as an index of DNA abnormality, and effect on possible prognosis must await longer term survival.

The results of the flow cytometric analysis, further emphasise the heterogeneity of cell populations within an individual tumour and confirms the findings of other workers (Rognum et al, 1982).

The estimation of DNA/RNA content, combined with a measure of the percentage of dead and dying cells is a relatively novel technique (Quirke et al, 1985), and agrees with estimates of cell death within solid tumours (Iversen, 1967; Refsum and Berdal, 1967). The prognostic significance of the numbers of such cells within a rectal carcinoma is as yet unclear. As for assessment of tumour pre-operatively, however, although an improvement on histological grading, cell death was not as accurate as determination of DNA aneuploidy.

The failure in this study to find difference, either by histopathology or flow cytometry between randomised superficial and deep samples, tends to disprove the concept that deeper advancing edges of colorectal cancers are more poorly differentiated than their more superficial counterparts.
In this small number of cases, no correlation between histological grade, Dukes' stage and DNA content was apparent, which confirms the findings of others (Tribukait et al, 1983; Quirke et al, 1985). This lack of a relationship is not entirely unexpected as Dukes' stage is an index of progression and not of growth rate or biological aggressiveness.

The importance of the ploidy status of a rectal cancer has been questioned recently. Goh et al (1987), whilst accepting that DNA aneuploidy is related to survival, by multivariate analysis, suggest that this plays only a small part in overall staging and prognosis. Obviously an advanced tumour has a poor prognosis and the relevance of these findings to this study are questionable. A study by Jones et al (1988) has reported similar findings to those of Goh et al. Other authors (Kokal et al, 1986) conclude, however, that ploidy of colorectal tumours is the single most important prognostic factor. Others (Banner et al, 1985) have correlated aneuploid pre-operative biopsies with advanced stage of the tumour at laparotomy.

This study was designed to quantitate pre-operative assessment of rectal tumour biopsies, which as stated, by histological assessment are unreliable. This fact has been further substantiated by the results of this investigation. Flow cytometric analysis of pre-operative biopsies is quantitative and is significantly better at predicting the cell status of the main tumour. Multiple biopsies must, however, be measured. Flow cytometry, by removing the
subjectivity of the analysis of pre-operative biopsies of rectal cancer would thus appear a superior method to the present histopathological assessment, and by so doing, allows the clinician a more accurate parameter on which to base treatment decisions. Flow cytometry would therefore appear of value in the perioperative assessment of rectal tumours. Those high risk patients with aneuploid tumours so identified could then be considered for more extensive surgery, for adjuvant therapy, or a combination of the two.
**TABLE 5.1.**

Differentiation of single pre-operative biopsy and differentiation of resected tumour (n = 50) (%)

<table>
<thead>
<tr>
<th></th>
<th>V.A.</th>
<th>Well</th>
<th>Moderate</th>
<th>Poor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single biopsy</td>
<td>13 (21)</td>
<td>6 (10)</td>
<td>31 (52)</td>
<td>10 (17)</td>
</tr>
<tr>
<td>Resected tumour</td>
<td>9 (15)</td>
<td>4 (7)</td>
<td>27 (46)</td>
<td>19 (32)</td>
</tr>
</tbody>
</table>

Agreement in 35/59 (59)

(V.A. villous adenoma)
**TABLE 5.2**

Differentiation of multiple pre-operative biopsies \((n = 4)\) and differentiation of resected tumour \((n = 76)\)

\[
\begin{array}{cccc}
\text{V.A.} & \text{Well} & \text{Moderate} & \text{Poor} \\
\text{Multiple biopsy} & 13 (17) & 4 (5) & 44 (58) & 15 (20) \\
\text{Resected tumour} & 11 (14) & 4 (5) & 37 (49) & 24 (32) \\
\end{array}
\]

Overall agreement 53/76 (70)
(cf. single biopsy \(X^2 = 1.16\) \(p = 0.28\))

(V.A. villous adenoma)
## TABLE 5.3

Level of agreement between preoperative biopsies and resected tumour by histological grading and two methods of flow cytometric quantitation

<table>
<thead>
<tr>
<th>Method</th>
<th>N</th>
<th>Agree</th>
<th>Disagree</th>
<th>Percentage Agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histological grading (well, moderate, poor)</td>
<td>36</td>
<td>17</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>Propidium iodide</td>
<td>17</td>
<td>16</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>Acridine orange/Ethidium bromide</td>
<td>19</td>
<td>19</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Overall agreement by flow cytometry 35/36 (97%)  
(cf. histological grading p=0.001)  
(Fishers Exact Test)
TABLE : 5.4.

Percentage mean number of dead and dying cells

(Area 4)

Resected specimen

<table>
<thead>
<tr>
<th></th>
<th>0-24</th>
<th>25-49</th>
<th>50-74</th>
<th>75-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
| Preoperative biopsy/les | 25-49 | 0     | 2     | 0      | 0
| 50-74 | 0    | 0     | 7     | 4      |
| 75-100| 0    | 0     | 1     | 3      |

14/19 (73%) correctly assigned

(cf. histological examination $X^2 = 5.17$ $p = 0.02$)
FIGURE 5.1

Propidium iodide stain. Diagramatic DNA histograms of diploid and DNA aneuploid tumours.
FIGURE 5.2
Propidium iodide stain. Actual DNA histograms from
a) DNA aneuploid rectal tumour and,
b) diploid rectal tumour
FIGURE 5.3
Diagramatic representations of DNA histograms produce by nuclear staining (propidium iodide) and DNA/RNA scattergrams produced by cell staining (acridine orange/ethidium bromide)
FIGURE 5.4

Acridine orange/ethidium bromide stain (AO/EB). Diagramatic representation of DNA/RNA scattergrams.
Cell subpopulations 1-4 as shown. DNA aneuploid tumours are divide into areas.

DIPLOID

NON-DIPLOID

Area 1. 1.5 - 2.5c
Area 2. 2.5 - 4.5c
Area 3. >4.5c
Area 4. Dead & dying cells
FIGURE 5.5

Sampling method of main resected tumour specimen.
FIGURE 5.6

Schematic diagram of a flow cytometer
FIGURE 5.7

Acridine orange/ethidium bromide stain. Actual DNA/RNA scattergrams of
a) diploid rectal tumour

b) DNA aneuploid rectal tumour showing increased number of cells $> 4.5c$
FIGURE 5.8

DNA/RNA scattergram of diploid rectal tumour (AO/EB stain). Area of 3 contains (10% of total number of cells counted.)
FIGURE 5.9

DNA/RNA scattergram of DNA aneuploid rectal tumour (AO/EB stain).

Area 3 contains 10% of total number of cells.
FIGURE 5.10

DNA/RNA histograms (AO/EB) and DNA histograms (PI stain) from 4 preoperative biopsies taken from rectal tumour. Flow cytometric analysis of corresponding resected tumour is depicted in figure 5.11
FIGURE 5.11
DNA aneuploid rectal tumour. DNA/RNA scattergrams (AO/EB stain) from 4 samples of a resected tumour a b c d correspond to sites of sampling depicted in Fig 5.5. Preoperative biopsies from this tumour are depicted in Fig 5.10
Chapter 6

The role of proteolytic enzymes in the local invasion of rectal and sigmoid colon tumours
INTRODUCTION

The second biological parameter addressed in this thesis is the ability of a rectal tumour to invade normal tissues, and by what mechanisms this may be achieved. Locally aggressive tumours carry a poor prognosis and thus if any factors can be identified which indicate that a particular tumour is locally aggressive, a further high risk group can be identified.

A malignant tumour, by definition, invades normal tissue. The exact mechanism by which this is achieved is, however, unknown. Since some malignant tumours release significant amounts of proteolytic enzymes (Dresden et al, 1972; Poole, 1973), it is possible that these are involved in spread of the tumour. Such a relationship has been demonstrated to experimental murine tumours (Liotta et al, 1980). Furthermore, in man, increased protease activity in epidermoid tumours of the head and neck appears to be associated with decreased survival (Abramson et al, 1975).

Collagenase activity has been demonstrated in colorectal tumours (Dresden et al, 1972; Tighe et al, 1981), and in the first part of this chapter, the relationship between peptidase activity in tumours of the rectum and rectosigmoid and their degree of spread was investigated.

Collagenolysis under normal conditions is a two-stage procedure. Initial cleavage by extracellular enzymes is followed by phagocytosis.
of the fragments and intracellular lysosomal digestion. Therefore, the activity of two extracellular peptidases, collagenase and collagenase-like peptidase (CLP) and two intracellular lysosomal enzymes, cathepsin B (cat B) and cathepsin H (cat H) was measured in tumour tissue and normal colonic wall from the same patient. Proteolytic enzyme levels were correlated with the stage of the disease, in an effort to define any possible prognostic significance.

These enzyme systems in vivo are unlikely to operate in isolation, and feedback mechanisms probably exist which control activity. Since it has been demonstrated that the acute phase reactant proteins (APRP) may play a role in inhibition of these proteolytic enzymes (Koj, 1974; Darcy, 1964), serum concentrations to two APRP's alpha-1-acid glycoprotein (AGP) and C-reactive protein (CRP) were also measured and correlated with tumour peptidase activity.

The second part of the study deals with the possible use of exogenous protease inhibitors, which may inhibit malignant cell invasion (Latner et al, 1973), and their use in reducing the activity of proteolytic enzymes in colorectal tumours. Aprotinin (Trasylol) is reported as inhibiting collagenase activity (Latner et al, 1973; Young and Wheeler, 1984) and specific inhibitors of cathepsin B exist, including the anti-inflammatory drugs, which are reported of use in patients with rheumatoid arthritis where this enzyme appears implicated in the disease process (Haatja et al, 1978). Therefore
the potential use of these compounds against two tumour proteases has been investigated in vitro.
Methods

Peptidase activity and serum APRP were measured in 50 patients with a histologically proven carcinoma of the rectum of sigmoid colon. The pathological details of these tumours are listed in Table 6.1. Local spread in this study was defined as microscopic evidence of malignant infiltration outside the bowel wall, or involvement of adjacent structures. Venous invasion, intra- or extra-mural was assessed histologically. Both were assessed by experienced pathologists.

Immediately after resection, samples of tumour were taken from the macroscopically defined growing edge and also from normal colonic wall as far distant from the tumour as possible. The samples were washed in normal saline and flash frozen separately in liquid nitrogen. The frozen samples were stored at -70°C for subsequent analysis.

Blood samples were collected from each patient, immediately prior to surgery for estimation of serum APRP levels. These samples were allowed to clot at room temperature, centrifuged at 3000 rpm and the serum stored at -25°C.
Analysis of Peptidase Activity

For measurement of peptidase activity in normal colonic wall and tumour, the specimens were rapidly thawed and homogenised. In vitro activity was then measured for Cathepsin H and B by the fluorimetric method of Barrett (1980). Collagenase-like peptidase was assayed by the method of Kojima et al (1979) and collagenase by the method of Masui et al (1977). All the above methods utilised synthetic polypeptides, the details of which are given in Appendix 6.1. The assay for CLP utilised a synthetic substrate (Succinyl-Gly-Pro-Leu-Gly-Pro) - 4-methylocoumaryl-7-amide. CLP in vitro liberates the methylocoumarylamide (MCA) from the peptide, which can be dissolved in an organic solvent and measured by direct fluorimetry (Fig. 6.1.).

Collagenase activity in this study was measured during a synthetic polypeptide, linked to 2, 4-dinitrophen-(DNP-Pro-Gly-Ile-Ala-Gly-Gln-D-Arg-OH). Collagenase splits this molecule (Fig. 6.1.) and subsequent extraction by ethyl acetate liberates the DNP fragment which was measured spectrophotometrically.

Cat H and Cat B were measured by a fluorimetric method, again using synthetic peptides, Benzyloxy carbonyl (Z) - Phe-Arg-4-methyl-7-coumarylamide (MCA) for Cat B and Arg-MCA for Cat H (Fig. 6.1.). All the synthetic peptides were purchased from the Peptide Institute (Osaka, Japan).
To standardise activity, protein content of the sample was measured by the Folin-Lowry method (Lowry et al, 1951) and enzyme activity expressed as nmol (mg protein)\(^{-1}\) min\(^{-1}\). The assays were reproducible and interassay variation was minimal. Mean coefficients of variance (and s.e.m.) between assays were: cathepsin B, 5.48 (0.76); cathepsin H, 6.04 (1.28); CLP, 10.74 (1.54); collagenase 2.84 (0.36). Intra-assay variation overall was <10 per cent (Table 6.2.).

**Measurement of Acute Phase Reactant Proteins**

Alpha-1-acid glycoprotein and C-reactive protein were measured by single radial immunodiffusion (Mancini et al, 1965), the methodology is detailed in Chapter 3.

**The Effect of Exogenous Inhibitors on Collagenase and Cathepsin B**

To evaluate the effect of exogenous protease inhibitors on in vitro activity of tumour collagenase and cathepsin B, therapeutic dosages of aprotinin, for collagenase, and of ibuprofen, indomethacin, chloroquine and leupeptin for cathepsin B, were added to the tumour homogenate prior to assay of the respective proteases. The assays were then performed as already described. The range of dosages utilised (n = 6) are listed below.
Aprotinin $1 \times 10^{-2} - 10$ units/ml
Ibuprofen $24 \times 10^{-2} - 24 \times 10^3$ ng/ml
Indomethacin $6 \times 10^{-2} - 6 \times 10^3$ ng/ml
Chloroquine $6 \times 10^{-1} - 6 \times 10^4$ ng/ml
Leupeptin $3 \times 10^{-1} - 3 \times 10^4$ ng/ml

Each drug was tested on six tumour samples. Inhibition was measured as mean percentage (+SD) of control activity in the tumour sample for each dosage utilised.

**Statistical Methods**

The data proved non-parametric and therefore the results are expressed as median and semi-interquartile range, and analysed by the Wilcoxon Rank Sum Test for paired, and the Mann-Whitney U Test for unpaired samples. Spearman rank correlation was utilised in the analysis of the relationship between serum APRP and tumour peptidase activity. Drug inhibition was expressed as mean percentage (+SD) of control sample.
RESULTS

**Peptidase Activity** (Fig. 6.2.) (Appendix 6.2.)

A significant elevation of activity of cathepsin B, CLP and collagenase was found in tumour tissue compared with that in normal colonic wall. Median values expressed as nmol (mg protein)^-1 min^-1 were respectively: Cat B 0.71 and 0.42 (p<0.001); CLP 25.24 and 12.25 (p<0.001); collagenase 0.49 and 0.31 (p<0.001). There was wide individual variation in peptidase activity both in tumour tissue and colonic wall. To standardise a relative increase in activity in the tumour compared to normal tissue, the ratio of activities tumour/colonic wall was calculated (Figure 6.3.) (Appendix 6.3.). Median values of this ratio were 0.9 and 1.6 for cathepsin H and B respectively, 1.7 and 1.4 for CLP and collagenase respectively. This ratio was then correlated with tumour characteristics.

**Relationship to Tumour Spread**

There was no clear relationship between peptidase activity and Dukes' stage (Fig. 6.4.), or degree of differentiation (Fig. 6.5.). There was, however, a highly significant elevation of cathepsin B activity in tumours which exhibited local spread compared with those that did not, (Fig. 6.6.) (median values 2.76 and 1.36, U = 304, z = 4.68, p<0.0001). Similarly, though less significant, was the
case with tumours which showed evidence of venous invasion (Fig. 6.7.) (median values 1.82 and 1.18, U = 57 p<0.01).

**Peptidase Activity and Serum APRP**

Complete results of serum APRP were available in only 42 patients.

There was no correlation between preoperative serum levels of AGP and activity any of the tumour peptidases. (Spearman rank correlation: AGP and Cat B, 0.004, p = 0.97; AGP and Cat H, =0.148, p = 0.35; AGP and CLP, -0.203 p = 0.19; AGP and Coll =0.256 p = 0.10).

There was, however, an inverse correlation between activity of cat H, CLP and collagenase in the tumour and preoperative levels of CRP which was statistically significant (Fig. 6.8.). Utilising Spearman rank correlation $r_s$ values for CRP and these three peptidases were: $-0.359$ (p<0.05); $-0.332$ (p 0.05); $-0.302$ (p = 0.05) respectively.

**Results of Protease Inhibitors**

The results of the effects of exogenous protease inhibitors on collagenase and cathepsin B are listed in Table 6.3.

Aprotinin in vitro did not inhibit collagenase activity, the range of Inhibition expressed as percentage of control activity (+SD) was
99.5 (11.7) - 112.1 (7.0). Only leupeptin inhibited cathepsin B activity in vitro, the range of inhibition was 2.7 (0.9) - 102.2 (8.2). There was a strong inverse correlation between dose of leupeptin and cathepsin B activity (r = -0.9, p = 0.01).

At dosages above 30 ng/ml, virtual complete inhibition of cathepsin B was observed.
DISCUSSION

This study has demonstrated an increased activity of three proteolytic enzymes in carcinomas of the sigmoid colon and rectum compared with activity in normal colonic wall. Although no clear relationship emerged between enzyme activity and Dukes' stage or degree of differentiation, markedly increased activity of cathepsin B was evident in locally invasive tumours and those with venous invasion. An apparent inverse correlation between tumour proteases and serum CRP has also been demonstrated.

Much of the early work on tumour collagenases was based on the method of Gross and Lapierre (1962) which utilised tissue culture techniques to establish the presence of proteases. These methods were, however, difficult to quantify. Since the introduction of techniques which employ specific synthetic polypeptide substrates to measure mammalian proteases (Barrett, 1980; Kojima et al, 1979; Masui et al, 1977), in vitro analysis of homogenised tissue samples can be used to estimate enzyme activity. These assays are accurate and reproducible, the assay for cathepsin B being particularly specific (Barrett, 1980). Doubt has been cast on the accuracy of synthetic substrates in estimating collagenase activity in tumour homogenates (Woolley et al, 1980), however Masui (1977) has confirmed the accuracy of his method by correlating it with collagenase activity measured by direct assay of radioactive $^{14}\text{C}$ collagen fibrils (Terato et al, 1976).
In vivo neutral collagenases are found in forms other than the active one, as a pro-enzyme or as a complex with an inhibitor. In the latter form the enzyme is difficult to assay (Sellars et al, 1977), however, in this study an attempt was made to activate latent collagenase present by pretreatment of the homogenate with trypsin. Presumably, however, the amount of active protease is of most relevance to tumour invasion.

To overcome differences in composition of the samples, for example due to increased oedema in the tumour, protein content was measured as the standard for enzyme activity, rather than weight of tissue. However, no allowance could be made for differences in inflammatory cell infiltrate in the biopsies, which can themselves produce proteases.

Since Fischer (1925) first described the capacity of tumour tissue in organ culture to release factors capable of degrading extracellular protein, interest has been shown in defining the role of proteolytic enzymes in the spread of malignant tumours. Certainly alteration in collagen content of the tissues occurs during and possibly preceding malignant change (Orr, 1938; Gillman et al, 1955) and it is further postulated by some authors that these stromal changes may have a role in tumorigenesis (Gillman et al, 1955).

The source of these proteolytic enzymes is conjectural. It has been observed (Shamberger and Rudolph, 1967) that rapidly growing cells
at the periphery of a tumour has the highest levels of lysosomal enzyme activity. Bauer (1979) however has suggested that tumour cells secrete a chemical agent which influences transformed stromal fibroblasts to produce these peptidases.

Numerous authors (Robertson and Williams, 1969; Dresden et al, 1972; Poole, 1973; Lloita et al, 1980; Tighe et al, 1981; Woolley, 1982) have reported increased proteolytic activity in a variety of tumours including colorectal, and the results of this study support these findings.

There was no relationship between peptidase activity and the degree of tumour differentiation. However in the light of the recent controversy surrounding the histological assessment of tumour differentiation (Thomas et al, 1983), the significance of this finding is open to question. Similarly there was no association between enzyme activity and Dukes' stage.

In tumours which were found to be locally invasive a significantly higher level of cathepsin B was evident compared with tumours confined to the bowel wall. This increased activity was also evident in those tumours with evidence of vascular invasion. Activity of the other peptidases was not significantly different between the groups. The role of cathepsin B in collagenolysis is not clearly defined. In vitro this enzyme will digest collagen and proteoglycans, the two major components of the extra-cellular matrix. Activity is, however, maximal at low pH which is not
available in the extracellular environment, although some activity remains at neutral pH. A relatively acid pH is found within the cell, and one explanation for the role of cathepsin B is that it degrades phagocytised portions of collagen molecules which have been cleaved by extracellular peptidases (Etherington, 1976). An alternative mode of action is suggested by Graf et al (1981) who regard cathepsin B as an activator of latent collagenase. Cathepsin B has also been implicated in tumour metastasis. Sylven (1968) has demonstrated that lysosomal cathepsin B facilitates cell detachment from glass and would appear to overcome the normal restraints of cell movement and may thus aid metastasis. Previous studies have suggested a role for cathepsin B in the spread of carcinoma of the breast (Recklies et al, 1980), and raised levels have been detected in invasive gastric cancers (Vasishta et al, 1985, Vasishta et al unpublished observation). This study indicates that cathepsin B may be the key enzyme in local spread of colorectal tumours. If this is the case, a specific inhibitor may have a role in the management of patients with local tumour infiltration. Furthermore, elevated levels of cathepsin B may indicate a further category of poor risk patients who require local adjuvant treatment.

Natural inhibitors of these enzymes particularly collagenase exist in the serum and possibly in extracellular fluid. In the serum alpha-1-macroglobulin and beta-2-anticollagenase are the two most widely documented (Woolley et al, 1980). In tissue culture, tumours grown in serum-free medium show increased production of proteases.
Acute phase reactant proteins are produced primarily by the liver in response to a variety of stimuli including malignancy, and there is in vitro evidence to suggest that these compounds can inhibit protease activity (Koj, 1976; Darch, 1964).

This study has demonstrated an inverse correlation between serum levels of CRP and three of the peptidases measured, Cathepsin H, CLP and collagenase. Whilst a correlation between two variables does not necessarily imply a causal relationship, this inverse correlation would certainly support the experimental evidence which suggests that acute phase reactants inhibit tissue activity of some peptidases. It is, however, interesting to note that there was no inverse correlation of CRP with cathepsin B activity. That this peptidase appears related to local invasion may, in part, be due to this lack of natural inhibition. Elevated levels of CRP were found in a high proportion of patients with disseminated disease or locally extensive tumours which is detailed in Chapter 3. If high levels of CRP do inhibit peptidase activity in the tumour, this may partly explain one apparent contradiction in this study, in that patients with disseminated disease (Dukes' 'D') in whom increased enzyme activity would be expected, had relatively low levels, particularly of CLP (Fig. 6.4.). This apparent inhibition probably occurs too late to influence the progress of the disease. In patients with locally confirmed tumour surrounded by a vigorous inflammatory response, very high levels of CRP are found, as detailed in Chapter 3, presumably in response to the acute inflammation. This relatively early acute phase response may have a role in confining
the disease locally and thus aiding local host defences. These patients have a relatively good prognosis (see Chapter 2). These hypotheses are, however, obviously oversimplifications of complex biochemical interrelationship occurring at the host-tumour interface.

There is theoretical and experimental evidence to suggest that protease inhibitors can inhibit malignant cell invasion (Latner et al, 1973) and that aprotinin can inhibit collagenase in vitro (Young and Wheeler, 1984). This study has failed to confirm the inhibition of collagenolytic activity by aprotinin in vitro. This is perhaps not surprising as aprotinin probably achieves inhibition of collagenase by blocking the activation of the latent enzyme to its active form in vivo (Ikenaka et al, 1974). In this assay, only activated collagenase was measured.

Cathepsin B activity is raised in patients with rheumatoid arthritis, and the beneficial effects of anti-inflammatory drugs are thought to be partially related to inhibition of this enzyme (Haatja et al, 1978). Chloroquine in particular is reported as a specific cathepsin B inhibitor. It was therefore disappointing to find no commonly used drug which was able to produce in vitro inhibition of cathepsin B at dosages corresponding to the therapeutic range.

Leupeptin, in vitro, however appeared to be a specific and virtually complete inhibitor of cathepsin B activity. Leupeptins are oligopeptide aldehydes produced by specific strains of streptomyces
(Umezawa and Aoyagi, 1977) and are potent inhibitors of lysosomal enzymes (Aoyagi et al, 1989).

As cathepsin B appears important in local invasion, leupeptins may thus have a potential role in the future management of patients with local extensive rectal cancer. There was no correlation between cathepsin B activity and metastatic (Dukes' 'D') lesions, and experimentally, leupeptins have failed to reduce the production of metastases in an animal model (Giraldi et al, 1977), and thus any potential benefit may be confined to limiting local tumour invasion.

This study has demonstrated that peptidase activity in tumours of the sigmoid colon and rectum is increased as in the case of cathepsin B, this increased activity appears related to local invasion. In conjunction with more accurate assessment of these patients by perioperative staging, the role of specific peptidase antagonists, in particular leupeptins, may prove of value in future management.

Furthermore, by refining the methodology it is feasible that assays for peptidase activity, in particular for cathepsin B, could be performed on pre-operative biopsies of rectal tumours. This may further help identify those high risk patients with locally invasive disease who warrant consideration of adjuvant therapy.
<table>
<thead>
<tr>
<th>Pathological Details of Tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dukes' Stage</strong></td>
</tr>
<tr>
<td>n=50</td>
</tr>
<tr>
<td>A 1 (2)</td>
</tr>
<tr>
<td>B 18 (36)</td>
</tr>
<tr>
<td>C 21 (42)</td>
</tr>
<tr>
<td>'D' 10 (20)</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
</tr>
<tr>
<td>Well 6 (12)</td>
</tr>
<tr>
<td>Moderate 30 (60)</td>
</tr>
<tr>
<td>Poor 14 (28)</td>
</tr>
<tr>
<td><strong>Local Spread</strong></td>
</tr>
<tr>
<td>No LS 37 (74)</td>
</tr>
<tr>
<td>LS 13 (26)</td>
</tr>
<tr>
<td><strong>Venous Invasion</strong></td>
</tr>
<tr>
<td>No VI 26 (51.5)</td>
</tr>
<tr>
<td>VI 24 (48.5)</td>
</tr>
</tbody>
</table>
### TABLE 6.2

**Mean coefficient of variance (%) (and SEM) for**

**Duplicate Samples, Inter- and Intra-assay Variation**

<table>
<thead>
<tr>
<th></th>
<th>Cat B</th>
<th>Cat H</th>
<th>CLP</th>
<th>Coll</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duplicates</td>
<td>3.30(1.9)</td>
<td>11.18(3.95)</td>
<td>14.11(5.01)</td>
<td>3.20(2.70)</td>
</tr>
<tr>
<td>(n=50)</td>
<td>(n=50)</td>
<td>(n=50)</td>
<td>(n=50)</td>
<td>(n=50)</td>
</tr>
<tr>
<td>Intra-assay</td>
<td>2.80(1.4)</td>
<td>5.70(2.50)</td>
<td>2.40(1.30)</td>
<td>9.4(4.20)</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=9)</td>
<td>(n=8)</td>
<td>(n=6)</td>
<td>(n=12)</td>
</tr>
<tr>
<td>Inter-assay</td>
<td>5.48(0.76)</td>
<td>6.04(1.28)</td>
<td>10.74(1.54)</td>
<td>2.84(0.36)</td>
</tr>
<tr>
<td>(n=6)</td>
<td>(n=8)</td>
<td>(n=6)</td>
<td>(n=6)</td>
<td>(n=15)</td>
</tr>
</tbody>
</table>
### TABLE 6.3

**Effect of Exogenous Protease Inhibitors on In Vitro Activity of Tumour Collagenase and Cathepsin B**

<table>
<thead>
<tr>
<th>Collagenase</th>
<th>Concentration (n=6)</th>
<th>Range of Inhibitor (% control sample) +SD</th>
<th>Dose Response Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>$1 \times 10^{-2}$-10 units/ml</td>
<td>99.5 (11.7) - 112.1 (7.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Cat B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>$24 \times 10^{-2}$-24 $\times 10^3$ ng/ml</td>
<td>96.3 (4.5) - 99.7 (5.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>$6 \times 10^{-2}$-6 $\times 10^3$ ng/ml</td>
<td>103.0 (3.5) - 109.7 (6.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>$6 \times 10^{-1}$-6 $\times 10^4$ ng/ml</td>
<td>97.0 (6.7) - 103.3 (3.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>$3 \times 10^{-1}$-3 $\times 10^4$ ng/ml</td>
<td>1.3 (1.0) - 102.2 (6.2) r=-9, p=0.01</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 6.1
Measurement of peptidases utilising synthetic polypeptides
FIGURE 6.2
Median values and semi-interquartile ranges (SIR) (nmol (mg protein)\(^{-1}\) min\(^{-1}\)) of peptidase activity in tumour tissue (T) compared with normal colonic wall (W) from the same patient.
(Wilcoxon Rank Sum Test)
Relative increase in activity in tumour tissue compared with normal colonic wall, expressed as median (+SIR) of the ratios of tumour activity/normal colonic wall activity (T/W). (CB, cathepsin B, CH cathepsin H)
FIGURE 6.4

Relative activity (T/W) of Cat B, CLP and collagenase

in relationship to Dukes' staging
FIGURE 6.5

Relative activity (T/W) of cat B, CLP and collagenase
in relationship to degree of differentiation.
FIGURE 6.6

Relative activity (T/W) of Cat B, CLP and collagenase in tumour with local spread (L/S) compared with tumours with no spread (no LS). Median and SIR

\[ p < 0.0001 \]

\[ \text{CLP} \text{ NS} \]

\[ \text{Coll} \text{ NS} \]
FIGURE 6.7

Relative activity (T/W) of Cat B, CLP and collagenase in tumour with evidence of venous invasion (VI) and those with no venous invasion (no VI). Median and SIR
FIGURE 6.8

Tumour peptidase activity of Cat H, CLP and collagenase and preoperative serum levels of CRP (mg/l). (overlapping points have been omitted for clarity)
CHAPTER 7

The Assessment of Tumour Growth Rate
by Production of Multicellular Tumour Spheroids
from Colorectal Cancer
INTRODUCTION

The final aspect of biological behaviour assessed in this thesis is the growth rate of the tumour and its relationship to stage of the tumour and prognosis.

To achieve the aim of this study, an in vitro, model of tumour growth, multicellular tumour spheroids was developed from primary colorectal tumours. The growth rates of spheroids from individual patients were correlated with the stage of the disease and the subsequent outcome. The model also provided a useful tool for the assessment of adjuvant chemotherapeutic agents in individual tumours dealt with in the latter part of this chapter.

Measurement of tumour growth rate in vitro, may be an important parameter in determination of high risk patients with biologically "aggressive" tumours. Such patients are candidates for a more radical operative approach combined with adjuvant therapy.

The ideal in vitro tumour model does not, as yet, exist, however multicellular tumour spheroids, as outlined in the Introduction to the thesis approach this ideal.

As yet, attempts to grow MTS from primary colorectal cancers has proved largely unsuccessful. The initial section of the study, therefore, deals with the attempt to produce a spheroid model directly from individual tumours, and the validation of the
methodology. It was important to establish that the MTS growing in culture comprised tumour cells and not merely connective tissue cells.

Adjuvant chemotherapy of large bowel cancer has to date, proved largely disappointing. Tumours of the colorectum are intrinsically chemoresistant, with a high degree of clonal heterogeneity. Coupled with this is the lack of suitable drugs. The fluorinated pyrimidines appear most successful, 5-fluoro-uracil (5FU) being the most widely used. Overall results have proved inconclusive (Higgins et al, 1978; Lawrence et al, 1975) although recently, the Gastrointestinal Tumour Study Group (1985) has reported encouraging results using radiotherapy and combined chemotherapy, and I Taylor et al, (1985) have reported success in the administration of 5FU via the portal vein in the perioperative period, in reducing the number of subsequent hepatic metastases. On average, however, only 20% of patients are likely to respond clinically to 5FU (Carter, 1976). Ideally, this 20% should be identified before commencement of therapy, to avoid unnecessary toxicity in the 80% who will not respond. Various in vitro models have been developed in an attempt to achieve this aim, and they have several different utilised methods, which have previously been alluded to in the introduction.

Multicellular tumour spheroids (MTS) (Fig 7.1 - 7.2) are three dimensional aggregates of tumour cells, first described by Sutherland et al (1971), which demonstrate the heterogeneity of
cellular oxygen status and proliferative state found in solid tumours. Indeed MTS in culture demonstrate many of the characteristics of solid tumours in vivo, including their response to chemotherapy (Yuhas et al, 1978). Spheroids have been produced from many solid tumours (Yuhas et al, 1978; Yuhas et al, 1977; Haji Karim and Carlsson, 1978; Conger and Ziskin, 1983; A C Jones et al, 1982) and also from cell lines derived from colorectal tumours (Barone et al, 1981; Lees et al 1981). This latter approach, however, although allowing some indication of sensitivity of colorectal cancer on the whole, negates the concept of determining individual tumour sensitivity, which is necessary if chemotherapy is to play any useful role in the future clinical management of rectal cancer. Ideally this information should be available pre-operatively.

The first part of this study, describes the development of a method to produce multicellular tumour spheroids directly from primary colorectal cancers, both from the main specimen, and where available from pre-operative sigmoidoscopic biopsies, and also with validation experiments to ensure that the spheroids produced, comprise colorectal adenocarcinoma cells, and not sundry connective tissue elements. The second section deals with the correlation between in vitro growth rate and biological "aggressiveness" of the tumour. The last section addresses the potential use of spheroids as predictors of individual tumour sensitivity to chemotherapeutic agents.
METHODS

1. Production of Multicellular Tumour Spheroids

Tumour tissue from 33 specimens of colorectal carcinomas was obtained immediately following resection. In seven patients with a rectal carcinoma, pre-operative biopsy material was removed via the rigid sigmoidoscope with standard biopsy forceps. A portion of tumour was imprinted on to two microscope slides for Feulgen microdensitometry of 200-250 tumour cell nuclei using a scanning microdensitometer (M-85 Vickers Instrument, York) (Dixon and Stead 1977), and tumour was also removed for measurement of cellular DNA content by flow cytometry (Ortho Cytofluorograph).

The tumour tissue was immediately placed into Hanks balanced salt solution (HBSS) containing gentamicin at a concentration of 4 mg/ml. Tumour tissue could be stored for up to 12 hours in this solution if maintained at 4°C.

To prepare spheroids, the tumour tissue was washed in fresh HBSS, cleaned of mucinous debris, and extraneous tissue dissected away. The sample was then rinsed and incubated in fresh HBSS containing streptomycin 5 mg/ml, penicillin 5 mg/ml and gentamicin 4 mg/ml for five minutes in a 30 ml universal container.
Samples were then dissected from the main tumour block to provide a 2-3 mm$^3$ block for histology, preserved in 10% neutral formalin.

After a final rinse in fresh HBSS the tumour was disaggregated, firstly by mechanical chopping with a scalpel and scissors, and then by serial passage through graduated fine bore needles. The suspension was filtered through a fine wire mesh to remove debris and cell clumps. If necessary a final precipitation separation was performed to produce a single cell suspension. Occasionally, for very tough tissue fragments, trypsin digestion (0.25% trypsin in HBSS for 25 minutes at 37°C) was necessary. A total cell count of the single cell suspension was performed using a modified Neubauer haemocytometer, and a viable cell count performed using Trypan Blue exclusion (Flow Laboratories Ltd).

The cell suspension was centrifuged at 170g for eight minutes, the supernatant, containing red cells and debris was discarded, and the cell pellet suspended in complete medium (Medium 199, 15% newborn calf serum, 1mM glutethamide, 1.7 g/L sodium bicarbonate, 20 ml Hapes, non-essential amino acids, gentamicin 4 mg/ml - all from Gibco Bioccult Ltd).

Approximately 2 x 10$^5$ cells were then inoculated into tissue culture flask (25 cm$^3$ - Sterillinn) containing complete medium, base coated with 1% agar containing 10% complete medium.
The cells were allowed to settle, incubated at 37% in an atmosphere of 95% oxygen, 5% carbon dioxide. Subsequently the flasks were gently rocked from side to side to produce a central band of tumour cells. The flasks were then allowed to stand for 24 hours at 37°C to facilitate cell adhesion. If there were large numbers of viable cells in each flask, aggregation would occur spontaneously by chemotaxis. Conversely, if the cells were widely separated further agitation of the flasks was necessary to encourage aggregation.

Once dense bands of cells had formed, these were reduced to smaller individual aggregates by vigorous shaking, and further complete medium was added. After a further 24 hours incubation, the cell aggregates had formed into protospheroids, and were transferred to larger flasks (75 cm³ - Sterilin), with 20 ml complete medium. When the spheroids had fully formed and appeared stable, they were transferred to 700 ml spinner flasks (Techne-Cambridge) containing 500 ml complete medium for further growth. The medium was changed three times a week. By seven days, most spontaneously degradable spheroids had dispersed, and the majority left were approximately 100 μm in diameter. Spheroids were then maintained in spinner flasks, the medium was changed every two days, when spheroid diameters (mean of two diameters at right angles) were measured, using an inverting microscope.
(Artek 982) with a calibrated eyepiece, at a density sensitivity of 6.88.

For histological examination, spheroids were washed in HBSS, fixed for 1.5 hours in Bouin's solution and removed to 70% ethanol at 4°C for storage. The spheroids were subsequently embedded in 1% alginate drops to facilitate further handling. After dehydration in a graded alcohol series (70-100%), and cleaning in Xylene, each alginate ball, containing up to five spheroids was vacuum embedded in Ralwax (R A Walsh, London), mounted in a paraffin block and serial 5 μm sections cut. These were placed on slides, dewaxed and stained with haemotoxylin and eosin (H & E) (Fig. 7.3.).

**Validation Studies**

Having developed a method for producing spheroids directly from individual colorectal tumours, it was essential to answer two questions:-

a) Were the spheroids composed to tumour cells and

b) Does growth of MTS in vitro bear any relationship to in vivo characteristics of the parent tumour?

To answer the first question the following studies were performed.
i) **Growth of non-malignant tissue as MTS**

Portions of tissue were obtained from the operative specimen in three patients with histological proven villous adenomata, and two biopsies of normal colonic mucosa. The tissue was treated as described above in order to determine whether MTS would form from non-malignant colonic tissue.

ii) **Histological comparison of parent tissue and spheroid**

In 12 patients, routine sections (H & E) taken from the resected specimen for routine histopathological examination were compared with sections of the corresponding spheroids, by a pathologist experienced in gastrointestinal pathology.

iii) **Comparison of cellular DNA content**

DNA content, of parent and spheroid cells was measured in 10 tumour and five pre-operative biopsies by microdensitometry (Dixon and Stead 1977) or by flow cytometry using the propidium idodide method (Dyson et al, 1984) (See Chapter 5).
Growth of MTS as xenografts in Immune deprived mice

As final confirmation, three to five spheroids produced from each of 10 different tumours, were introduced subcutaneously into the flanks of 10 mice previously rendered immune-deprived by thymectomy and whole body irradiation, via a wide bore cannula. The mice were kept in sterile conditions and any nodules which developed were excised after sacrifice and embedded in paraffin, sectioned and stained with haemotoxylin and eosin. As macrophages may grow in immune-suppressed animals as nodules, which can mimic tumour deposits, any found were also stained with specific macrophage stains to alpha-1-antitrypsin, alpha-1-antichymotrypsin and lysozyme which can identify any macrophage or macrophage related cells. Polyclonal antibodies to the above enzymes raised in rabbits, were obtained (DAKA, Denmark), and the technique used was an indirect immunoperoxidase method. The tissue was blocked with 3% hydrogen peroxide in methanol, treated with the primary antibody, then an antirabbit peroxidase, stained with diaminobenzidine, and finally counterstained with haemotoxylin. Any macrophages present would stain dark brown.
Relationship to In Vivo Tumour Growth

To answer the second question, spheroid growth rates in culture, from 19 tumours were compared with the extent of tumour spread, the histopathological status of the tumour and the early postoperative survival of the corresponding patients. The pathological details of the 19 patients are listed in Table 7.1.

Evaluation of Chemosensitivity

For evaluating chemosensitivity of the spheroids, 10 stable spheroids of from 200-300 μm in diameter were selected for each flask. The spheroids were placed in HBSS, and received one hour exposure to clinically achievable peak plasma drug concentration. Following this the spheroids were placed in fresh medium and growth measured every 48 hours. The drugs and dosages are detailed in Table 7.2.

Statistical Methods

Growth rates of spheroids from individual tumours demonstrated a normal distribution and are thus quoted as mean ± standard deviation. However, due to the wide variation in growth rates between tumours, the data obtained could not be analysed by parametric statistics. Therefore, for analysis of differences between groups, non-parametric tests
were utilised (Mann Whitney U test, Kruskal-Wallis test, Fishers Exact Test).

**Addendum on measurement of MTS growth in culture**

Growth of multicellular tumour spheroids in this study was measured as increase in diameter (mean of two diameters at right angles). Conger and Ziskin (1983) and earlier, Landry et al (1982) have demonstrated that radial growth of MTS in culture is linear with time after an initial period of geometric growth. This initial period of growth is characterised by the aggregation of single cells into small clumps and their quasi-exponential growth (Sutherland and Durand, 1976) until a critical volume is reached. The second phase of growth is linear (Yuhas and Li, 1978), and is directly related to the depth of the dividing shell and the fraction of cells in cycle. Finally as they enlarge, due to accumulation of toxic waste products within the spheroid, a critical concentration is reached at the edge of the cycling rim causing progressive diminution in its thickness, and a slowing of growth which then reaches a plateau (Folkman and Hochberg, 1973). Over the linear phase of MTS production increase in diameter provided the simplest and most direct measure of spheroid growth, as demonstrated both mathematically and experimentally (Landry et al, 1982; Conger and Ziskin, 1983). Thus in this study, growth was measured as increase in

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diameter. Volume of spheroids was calculated from the formula for ellipsoids:

\[ v = \frac{4}{3} (ab)^{3/2} \]

Where a and b are the perpendicular radii of the visually measured spheroids.
RESULTS

Production of MTS

Using the method described previously, of the 33 operative specimens, multicellular spheroids were successfully produced from 26 (79%) and of the seven pre-operative sigmoidoscopic biopsies, five (71%) successfully produced spheroids.

The number of spheroids produced from an individual tumour sample varied from a minimum of 29 to a maximum of 415.

To produce spheroids of approximately 200 um in diameter took from two to six days depending on growth rate.

Full growth curves were obtained for spheroids from 19 operative specimens, the mean (±SD) growth rates are listed in Table 7.3., and graphically in Figure 7.4. These mean growth rates were calculated over the initial 10 day period following stabilisation of the spheroids, during the linear phase of growth, and were measured from a minimum of 10 spheroids for each tumour sample. Growth rates from individual tumours varied widely from a minimum of 4 um/day to 52 um/day, over the 10 day period. Volume doubling time ranged from 5.6 to 17.0 days.
Validation Studies

i) Growth of non-malignant tissue

No non-malignant tissue, which has been treated exactly as described in the methods sections, produced spheroids. Tissue would grow in culture as single cells for two to three days, occasionally small aggregates would form, but spheroids would not. Fibrous tissue elements and red blood cells, if incorporated into tumour spheroids caused disruption.

ii) Histological Evidence (Figs. 7.5. - 7.6.)

Histological sections examined by light microscopy comparing the cytological appearances of spheroid and parent tumour proved identical to all 12 cases. Tumour differentiation also appeared to be maintained. A well differentiated adenocarcinoma produced well differentiated spheroids (Fig. 7.5.) with even glandular structures faithfully reproduced. Conversely a poorly differentiated tumour produced poorly differentiated spheroids (Fig. 7.6.).
Spheroids derived from individual tumours had identical DNA profiles to the sample of origin (Fig. 7.7.). However some samples from different areas of the same tumour demonstrated heterogeneity of DNA content. Figures 7.7a. and 7.7b. illustrate the DNA profile of the disaggregated spheroids and the original tumour respectively. Allowing for a much smaller number of cells measured from the spheroids, the profiles are identical. Figures 7.7c. and 7.7d. depict the DNA profiles of spheroid and sample respectively, from the same tumour as 7.7a. and 7.7b., but from the pre-operative biopsy. There is a well demonstrated, non-diploid cell population in the pre-operative biopsy sample, which was reflected in the spheroids derived from that tissue not present on the main operative specimen. Figure 7.8. shows that DNA profiles of spheroids from the five pre-operative biopsies which grew in culture compared with spheroids from the resected tumour, measured by microdensitometry. Although the profiles were similar, there were slight differences, which again highlights the heterogeneous nature of colorectal cancer.
iv) **Growth of MTS as xenografts in immune deprived mice**

Of the 10 mice inoculated with tumour spheroids, three produced nodules at the site of implantation (Figs. 7.9. - 7.10.) and one produced metastases to the liver (Fig. 7.11.).

Histological examination of sections through these nodules demonstrated poorly differentiated adenocarcinoma. In one section (Fig. 7.9.), two nodules are shown at low power. One nodule has retained its original spheroid structure with an outer wall and necrotic centre, however, the other nodule appears much more cellular. On closer inspection (Fig. 7.10.), at higher power, numerous blood filled channels can be seen traversing the spheroid, and it would appear that this nodule has developed a blood supply from the mouse's circulation.

Examination of the liver nodules (Fig. 7.11.) confirms metastatic poorly differentiated adenocarcinoma, in all sections.

Ten sections stained for alpha-1-antitrypsin, alpha-1-antichymotrypsin and lysozyme (Figs. 7.12. - 7.14.) were all negative, and thus these nodules do not represent clumps of macrophages.
Spheroids derived from individual samples had an identical light microscopic appearance and DNA profile to their tumour sample of origin, and furthermore grew and metastasised as xenografts in immune-deprived mice. This would therefore confirm that the spheroids produced in this study consisted of adenocarcinoma cells.

**MTS Growth Rate and Relationship to In Vivo Tumour Behaviour**

The median growth rates of spheroids from the 19 patients were correlated with Dukes' stage (Fig. 7.15), presence of absence of local spread (LS) and presence or absence of venous invasion (VI) (Fig. 7.16.), and survival (Fig. 7.17.).

Although a trend was evident with Dukes' stage (Fig. 7.15.), with apparently faster growing spheroids found in patients with Dukes' 'C' or 'D' lesions, this failed to reach statistical significance (p = 0.07, Kruskal Wallis).

In the six patients with evidence of local extension however, spheroid growth rates were significantly higher than in the 13 with locally confined growth (LS present:median (+ range) um/day : 38 (27-52) LS absent 19 (4-30). MW = 1 p<0.01). A similar if less apparent difference was seen in the 10 patients with evidence of
venous invasion (VI) compared to the nine with no vascular involvement (no VI). Although faster growth rates were associated with venous invasion, this just failed to reach statistical significance. (VI, median (+ range) um/day : 30 (15-52), no VI 20 (4-30) MW = 20 p = 0.05) (Fig. 7.16.).

The most interesting correlation, however, was that with survival. The original 19 patients have now been followed for a minimum period of 18 months. Of the original 19, seven (37%) have died of disease and 12 (63%) remain alive. Five tumours had MTS growth rates of >30 um/day - all five have died of disease. Of the 14 patients with growth rates <30 um/day, two have died of disease (p=0.01, Fishers' Exact Test). The length of postoperative survival, of the seven patients who subsequently died correlates inversely with spheroid growth rate (Fig. 7.17.) (Spearman's rank correlation Rs = -0.80, p 0.02). Not all the patients who have died had disseminated disease at initial laparotomy. The shortest survivor (three months) had in fact locally extensive disease within the pelvis. Despite a palliative excision, he rapidly developed widespread intra-abdominal disease and succumbed. The two longest survivors of the seven (15 months), both had widespread liver metastases at operation. Thus spheroid growth rate in culture did appear to reflect certain characteristics of
the parent tumour in vivo, in particular rapid growth rates correlated with local tumour spread and early postoperative death, although it is appreciated that overall numbers were small. Nevertheless this model appears able to define biologically aggressive tumours, and thus high risk patients.

Response of MTS to Chemotherapeutic Agents

The effect of chemotherapeutic agents on MTS growth was measured as percentage regression which was defined as the maximum percentage decrease in size compared to immediate pretreatment size, following one dose of the agent.

Initially six patients' spheroids were treated with 5FU at a concentration of $10^{-2}$ ug/ml. Regression varied from 0-100%. Spheroids from three further patients were then treated with a range of chemotherapeutic agents at dosages corresponding to peak plasma level in patients (Figs. 7.18. - 7.19.). Spheroid growth demonstrated a widely variable response.

Actinomycin D and vinblastine appeared ineffective to halting spheroid growth in any patient. Methotrexate appeared to slow growth in two of the three patients, as
did adriamycin. 5FU caused regression in all three patients, and in two caused maximal regression.

In one patient, however, (JS) bleomycin appeared to be the most effective agent in halting spheroid growth. One further point which emerged was the time taken for the spheroids to commence regrowth, approximately 5-6 days after a single dose of agent, which may prove of importance in the timing of the doses clinically.

If spheroids from pre-operative biopsies are to prove of use in the determination of chemosensitivity of the main tumour, then their behaviour in response to the agent must closely follow that of the parent tumour. MTS from three of the five pre-operative biopsies had growth rates similar to those from the corresponding parent tumour (Fig. 7.20.), and this was reflected in their response to 5FU. In the remaining two pre-operative biopsies, however, slower growing spheroids were produced compared with those from their resected counterparts (Fig. 7.21.). The faster growth rate of MTS from the operative specimens was associated with a greater sensitivity to 5FU (Fig. 7.20.). This potential difference in response from different areas of the same tumour emphasise that multiple tumour samples need to be considered in chemosensitivity assays.
DISCUSSION

This study has demonstrated that MTS can be produced from primary colorectal cancers and pre-operative biopsy material with a high degree of efficiency. Colorectal tumours are difficult to grow in tissue culture, the reported success rates for human tumours varies widely, (Laboise et al, 1981; Rupniak and Hill, 1980; Agrez et al, 1982; Trotter et al, 1984) and plating efficiency rarely exceeds 50% (Warenius and Bleehen, 1982). The major problem with colorectal tissue appears to be infection of the specimen. In this study the antibiotic combination used combined with frequent changes of medium overcame the majority of bacterial infections. Fungal infection, however, proved disastrous when it occurred, and accounted for the majority of failures to produce spheroids.

The number of spheroids produced varied markedly from sample to sample, and was directly related to the amount of tumour tissue present in the sample. Samples which contained a high proportion of fibrous stroma or necrotic tumour produced fewest spheroids, and vice versa.

Tumours which produced large quantities of mucin were difficult to grow as MTS. Mucin production within the
spheroid tended to disrupt it, whereas mucin production around the outside of the spheroid appeared to restrict growth.

Multicellular tumour spheroids appear a useful model for the study of solid tumour behaviour which is unavailable in monolayer culture. Spheroids demonstrate intimate cell to cell contact, chronically hypoxic cell populations (Sutherland and Durand, 1972), and cell cycle times which are comparable with exponential monolayer rates, though essentially non-dividing. (Durand and Sutherland, 1973). They thus combine the relevance of organised tissues with the accuracy of in vitro methodology.

MTS growth rates in culture have been extensively documented (Yuhas and Li, 1978; Landry et al, 1982). Yuhas and Li (1978) investigated the growth of several solid tumours and found a wide range of growth rates in culture. They discovered, using autoradiographic labelling with $^{125}\text{I}$ iodouridine deoxyribose ($^{125}\text{I}$ Id Urd) that growth rates correlated with the number of cycling cells in the spheroid, the growth fraction, which also correlated with the depth of the outer dividing shell. Furthermore, increase in MTS diameter correlated directly with the uptake of $^{125}\text{I}$Id Urd and appeared to be the simplest and most accurate method of measuring
growth rates in vitro. For this reason MTS growth rates in this study were measured as the increase in maximum diameter. Landry et al (1982) have further confirmed the validity of this approach by developing a mathematical model, which takes into account the geometrical characteristics of multicellular tumour spheroids. This model incorporates known cellular and spheroid parameters, such as cell size, cell doubling time and cell shedding rate, to explain the linear growth of MTS in culture. The model fits well with the experimental data on MTS growth in vitro. Landry et al's idea of a discrete cycling rim at the spheroid periphery, where cell growth rate is similar to monolayer growth rates in exponential growth phase, and outside of which no cycling cells exist, represents a useful concept for modelling spheroid growth. Such a precise spatial distribution of cell states in spheroids must represent a geometrical oversimplification. However, this growth equation mimics very closely the in vivo development of tumour. In vivo tumours can be considered as an assembly of subunits, which, like spheroids receive nutrients radiating from the closest vessels (Sutherland and Durand, 1976; Folkman and Hochberg, 1973). Also analogous to MTS, the tumour growth fraction decreases away from the nutrient supply. As spheroids enlarge, the linear phase of growth reaches a plateau. Shymko and Glass (1976) suggested that throughout the interior
of spheroids, growth inhibitory factors are produced by the cells. As the MTS enlarge, these products accumulate within the spheroids until a critical concentration is reached at the edge of the cycling rim causing a progressive diminution in thickness. Saturation phase of spheroid growth may however, merely correlate with the concentration of critical metabolites, such as glucose and oxygen (Landry et al, 1982). Little, however, is known about the initial phase of spheroid growth. Clumping of cells through random collisions was suggested (Sutherland and Durand, 1976), however Landry et al (1982) have proposed that the process is due to specific membrane attachment properties, characteristic of mitotic cells present in suspension. Further expansion then occurs as a result of exponential growth, with concomitant surface cell shedding and additional aggregation. Observations of colorectal tumour cells in the early stages of spheroid formation in this study confirm the latter suggestion. If large numbers of cells were present, cells migrated towards each other and spontaneously aggregated without the need for agitation, perhaps by chemotaxis. Only where cells were widely separated was agitation required to initiate growth.

Extensive validation studies were undertaken to ensure only malignant cells were forming spheroids. Normal colonic mucosa, and cells from villous adenomata would
not form spheroids. The inability of cells from non-malignant tissue to form MTS has been confirmed by Yuhas et al (1977). The histological appearances, maintenance of cellular differentiation and flow cytometric data adds further evidence to the nature of the spheroids produced in this study. Final confirmation, however, came from the ability of the colorectal MTS to grow and metastasise as xenografts in immune-suppressed mice. It would appear that the "take" rate of xenografts in this study was low, but this rate of 40% is comparable to other xenograft success rates (Steel et al, 1983). Two interesting points were noted. Firstly, in one section of a subcutaneous nodule (Fig. 7.10.), it was apparent that the implanted spheroid had developed a blood supply, perhaps due to release of tumour angiogenesis factors; although these factors were not measured in this study. It does not stretch the imagination too far to extend this observation to explain the mechanism of seeding of micrometastases in man. Secondly, in one mouse, the implanted spheroid produced metastases, these being confirmed as adenocarcinoma cells. The ability of spheroids from an original poorly differentiated adenocarcinoma to metastasise provides final conclusive evidence that MTS are composed of malignant cells. What is not clear, however, is whether these cells represent one or more clones of cells from the tumour. Clonal heterogeneity is a common feature
of colorectal tumours, which suggests a degree of genetic instability. Metastases from these tumours often display a different DNA pattern to the primary tumour (Friedlander et al, 1984). It is known that lung metastases from colorectal cancer can grow five to six times faster than the primary growth (Wellin et al, 1963). It is possible that only these most "aggressive" clonogenic cells will form spheroids, however, these may be the very cells which are most likely to produce metastases and therefore, this may be a positive advantage when evaluating chemosensitivity, as it is these "aggressive" cells which must be destroyed if the patient is to be cured of his disease.

The major criticism of any in vitro techniques to measure tumour growth and chemosensitivity is the lack of correlation with in vivo tumour behaviour. It was therefore of paramount importance to observe the correlation between in vitro growth rates of MTS and behaviour of the corresponding tumour in vivo. Perhaps of most importance was the correlation with early postoperative death. This finding has important therapeutic and prognostic implications. It would appear that MTS growth rate in vitro, does correlate with biological aggressiveness in vivo. If this finding is confirmed by more extensive studies, growth of MTS from rectal carcinomas could provide an Important
criterion in the perioperative assessment of individual tumours, fast growth rates in vitro, correlating with a highly active tumour.

It was surprising, therefore, to observe the lack of correlation with Dukes' stage of the parent tumour. One possible explanation is that Dukes' stage provides only a static measure of tumour spread at one point in the natural history of the disease. It can give no information on cell kinetics or tumour growth rate. Spheroid growth rates, which reflects tumour cell cycling times may thus prove to be a better prognostic indicator. This view is further supported by flow cytometric analysis of colorectal cancer which has demonstrated that a tumour with a rapid cell cycling time has a poor prognosis (P Quirke - unpublished observation). It also proved of interest that MTS growth rates correlated with two other important prognostic features, local tumour spread and venous invasion. Significantly higher growth rates were seen in patients who had either local extension or venous invasion, both of which are known to carry a poor prognosis. Such correlations confirm the ability of MTS growth rates in vitro to reflect the parent tumour behaviour in vivo, and potentially identify high risk patients who require further therapy.
In vitro evaluation of drug sensitivity has many drawbacks, however, the spheroid model provides an ideal opportunity for assessment of any sensitivity. As already stated, the structure of MTS are very similar to micrometastases from colorectal tumours, and it is at this stage of microscopic spread that chemotherapeutic agents are most likely to be effective. Furthermore MTS growth rates bear a close relationship to behaviour of the parent tumour. The chemotherapeutic response of MTS in vitro, cannot, however, reflect the body's immune response to the tumour following treatment, nor can it allow for pharmacokinetic problems associated with distribution of the drug within the tumour in vivo.

Multiple sampling of the tumour and where appropriate metastases, may help overcome the problem of clonal heterogenicity. The inherent chemoresistance of colorectal cancer may arise from this heterogenicity. Resistant clones of cells may be found in these tumours, treatment with cytotoxics would therefore eliminate sensitive cells, but these would be replaced by expansion of the resistant subpopulation. Furthermore, during treatment, mutation may occur producing metabolic or structural changes which confer subsequent drug resistance.
MTS may provide a method to identify the development of such resistance. By measurement of cellular DNA following treatment with cytotoxic agents cell subpopulations can be monitored within the spheroid and resistant clones identified. This may allow a less empirical approach to the design of chemotherapy regimes and perhaps aid in the evaluation of agents which are less likely to lead to the acquisition of secondary chemoresistance. There is certainly evidence from this study that clonal heterogenicity exists within the same tumour. Of the five tumours where pre-operative biopsies produced spheroids, three had growth rates similar to that of the resected specimen, however, two had slower growth rates, and this was reflected in the response to 5FU (Figs. 7.20. - 7.21.). The faster growth of MTS from the operative specimens was associated with a greater responsiveness to 5FU (Fig. 7.21.). 5FU acts by disturbing functional mechanisms concerned with the cell cycle. The phenomenon of "thymineless death" has been invoked to explain the cytotoxic effects of 5FU, with the blockade by thymidylate synthetase reaction leading to inhibition of DNA synthesis, while cellular production of both RNA and protein continues (Cohen et al, 1958). An imbalance of growth occurs that is not compatible with cell survival. This mechanism of action is consistent with the greater activity of 5FU against the faster
proliferating MTS derived from the resected tumour compared with those from the corresponding pre-operative biopsies. Alteration in growth rates of human colorectal tumours occurs frequently (Welin et al, 1963), and irregular growth patterns have been observed for human colorectal tumour xenografts in athymic mice (Pickard et al, 1975). No spontaneous changes in the growth rates of the faster growing spheroids to that of their slower growing counterparts occurred, or vice versa, nor was there any evidence of a differential adaptation to the in vitro conditions for MTS from pre-operative biopsies compared with the parent tumour.

When spheroids from three patients were exposed to a panel of chemotherapeutic agents at dosages corresponding to peak plasma levels, a differential response was noted (Fig. 7.18.-7.19.), with some drugs not delaying MTS growth, whilst others caused marked growth regression. Although numbers were small, there appeared to be a basis for rationalising drug treatment on the results of these assays. The overall in vitro response rate to 5FU in this series of patients (n=9) was 30%, which agrees with other quoted figures of response rates of colorectal tumours to 5FU (Carter, 1976; Trotter et al, 1983).
The reported results for the chemotherapy of colorectal cancer in patients are to date poor. However, two recent reports are encouraging. One by I Taylor et al (1985), reports on the perfusion of the liver during the perioperative period with 5FU. This demonstrated a reduction in the subsequent development of liver metastases. The other report from the Gastrointestinal Tumour Study Group (1985) demonstrating significant improvement in survival with combination chemotherapy and radiotherapy, suggest that in the future chemotherapy may have an important role in the management of colorectal cancer. The present study has demonstrated that multicellular tumour spheroids are potentially a useful model for the evaluation of individual tumour chemosensitivity. However, there is evidence of intra-tumour differences in the growth, sensitivity and cell kinetics of MTS produced from colorectal tumours, and whilst the real cause of these differences remains unclear, the results emphasise that both multiple and serial samples should be considered in chemosensitivity assays. Whether in vitro MTS response correlates with tumour response in the patient remains to be proven.

In the future, however, the ability to produce MTS efficiently from multiple pre-operative biopsies may well have a useful role in the perioperative assessment of rectal cancer, and in conjunction with the other
biological assessment may identify high risk groups and thus allow the surgeon to select which patients require adjuvant chemotherapy and, if so, which may be the most appropriate agent to choose. Furthermore, the spheroid model is a potentially useful method for assessing new forms of therapy, such as immunotherapy.
### Table 7.1.

**Pathological Details of Patients**

(n = 19)

<p>| | | |</p>
<table>
<thead>
<tr>
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<tr>
<td>A</td>
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</tr>
<tr>
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### Table 7.2.

**Chemotherapeutic Agents Evaluated**

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<th>Drug</th>
<th>Supplier</th>
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<td>Actinomycin D (Act D)</td>
<td>Merck Sharp Dohme</td>
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<tr>
<td>Adriamycin (Adr.)</td>
<td>Farmitalla Carlo Erba</td>
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<tr>
<td>1,3-bis-2-chloroethyl-1-nitrosourea (BCNU)</td>
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<td>Bleomycin</td>
<td>Landbeck</td>
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<tr>
<td>Cis-platinum (Cis)</td>
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<tr>
<td>Methotrexate (meth)</td>
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</tr>
<tr>
<td>5-Fluorouracil (5FU)</td>
<td>Roche</td>
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<tr>
<td>Vinblastine (Vinb.)</td>
<td>Lederle</td>
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</tr>
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</table>
Table 7.3

Growth Rates of MTS from Operative Specimens

\( n = 19 \)

(um)

Mean of 10 spheroids (+ SD))

\( T = \) stabilization of MTS

<table>
<thead>
<tr>
<th>Days</th>
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<th>MT</th>
<th>EH</th>
<th>DK</th>
<th>CP</th>
<th>CH</th>
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<td></td>
<td>(16)</td>
<td>(15)</td>
<td>(14)</td>
<td>(15)</td>
<td>(24)</td>
<td>(29)</td>
<td>(33)</td>
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Table 7.4.

Growth Rates of MTS (μm/24 hrs) in Relation to
Dukes' Stage, Local Spread and Venous Invasion
(n = 19)

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* p = 0.07 (Kruskal Wallis)
+ p = 0.01 (Mann Whitney U test)
# p = 0.05 (Mann Whitney U test)
FIGURE 7.1

Multicellular tumour spheroid viewed through inverting microscope
FIGURE 7.2

High power view via inverting microscope of multicellular tumour spheroid, showing outer shell of dividing cells and relatively acellular, necrotic core.
FIGURE 7.3

H + E section of colorectal tumour spheroids. (x40)
FIGURE 7.4
MTS growth rates (n = 19) from individual tumours. Mean growth rate calculated over initial 10 day period.
FIGURE 7.5.

Histological sections (H+E) of

a) primary well differentiated rectal tumour, and

b) spheroid derived from that tumour. (x160)
FIGURE 7.6.

Histological sections (H+E) of
a) primary poorly differentiated rectal tumour and,
b) spheroid derived from that tumour. (x160)
FIGURE 7.7

Flow cytometric analysis (propidium iodide method)

of cells from parent tumour and corresponding MTS

a) resected specimen MTS
b) cells from resected tumour
c) preoperative biopsy MTS
d) cells from preoperative biopsy
FIGURE 7.8

Frequency distribution of nuclear DNA contents of lymphocytes (solid block areas) and carcinoma cells (shaded areas) from smears of spheroids from preoperative biopsies (pre) and resected (post) rectal tumours.
FIGURE 7.9.

Low power (x10) section (H+E) of tumour nodules growing subcutaneously after implantation in immune-deprived mouse.
FIGURE 7.10.

Higher power view (x40) of nodules depicted in Figure 7.9., demonstrating blood channels developing in tumour nodule on the right side of the picture.
FIGURE 7.11.

Histological section (H+E) through mouse liver demonstrating deposit of secondary tumour from implanted colorectal spheroid (x60).
FIGURE 7.12.

Histological section of mouse liver with tumour nodule stained for $a_1$ antitrypsin (x60).
FIGURE 7.13.

Histological section of mouse liver with tumour nodule stained for $a_1$ antichymotrypsin (x80).
FIGURE 7.14.

Histological section of mouse liver with tumour nodule stained for lysozyme (x60).
FIGURE 7.15

MTS growth rates (median + semi-interquartile range) and Dukes' stage (n = 19) (Kruskal Wallis).

Dukes' Stage
FIGURE 7.16

MTS growth rates (median + semi-interquartile range) in relation to local spread and venous invasion

(* Mann Whitney U test)  (n = 19)
FIGURE 7.17

Growth rates of MTS and postoperative survival of patients (n = 7) who subsequently died of disease (Spearmans' Rank Correlation)
FIGURE 7.18
MTS growth rates from one patient exposed to one dose of
cytotoxic agents corresponding to peak plasma level
FIGURE 7.19

MTS Growth rates from two further patients exposed to one dose of cytotoxic agent corresponding to peak plasma levels
FIGURE 7.20
Growth curves following 5FU treatment of MTS from 3 patients; Solid lines - spheroids grown from preoperative biopsies; Broken lines - spheroids from resected tumours. Error bars shown on control spheroids only.
FIGURE 7.21

Growth curves following 5FU treatment of MTS from two patients: solid lines resected specimen; broken lines preoperative biopsy. Error bars shown only on control spheroids.
CHAPTER 8

Overall Conclusions and Implications for the Treatment of Rectal Neoplasia
This thesis has endeavoured to identify those patients at high risk, in whom surgical treatment alone is unlikely to prove curative.

This has involved two main directions of investigation. Firstly, by use of more sophisticated radiological techniques, and by measurement of serum markers, the initial section of this thesis has demonstrated that both local tumour extension, and distant, overt spread to the liver can be more accurately assessed prior to surgical intervention. Both groups of patients, those with local spread, and those with liver metastases, are, in the majority of cases, beyond cure by surgery alone.

The second part of the thesis has attempted to define certain biological parameters, namely the degree of cellular differentiation, the ability of the tumour to invade normal tissues, and the growth rate of the tumour, as measured by the spheroid model, which again attempt to identify patients with biologically "aggressive" tumours which potentially require therapy in addition to surgical excision.

In this chapter, therefore, the overall results of these various studies are discussed, and the implications for future treatment of rectal tumours, both surgical, and adjuvant are assessed.

In particular, two areas are addressed specifically. Firstly the potential selection of patients for adjuvant preoperative pelvic radiotherapy for locally extensive disease as outlined in the current

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MRC trial for fixed tumours. Secondly the selection of operative procedure, and the decision on whether the tumour should be resected, or some form of local therapy instituted. If the former, the decision on which type of resection should be performed.

The treatment of rectal carcinoma is at present in a state of flux. It is clear, however, that before major strides can be made in improving survival, the degree of tumour burden and the potential of the carcinoma to spread requires more accurate assessment than is the case at the present time. Despite improvements in surgical technique, the operative approach alone has failed to alter the natural history of the disease. Operative technique can, however, be improved.

Phillips et al (1984a) have highlighted the marked difference in local recurrence rates between different surgeons. This may in part be due to a difference in the amount of tissue removed during the procedure. We now know that tumours can spread beyond the extent visible at laparotomy (Quirke et al, 1986), and thus in the future efforts must be made to achieve as wide a local clearance as possible.

However, as previously outlined, surgery alone cannot provide the whole answer. Due to the late presentation of the majority of rectal tumours, the cancer has often spread beyond the scope of mere physical excision. Some form of adjuvant therapy is
necessary. To achieve this, a much more accurate assessment of the tumour burden, as a whole is necessary.

Certain characteristics of the tumour appear to be of greater prognostic importance than others. Local extramural extension in particular, as described in Chapter 2, appears of great importance, particularly with regard to local recurrence, but also to overall survival. Extramural extension, in rectal carcinoma, appears to carry a greater prognostic significance than early lymphatic involvement. Local radiotherapy, however, may be of use in such cases. What impact, therefore, have the findings of this thesis, on the potential selection of patients for preoperative radiotherapy, as administered in the present MRC trial? In this trial, at present, the criteria for entry are a fixed or partially fixed rectal tumour on clinical examination.

Chapter 3 of this thesis has demonstrated that clinical assessment of local tumour extension is at best, inaccurate, even if the tumour is palpable, a substantial number are too high for adequate assessment. Computerised tomography of the pelvis, however, is an extremely accurate method of detecting gross tumour extension outside the rectal wall.

The use of CEA and acute phase reactant proteins in isolation, proved less effective than computerised tomographic scanning in detecting local spread. The main use of these serum measurements would thus appear to be in refining the findings of computerised
tomography. CEA proved of value in assessing the degree of tumour burden, and in conjunction with the acute phase reactants proved especially accurate in defining those tumours fixed by inflammatory adhesions. However, to be of clinical value, measurement of such biochemical markers should be combined with the findings on abdominal computerised tomography.

As outlined in the introduction, pre, or perioperative radiotherapy may reduce local recurrence rates, and at present pre-operative DXT is being assessed in "fixed" tumours by a MRC trial at present underway. Let us examine the influence that a more accurate assessment of local tumour spread would have on the inclusion of patients in such a trial. The criteria for selection of patients for possible adjuvant pre-operative radiotherapy, based on the initial clinical assessment, are those proposed for inclusion in the current MRC trial of pre-operative radiotherapy, i.e. clinically fixed or partially fixed tumours.

On extended assessment by CT and APRP, the criteria for those patients likely to require pre-operative radiotherapy, at the inception of the study, were tumours with evidence of moderate or extensive extramural spread, as defined previously. The "gold standard" for comparison, were the pathological findings of moderate or extensive spread on examination of the specimen. As previously discussed, however, in Chapter 3, during the course of the study, the incidence of microscopic spread to the lateral excisional margins was found to be greater than was initially
evident on routine pathological examination (Quirke et al, 1987). For the comparison of clinical and extended assessment, therefore the original pathological criteria have been adhered to. However the implications of these findings are that all cases, where any degree of microscopic spread can be detected on the pathological examination, should be considered for perioperative radiotherapy. Computerised tomography cannot detect microscopic extramural spread. However in no case in this series of patients, where microscopic spread to the LEM was found, did the CT scan show a tumour confined to the rectal wall. Thus pelvic CT may underestimate the degree of lateral tumour extension. To be of use therefore, in future selection of patients for perioperative DXT, any patient who has evidence on pelvic CT in conjunction with appropriate serum levels of CEA and APRP, should be considered for such therapy.

On the basis of the MRC criteria of clinically fixed or partially fixed, tumours being included in the current pre-operative DXT trial, the clinical assessment including examination under anaesthetic outlined in Chapter 3, would have selected 22 (29%) of the 76 patients, for inclusion. However, only 17 (23%) of these patients fulfilled the pathological criteria ie exhibiting any degree of extramural spread (n = 29). The other five patients (7%) proved to have inflammatory fixation only, and would therefore have received unnecessary radiotherapy. Clinical assessment missed a further 12 (16%) patients, who fulfilled the pathological criteria for requiring DXT. In four of these patients, this was due to the fact
that the tumour was not palpable and thus could not be assessed. Thus overall, 17 (22%) of patients in this series would have been wrongly assigned to receive radiotherapy, based on clinical assessment alone.

Extended assessment by CT scanning of the pelvis, in conjunction with serum CEA and APRP levels proved significantly more accurate. Computerised tomography alone, identified 27 (93%) of the 29 patients with pathological evidence of extramural spread. Abdominal CT scanning also identified liver metastases in seven of these patients, who would therefore be excluded from receiving local pelvic radiotherapy. CT scanning of the pelvis did miss two patients (3%) with minor extrarectal spread who should have received radiotherapy, however in no patient would unnecessary radiotherapy have been advised, on the basis of pelvic CT findings.

Using a combination of serum levels of CEA and APRP alone for the decision on whether or not to include a patient in the DXT arm of the trial, proved less accurate than CT scanning alone. Pre-operative serum level correctly identified only 15 (52%) of the 29 individuals who require DXT on pathological criteria. However, measurement of CEA and APRP did identify 90% of patients with tumours fixed by inflammation and fibrosis.

The combination of pelvic CT scanning with serum CEA and APRP did not improve on pelvic CT alone in identifying patients with extramural extension, potentially suitable for local radiotherapy.
Compared with clinical assessment, however, pelvic CT proved significantly better than clinical examination in potential selection of patients for local irradiation ($X^2 = 14.56, p < 0.005$).

These findings have important implications for present, and future trials of radiotherapy in rectal cancer, as it would appear from this study, that if based on clinical assessment alone, many patients would be wrongly assigned to treatment. If this is the case, then the true results of such trials in controlling local spread of disease may be masked by the inclusion of unsuitable cases.

A more careful assessment, as outlined in this thesis, may therefore prove beneficial in future trials of adjuvant DXT, and also in the accurate selection of those patients who require such therapy, should the present trials demonstrate benefit on patient survival. Early and interim results using high dosage in the perioperative period have suggested that this may be the case.

Let us now turn to examine what impact the findings on the local status of the tumour may have on the operative procedure performed?

The decision on operative management of rectal neoplasm lies between resection or some form of local treatment. If the former, the choice then lies between a sphincter saving procedure, or an abdominoperineal excision. The criteria for the selection of operative procedure performed on the 76 patients in this study
were based on widely accepted surgical practice, current at the inception of this thesis. The decision was made by the clinician in charge of the case and relied on clinical examination to determine the height of the lesion and its fixity, histological examination of a pre-operative biopsy, information from the basic radiological techniques utilised in the initial clinical assessment and finally the surgeon's findings at laparotomy. Although the height of the lesion above the anal margin, and the build of the patient are of prime importance in the choice between abdominoperineal excision and sphincter saving resection, tumour fixation, detected clinically, or at operation may well tip the balance into what is regarded by some as a more "radical" resection, with sacrifice of the sphincters. Of the 76 patients in this study, at laparotomy, 10 patients were wrongly classified as having extensive extramural tumour spread. These patients had inflammatory adhesions and dense fibrosis on histological examination of the specimen. Seven of these patients (70%) were thus deemed by the operating surgeon, whose decision was based on clinical and operative findings, to require abdominoperineal excision in order to deal adequately with the local disease. However, in six (86%) of the patients who did undergo APER, a sphincter saving procedure would have been technically feasible. If the CT and serum CEA and APRP findings had been taken into account, all six patients who lost their anal sphincter, would have been identified pre-operatively and thus a more conservative operative procedure could theoretically have been performed.
Thus the accurate distinction of fixation detected clinically, between that due to local tumour infiltration, and fixity due to inflammation can be crucial in determining choice of operative procedures.

The six individuals outlined above who were assessed clinically, and at laparotomy to have "extensive" extramural spread would also have been "selected" for inclusion in the preoperative radiotherapy trial. The data from Chapter 2, demonstrated that patients with inflammatory fixation have a relatively good prognosis. In this series, 13% of patients had inflammatory fixation. The majority of these could, therefore, have received inappropriate treatment, both adjuvant pre-operative DXT and operative procedure, relying on present clinical selection. Such patients cannot be identified accurately, by clinical methods, and even at laparotomy, the distinction between inflammation and tumour spread is not easily made. Thus an approach afforded by a more extended staging system appears to offer a superior method of assessment.

Let us now examine the other end of the operative spectrum, the selection of patients for local procedures. The combination of pelvic computerised tomography and measurement of serum markers can accurately define depth of tumour penetration through the bowel wall, and thus it might be expected that such investigations would prove useful in the selection of patients for a local manoeuvre as the primary curative procedure. The methods previously used by those advocating local procedures (Papillon, 1975;
Madden and Kandalaft, 1971; Lock et al, 1978; York Mason, 1976) have been clinical. One main reason for these local techniques not being more widely adopted is the impression that selection cannot be sufficiently accurate. Unfortunately extended assessment, as outlined in this study, does not provide the complete answer. Although depth of tumour extension can be accurately gauged, identification of lymph node involvement by tumour proved inaccurate. Unfortunately, this makes extended staging untenable for selecting patients for a primary local manoeuvre. However, if it is felt that a local manoeuvre might be indicated for other reasons, such as a patient too old or weak to undergo laparotomy, an extended assessment could be useful in confirming the feasibility of such a procedure. Furthermore, an extended assessment is more likely to detect overt liver metastases. In elderly patients with more spread, some form of local therapy may be more appropriate. This is discussed later in the chapter.

Recently an alternative method has been proposed for the assessment of local tumour extension - transrectal ultrasound (Beynon et al, 1986). This modality appears, in early reports to give comparable results to computerised tomography of the pelvis, if performed by experienced operators. The main advantage of transrectal ultrasound is that of cost and ease of use. As yet the technique has not been widely evaluated, and requires considerable experience to interpret the scans. Stenotic tumours cannot be assessed fully, and although it is claimed that lymph node involvement can be identified, as with pelvic computerised
tomography, this, as yet too inaccurate to be relied on for patient selection for a primary local manoeuvre. The technique is, however, promising and warranted wider evaluation, however the main value appears to be in assessing the degrees of tumour extension through the layers of the bowel wall. It is less sensitive in detecting extramural spread which has been demonstrated to be a key factor in local recurrence.

What other features of the tumour impinge on the selection of local therapy? One important prognostic and biological variable, which affects local treatment selection - both operative and adjuvant, is the degree of differentiation of the tumour. It is well established that a poorly differentiated lesion has a worse prognosis than a well or moderately differentiated one (Grinnell, 1939; Dukes, 1940). Because of this poorer prognosis, the widely held belief is that a more radical operative approach is required. Thus Goligher (1984) recommends that any tumour, situated in the lower two-thirds of the rectum which proves to be poorly differentiated on pre-operative biopsy, requires the more "radical" procedure of abdominoperineal excision whether or not SSR is technically possible. This rather drastic approach has been modified recently by N S Williams et al (1983) who recommend that since significant distal intramural spread only occurs in poorly differentiated lesions, then a distal resection margin of 5 cm is required as opposed to 2 cm which they recommend for non-poorly differentiated tumours. However, for low tumours even this modified approach would mean loss of the sphincter in some patients.
If, however, some form of local excision is contemplated, current opinion (Lock et al, 1978; Whiteway et al, 1985) is that a poorly differentiated tumour should not be considered for such a manoeuvre.

It is clear, therefore, that if these practices are to be adopted, the surgeon requires to know accurately the degree of tumour differentiation. Current clinical practice is to take one or two superficial biopsies of the tumour at sigmoidoscopy. The study outlined in Chapter 5, confirms the findings of others (Qualheim and Gall, 1953; Thomas et al, 1983), that this assessment of differentiation is grossly inaccurate. Indeed as long ago as 1953, Qualheim and Gall concluded that there was no basis for using histological grading for prognosis, due to the heterogeneity found in colorectal tumours. The major problem in the histological assessment of rectal carcinomas is subjectivity. Even pathologists, widely experienced in assessing colorectal cancer will fail to agree in a high percentage of cases (Blenkinsopp et al, 1981). These findings were borne out by the results of this study.

If tumours are heterogeneous, it might be expected that the removal of multiple biopsies from the tumour, and a grade based on the majority of cellular differentiation would be an improvement. This, however, did not prove the case in the study conducted during this thesis. Even if a distinction could be made between poorly differentiated tumours, which are clinically the important lesions to detect, and non-poorly differentiated tumours,
on histological grounds, this would be an advance. Unfortunately, even interpreted by one specialist pathologist, the assessment of multiple biopsies for poor differentiation, did not differ significantly from that obtained from a single biopsy.

The question arises how this influences operative treatment. If the histological grade obtained from a single pre-operative biopsy was taken into account in the operative decision to determine the margin of distal clearance i.e. poorly differentiated lesions require a minimum of 5 cm clearance, then six (10%) of 59 patients in whom an adequate single biopsy was obtained would have undergone an inappropriate procedure. Similarly, if the histological grade from the single biopsy had been used to decide between abdominoperineal excision or sphincter-saving resection i.e. all poorly differentiated lesions in the lower two-thirds of the rectum require an abdominoperineal excision (Goligher, 1984), then nine patients (15%) would have had the wrong procedure.

The assessment of multiple samples would not, however, alter the number of patients who may have undergone an inappropriate surgical manoeuvre. If compared with the number of patients, who on the result of a single biopsy would have had a wrong procedure, a similar number, 9 (12%), would have undergone an inappropriate surgical procedure based on the results of multiple biopsies. These were assessed by an experienced pathologist using similar histological criteria to those employed on a single biopsy.
It may also be argued, from a prognostic point of view, that patients with poorly differentiated lesions constitute a high risk group. Surely such patients warrant consideration for some form of adjuvant therapy. Again the difficulty using present subjective criteria is inadequate identification.

Thus the results of this thesis indicate that histological examination of pre-operative biopsies, even multiple, cannot be recommended as a criterion for identification of high risk patients or as a guide to selection of either operative or adjuvant treatment.

There appears, however, a ray of hope. Flow cytometry, which, by accurately measuring tumour cell DNA content, provides a quantitative measure of tumour differentiation, may well be the answer. There is now accumulating a substantial body of evidence which confirms the prognostic significance of aneuploid colorectal tumours. (Wolley et al, 1982; Armitage et al, 1985; Banner et al, 1985). Furthermore some authorities (Banner et al, 1985) consider ploidy status of colorectal tumours to be the single most important prognostic variable. Banner et al thus recommend that flow cytometric analysis should replace histological assessment. If this approach is more widely adopted, the results of the study, outlined in Chapter 5, are important. The ability of flow cytometric analysis of pre-operative samples to reflect the ploidy status of the main tumour will allow a much more accurate perioperative assessment to be made.
On initial biopsy, therefore rectal tumours could be classified as diploid or non-diploid, and the latter group considered as poor risk and thus considered for a more radical approach, both with regard to surgery and selection for adjuvant therapy. By removing subjectivity, flow cytometry may provide a more rational assessment of tumour behaviour and prognosis. If the flow cytometric analysis of pre-operative biopsies, substituting non-diploid for poorly differentiated tumours was applied to the smaller group of 36 patients in whom ploidy was determined, then no patient in the study group would have undergone an inappropriate operation.

The importance of local tumour invasion has already been stressed. The studies outlined in Chapter 6 have suggested a possible mechanism by which rectal tumours may achieve local infiltration. Proteolytic enzymes play a major role in the genesis and spread of many tumours. As explained in the introduction to this thesis, whether these enzymes are produced primarily by the tumour or by tumour-stimulated fibroblasts, remains conjectural. It would appear, however, that proteolytic enzymes are involved in the spread of colorectal carcinoma. Whilst increased levels of several enzymes were found to be raised in this study, cathepsin B appears to warrant most attention. This enzyme has already been implicated in the local invasion of breast tumours (Reckles, 1980), and from the results of this study into rectal tumours, appears to be of specific importance in local tumour invasion. This further identifies a group of patients at high risk of recurrent disease.
The experiment, outlined in Chapter 6, has further demonstrated that a specific antagonist to cathepsin B in vitro, exists - Leupeptin. This non-toxic substance warrants further investigation as in the future it may play a role in therapy - either in patients with extensive pelvic spread, or in patients who develop local recurrence.

The assessment of proteolytic enzyme activity of a rectal tumour in the perioperative period may be able to identify a locally aggressive tumour, and one which should be treated accordingly. Such patients may well warrant consideration for adjuvant pelvic radiotherapy.

Activity of these enzymes appears to be most marked at the host-tumour interface, although increased activity is found throughout the tumour. In the experiments conducted in this study, enzyme levels were measured in the immediate postoperative period. It may prove possible, in the future to assess proteolytic enzyme activity on pre-operative biopsies. If this proves feasible then this will provide further information for assessment of the local status of the tumour. Based on this information, in combination with that gleaned from the extended staging protocol would aid the decision on the form of local treatment best suited to the individual.

The second main area of failure of treatment of rectal cancer, is widespread metastasis, primarily to the liver. Hepatic metastases occur in 80% of those dying from disseminated colorectal carcinoma.
There are, however, two separate problems. Firstly, there are those patients, who at the time of presentation, have macroscopic deposits in the liver, and secondly there are those patients with apparently normal livers at laparotomy who subsequently develop hepatic metastases.

Where liver metastases are already present at the time of presentation, unless localised, and potentially resectable, in the present state of knowledge, the patients are incurable. Thus those patients do not warrant adjuvant local therapy to the pelvis pre-operatively, and may be better served by a less radical operative approach. Up until now, the detection of microscopic liver disease has largely relied on clinical examination, biochemical tests of liver function, and ultrasonic examination of the liver. The data collected in Chapter 4, have mirrored the findings of previous studies outlined in the Introduction, in that a high percentage of liver metastases will be missed. Computerised tomography of the liver in this study proved the most accurate method of detecting macroscopic disease, however, even so, macroscopic metastases were missed.

The use of serum measurements of CEA did, however, prove useful in cases where computerised tomography missed macroscopic disease. In both these cases where metastases were found at laparotomy, but missed by pre-operative computerised tomography scanning, the CEA level was above 45 ng/ml. In neither case was there evidence of extensive intrapelvic spread, which would have
been an alternative reason for an elevated CEA. One further patient had a CEA level above 45 ng/ml. This patient had a normal liver computerised tomographic scan and no evidence of metastases at laparotomy. Unfortunately the patient died in the postoperative period. At postmortem small liver metastases were found in the liver substance. Thus combination of pre-operative estimation of serum CEA and acute phase reactant proteins with abdominal computerised tomography scanning appears able to refine the findings of each when taken in isolation. In the study, combination of computerised tomography and measurement of serum CEA and APRP correctly identified all 10 patients with overt liver secondaries, found at laparotomy. If identified prior to surgery, some form of local procedure could have been considered. All patients, however, underwent an abdominal procedure. Four (40%) of these patients would, retrospectively, have been suitable for local treatment. Three of these patients (75%) developed serious postoperative complications from their abdominal procedure and one died as a result. The remaining three patients all died of their disease within 12 months.

The present approach to patients with liver metastases at initial presentation, is at the moment nihilistic, with treatment aimed at maintaining quality of life. However, if metastatic disease is found, a more extended staging of the disease could prove useful in the future. Perhaps a more aggressive approach should be taken, where liver metastases are found.
The only hope of cure lies with resection. Unfortunately only 10% of patients with liver metastases are potentially suitable (August et al, 1984), however five year survival figures for those who undergo resection are approximately 20-25% (Wagner et al, 1984; Fortner et al, 1983). For the majority, where resection is not possible, the results of non-operative treatment are disappointing, with a partial response to chemotherapy occurring in approximately 20% (Carter, 1976; Kemeny et al, 1987). Unfortunately there are as yet no randomised clinical trials to assess the true efficacy of chemotherapy in these patients. One further role of extended staging may well lie in a more accurate assessment of patients included in such trials.

The second group of patients, those with an apparently normal liver at operation, who subsequently develop metastases, pose a different problem. Approximately 30% of patients with an apparently normal liver will develop liver secondaries at a later date (Finlay and McArdle, 1983b). It is suggested that these "occult" metastases are present, but undetected at the time of surgery.

Pre-operative liver computerised tomography and pre-operative serum CEA between them only identified one such patient, who died in the post operative period, who has been described previously. Unfortunately, on no computerised tomographic scan was any lesion identified which fulfilled the criteria for "occult" disease. As yet no other modality available can confidently predict the development of "occult" disease. The hepatic perfusion index
(Leveson et al, 1985) which detects subtle alterations in liver blood flow in patients with metastatic disease looks the most promising future development, however the early promise has not been fulfilled by longer follow-up (P J Robinson - unpublished data). Intraoperative ultrasound may be an alternative way forward. However, the extended assessment system, outlined in this thesis has failed to provide an answer to this difficult problem.

There is one potential note of optimism, however. I Taylor et al (1985) have reported the results of an adjuvant trial of intraportal 5-fluoro-uracil. The drug was administered to patients who underwent resection of colorectal tumours, in whom no evidence of metastatic disease of the liver was found at laparotomy. This trial demonstrated a significant reduction in the number of patients who subsequently developed metastases in the treated group. One possible explanation for this finding is that the 5FU destroyed "occult" metastases present in the liver, but not detected at laparotomy.

The apparent failure of chemotherapy in patients with macroscopic disease, combined with the results of I Taylor et al (1985), suggests that for successful chemotherapy, of liver metastases, the tumour should be attacked at an early stage, preferably when microscopic, to avoid problems of drug penetration into macroscopic metastases.
The other major problem with chemotherapy, is that of drug resistance. The heterogeneity of response by colorectal cancer to chemotherapy is well known. Response to 5FU, the most successful drug is still only approximately 20%. Thus to adopt an "across the board" policy to chemotherapy, even if confined to high risk groups, means that many patients with resistant tumours will receive ineffective and potentially toxic chemotherapy.

Perhaps the most important biological parameter is the rate of tumour growth. A rapidly growing tumour is likely to lead to an earlier demise of the patient. Thus the development of the multicellular tumour spheroid model as a measure of in vitro tumour growth, is potentially exciting. Although the numbers, as described in Chapter 7, were small, it would appear that the growth rate of MTS in vitro, from individual patients, relates to in vivo behaviour.

MTS growth rates inversely correlated with the patient's prognosis, with the most rapidly growing spheroids coming from patients whose prognosis subsequently proved to be extremely poor. Patients whose tumours produced rapidly growing spheroids in vitro, died earlier than those patients with slower growth rates. This appeared independent from the "tumour burden" at initial presentation.

Higher spheroid growth rates also correlated with other poor prognostic parameters such as those tumours which were locally invasive.
Thus the MTS model may provide an assessment of in vivo "biological aggressiveness" of individual tumours, thus identifying a further high risk group of patients. Moreover the in vitro characteristics of spheroids bear many similarities to the in vivo behaviour of micrometastases, such as "occult" hepatic metastases, in the pre-vascularisation phase. Spheroids may thus represent a near ideal model for determination of chemosensitivity of rectal tumours and also for evaluation of more recent forms of adjuvant therapy, such as immunotherapy and photodynamic therapy. The fact that spheroids can be produced from primary tumours may also allow an individual approach to the determination of tumour sensitivity and may therefore allow a more rational selection of patients for a particular type of therapy. This work is, however, at an early stage and requires further extensive evaluation. It may prove possible to test an individual tumour against a panel of chemotherapeutic, or other agents, to determine which, if any, is suitable for that particular case. Patients with faster growing tumours would be particularly suitable. As discussed previously in Chapter 8, these tumours respond better to cytotoxic agents.
Conclusions

There are several conclusions to be drawn from the studies outlined in this thesis:

1. Local tumour spread appears an extremely important prognostic indicator in rectal carcinoma. The retrospective investigation outlined in Chapter 2, confirms the importance of the mode of spread. A group of patients, however, with inflammatory fixation can be identified, who have an equivalent prognosis to those patients whose tumour is confined to the rectal wall.

2. Clinical rectal examination, even when performed by experienced clinicians proved an inaccurate assessment of the local status of the tumour, even if performed under general anaesthesia. In particular, patients with inflammatory fixation could not be identified. Even at laparotomy, this distinction proved impossible.

3. Pelvic computerised tomography proved a significantly superior method of assessing the degree of local tumour infiltration outside the rectal wall when compared with clinical assessment.

4. In isolation, measurement of pre-operative serum levels of CEA and APRP was not significantly better at assessing local
extramural extension, however these results, interpreted in conjunction with the abdominal CT findings, did help to refine the results of pelvic CT scanning, particularly with reference to the detection of inflammatory fixation. Pelvic CT scanning, although more accurate than clinical assessment, could not reliably identify involved lymph nodes.

5. Clinical assessment in conjunction with biochemical liver function was not an accurate method of identifying patients in the study group, with overt liver metastases, pre-operatively. When compared, computerised tomography proved significantly superior to liver ultrasound in detecting overt spread. Elevated serum CEA levels did correlate with the presence of hepatic metastases, and appeared able to confirm the CT findings. However, the major role of these serum markers appears to be in refining the abdominal CT results.

6. CEA levels in the absence of gross pelvic disease, and a normal liver CT may suggest "occult" spread, however none of the methods investigated in this thesis appears able to accurately identify "occult" metastases.

7. This study has further confirmed the unreliability of the histological assessment of pre-operative biopsies as a guide to tumour behaviour. Decisions on management should, therefore, not rely on this examination. Flow cytometric analysis of pre-operative biopsies appears a more accurate
method of assessment, and may provide a more rational basis on which to decide treatment options.

8. It appears that certain proteolytic enzymes, in particular cathepsin B play a role in the local invasion of tumours of the rectum and sigmoid colon. A specific inhibitor of cathepsin B, leupeptin was identified in vitro. Further studies into the situation in vivo may be warranted.

9. Perhaps the most important biological parameter of tumour aggressiveness, is growth rate. This study has demonstrated that growth rates of individual tumours can be measured in vitro using the spheroid model. These growth rates appear to correlate with prognosis. The spheroid model may well have a role in the future selection of patients for adjuvant therapy.
At the outset, the aim of this thesis was to assess accurately patients with a rectal neoplasm, in order to allow a more individual approach to treatment. By the modalities employed in this study, this aim has been achieved, and has enabled several high risk groups to be identified. The techniques evaluated in the thesis, thus allow a more rational basis for the multidisciplinary approach to the treatment of this common disease, which is necessary if survival is to be improved. This approach may already be taking effect. A significant prolongation of disease-free interval in patients who underwent "curative" resection of a rectal carcinoma, using combined radiotherapy and chemotherapy has been reported (Gastrointestinal Tumour Study Group, 1985). The way ahead for further improvement in survival lies in such a multimodality approach and co-operation between surgeons, radiotherapists and oncologists. A careful perioperative assessment, as outlined in this study, enables more accurate patient selection and allows selection of optimum treatment.

The necessity for careful staging of the disease was highlighted recently by the United Kingdom Co-Ordinating Committee for Cancer Research, who set up a Working Party which has published a clinico-pathological staging system for colorectal cancer (N S Williams et al, 1988) which embodies many of the concepts discussed in this thesis. Accurate staging is also of prime importance for future evaluation of new therapeutic modalities, and
future assessment of clinical trials. Accurate tumour staging is essential for the institution of such trials and for meaningful interpretation of the results.

This thesis has demonstrated that using present advances in technology, biochemistry, histopathological and in vitro culture techniques, rectal neoplasia can be assessed more accurately than has previously been possible. In the future such assessment will aid further understanding of the disease and thus hopefully allow therapy to be more successful.
Appendix : 3.1.

Pre-operative serum levels of CEA and APRP in patients with mobile (M), fixed by inflammation (FI) and fixed by malignant (FM) tumours

(+ = patients with liver metastases)

Colorectal tumours (n = 99)

a) Initial Group

1) AGP (g/L)

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### Appendix : 3.1.

**Initial Group** (n=99)

**II) CRP (g/L)**

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### Initial Group (n=99)

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<tr>
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<tr>
<td>FM</td>
<td>153.3 (2.7 - 277.0)</td>
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</table>

**Statistical Analysis**

**Mann Whitney U Test**

- M v FI \( U = 421 \) \( z = -1.330 \) \( p=0.1834 \)
- FI v FM \( U = 27 \) \( p<0.01 \)
- M v FM \( U = 118 \) \( z = -4.8319 \) \( p<0.0001 \)

**Excluding hepatic metastases**

<table>
<thead>
<tr>
<th>CEA (ng/ml)</th>
<th>Median (+range)</th>
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<tr>
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<td>FI</td>
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<tr>
<td>FM</td>
<td>33.9 (2.7 - 221.6)</td>
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**Mann Whitney U Test**

- M v FI \( U = 318 \) \( z = -1.7528 \) \( p=0.079 \)
- FI v FM \( U = 23 \) \( p<0.05 \)
- M v FM \( U = 0 \) \( z = -4.5692 \) \( p<0.0001 \)
Appendix: 3.1.

AGP Median (+ range)

M 0.09 (0.63 - 1.89)
FI 1.94 (1.34 - 2.79)
FM 1.54 (0.96 - 2.90)

Statistical Analysis
(Mann Whitney U Test)

M v FI  U = 20  z = -5.9575  p<0.0001
FI v FM  U = 72  p = NS
M v FM  U = 121  z = 04.7916  p<0.0001

Excluding hepatic metastases

Median (+range)

M 0.97 (0.63 - 1.89)
FI 1.94 (1.55 - 2.79)
FM 1.47 (0.96 - 2.88)

Mann Whitney U Test

M v FI  U = 13  z = -5.7891  p<0.0001
FI v FM  U = 20  p = 0.01
M = FM  U = 70  z = -3.2366  p=0.0012

Page 352
Appendix : 3.1.

**CRP Median (=range)**

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</tr>
<tr>
<td>FM</td>
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<td>4.0 - 79.0</td>
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</table>

**Statistical Analysis**

**Mann Whitney U Test**

- M v FI \( U = 26 \) \( z = -5.8890 \) \( p < 0.0001 \)
- FI v FM \( U = 42 \) \( p < 0.01 \)
- M v FM \( U = 162 \) \( z = -4.3280 \) \( p < 0.0001 \)

**Excluding hepatic metastases**

**Median (+range)**

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<tr>
<td>FM</td>
<td>17.5</td>
<td>10.0 - 79.0</td>
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</table>

- M v FI \( U = 26 \) \( z = -5.8267 \) \( p < 0.0001 \)
- FI v FM \( U = 24 \) \( p < 0.05 \)
- M v FM \( U = 43 \) \( z = -3.7598 \) \( p < 0.0001 \)
### Appendix: 3.1.

#### iii) CEA (ng/ml)

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### Appendix: 3.2.

CEA (ng/ml)

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<th>Slight (n=7)</th>
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**Appendix : 3.2.**

**CRP (g/L)**

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<td>18.5 (5.0 - 153.0)</td>
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<tr>
<td>FM</td>
<td>13.0 (0.0 - 79.0)</td>
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**Statistical Analysis (Mann Whitney U Test)**

- M v FI $U = 19, z = -4.3286, p < 0.0001$
- FI v FM $U = 119, z = -0.8364, p = 0.4025$
- M v FM $U = 120, z = -5.3882, p < 0.0001$

**Excluding Liver Metastases**

<table>
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<tr>
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<td>18.5 (5.0 - 153.0)</td>
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<tr>
<td>FM</td>
<td>17.0 (3.0 - 79.0)</td>
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**Statistical Analysis (Mann Whitney U Test)**

- M v FI $U = 17, z = -4.2997, p < 0.0001$
- FI v FM $U = 92, z = -0.5500, p = 0.5814$
- M v FM $U = 61, z = -5.1409, p < 0.0001$
Appendix: 3.2.

CEA (ng/ml)

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<td>FM</td>
<td>24.6 (2.8 - 250.0)</td>
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Statistical Analysis (Mann Whitney U Test)

- M v FI: U = 128, z = -1.4862, p = 0.1372
- FI v FM: U = 39, z = -3.1402, p = 0.0007
- M v FM: U = 135, z = -5.1916, p < 0.0001

Excluding Liver Metastases

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<td>4.2 (2.8 - 19.3)</td>
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<tr>
<td>FM</td>
<td>18.5 (2.8 - 203.1)</td>
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Statistical Analysis (Mann Whitney U Test)

- M v FI: U = 98, z = -2.0240, p = 0.043
- FI v FM: U = 38, z = -2.8327, p = 0.0046
- M v FM: U = 80, z = -4.8056, p < 0.0001
Appendix : 3.2.

Pre-operative assessment group (n=76)

Pre-operative levels of CEA and APRP

M = mobile
FI = fixed by inflammation
FM = fixed by malignancy
+ liver metastases
* lung metastases

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Appendix : 3.2.

CRP (mg/L)

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Page 359
Appendix : 3.2.

AGP (g/L) Median (+range)

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Statistical Analysis (Mann Whitney U Test)

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Excluding Hepatic Metastases

Median (+range)

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Statistical Analysis (Mann Whitney U Test)

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Appendix : 3.3.

**CEA and APRP**

*Initial Study: clinical, biochemical, pathological and haematological data*

(n=99)

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<th>Hb</th>
<th>WBC</th>
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<th>AP</th>
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<td>15.3</td>
<td>7.2</td>
<td>18</td>
<td>7.2</td>
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</tbody>
</table>
Appendix : 3.4.

Logistic Regression Data

The logistic regression analysis was performed on the University of Leeds Amdahl 470 mainframe computer, and utilised their SAS statistics software.

The final reduced model derived from the initial 99 patients was as follows:-

Three group names "M", "FI", "FM".

Covariates specifying the full model:-

- age, sex, site of tumour (rectum or other), weight loss (>5kg over prior three months), Dukes' stage, CEA, AGP, CRP, Haemoglobin, white cell count (x10^9/L), percentage lymphocyte count, and alkaline phosphatase (IU/L). Non numeric data was binary coded.

The analysis was based on 99 cases.

Group 1 - only a constant (CON) term included.
Group 2 - covariates are CON, CEA.
Group 3 - covariates are CRP, AGP.
Total number of fitted parameters = 5
Maximum number of iterations = 30
Maximum number of step reductions = 5

Tolerance for convergence EPS = 0.00001.
Number of iterations between each Hessian update = 3

Sample size of each group:-

M  37
FI 16
FM 16

After commencement of iterative procedure:-

- convergence after eight iterations
- final log likelihood ratio is -47.00

<table>
<thead>
<tr>
<th>Group</th>
<th>FI 1.</th>
<th>CON</th>
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<th>1.0156</th>
<th>0.140737865806D</th>
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<tr>
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<td>CRP</td>
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<td>0.0325</td>
<td>0.125882186451D</td>
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</table>
Appendix : 3.4.

The equivalent standard logistic model was:-

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<th>FM</th>
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<td>0.0000</td>
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<tr>
<td>SITE</td>
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<td>0.0000</td>
</tr>
<tr>
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<td>0.0000</td>
</tr>
<tr>
<td>DUKES' STAGE</td>
<td>-0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>CEA</td>
<td>-0.0000</td>
<td>0.0206</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>HB</td>
<td>-0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>WBC</td>
<td>-0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>% LYMPH</td>
<td>-0.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>AP</td>
<td>-0.0000</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Lagrange multiplier statistic was 14.599
Appendix : 3.5

Method of Measurement of CEA and Acute Phase Reactants

CEA

CEA PRIST kits are commercially available. Each kit contains:

- 20 ml CEA - free diluent (porcine serum)
- 10 ml Anti-CEA antibodies, antibodies raised in rabbit, lyophilised, lugg, 260 kBg (7.0uCi)
- 10 ml Tween solution
- 5 x 5 ml Predispensed CEA standard (isolated from liver metastasis from colon carcinoma)
  - lyophilised - 0.5, 1.5, 5.0, 15, 50ug/L after reconstitution.
  - Calibrated against WHO Ref. prep 2/22/J. National Institute for Biological Standards and Controls (London) 100ng CEA equivalent to 1 unit CEA (WHO).
- 90 x anti CEA antibody discs in buffer

Antibodies raised in sheep

The CEA standard solutions are each dissolved in 100u/L redistilled water. The anti CEA antibody $^{125}$I is dissolved in the 5 ml Tween
solution. The serum samples when thawed, are diluted 1:5 with CEA-free diluent.

All standards and unknown samples are run as duplicates. A standard curve is produced for each test run. The steps of the estimation are as follows:

1. Add one anti-CEA antibody disc to the bottom of each test tube, except the two which act as total activity tubes.

2. Pipette 100μL reconstituted standard (0.5, 1.5, 5.0, 15, 50μg/L) on to the discs in tubes 3-12 (allows duplicates).

3. Pipette 100μL unknown sample on to discs in subsequent tubes.

4. Cover tubes with aluminium foil and agitate gently for three hours at constant temperature.

5. Remove all liquid from tubes, add 2.5 ml 0.9% sodium chloride and allow to stand for 10 minutes. Remove and repeat rinsing procedure twice. Allow discs to dry.

6. Pipette 50μL of anti-CEA antibody ¹²⁵I solution into all tubes. The tubes are immediately covered and incubated overnight for 16-20 hours, at constant room temperature.
7. After incubation, the discs are rinsed as above, and the found radioactivity measured via a scintillation counter. Blank tubes are measured for background activity.

Results are calculated as follows:-

\[
\% \frac{T}{T} = \frac{B \text{ of standard or unknown}}{T} \times 100
\]

Where \( T \) = total activity

\( B \) = counts of standard/unknown

Background activity is subtracted. The value obtained from the CEA standard is plotted against CEA concentration ug/l on logarithmic graph paper to construct a standard curve. The concentration of CEA in the unknown samples can be read directly from this curve, and corrected for the dilution.

The normal CEA level in our laboratory, obtained from 50 normal, healthy volunteers is 10ng/ml. The mean coefficient of variance in this series was 10%.
**Appendix : 3.5.**

APRP – APRP were measured using a standard radial immunodiffusion method detailed below:

**RADIAL IMMUNODIFFUSION (R.I.D.) METHOD**

1. Preparation of plates.

Reagents

1. 0.1M **BARBITONE BUFFER pH 8.6**

   Sodium barbitone 20.6g
   Sodium azide 1.0g
   Dissolve in 800 ml distilled water
   pH to 8.6 with concentrated HCl
   Make up to 1 litre with distilled water.

2. **WORKING BUFFER**

   1 volume 0.1M barbitone buffer + 3 volumes distilled water.

3. a) **1% AGAROSE**

   1g agarose per 100 ml working buffer.

b) **1% AGAROSE 3% PEG**

   1g agarose

Page 375
3g polyethylene glycol (PEG)
Per 100 ml working buffer

4. 2.5% AGAROSE

1.25g agarose

50 ml distilled water

Reagents 3 and 4 were prepared by heating on a heated stirrer until completely dissolved and then placed in a water bath at 55-60°C. After use, excess agarose can be left to cool and stored at 4°C. For re-use, autoclave to melt the agarose.

PROCEDURE

1. Add a small amount of sodium azide (about 0.1%) to the molten agarose, to prevent microbial growth.

2. Clean 10cm x 10cm glass plates by soaking in methanol. Dry surface by polishing with a tissue.

3. Holding plate by a corner, warm it in a bunsen flame. Drop about 1ml of the 2.5% agarose onto the plate and spread this quickly over the whole surface using a microscope slide. Remove excess agarose by scraping the microscope slide across the plate. Repeat at right angles to original direction of scraping, to leave a thin film of agar on the surface. This
coating helps the 1% agarose to adhere to the plate. Place plate on levelling plate.

4. For each plate, measure out 16 ml of the 1% agarose into a universal and add the specified amount of the appropriate antiserum. Mix gently but thoroughly, avoiding the formation of bubbles and not allowing the agarose to cool. Pour carefully, but swiftly, onto the centre of the skinned plate, pushing it to the edges with the rim of the universal. Allow to set (about five minutes).

5. Store plates in a plate-holder in an airtight box kept humid with damp tissues.

6. Just before use, punch 49 holes of 2.5 mm diameter in the plates, according to the template. Any agar left in the holes, due to skinning too thickly, can be removed using a glass capillary.

Cut off top left-hand corner of gel to mark beginning of first row.

Remove any water which has accumulated in the wells with the corner of tissue.

Apply 5 μl of the samples and standards to the wells using eg a SMI(R) Micropettor. Hold pipette vertically above plate and drop
sample into centre of well ensuring that no drops are left on rim of capillary tube.

Rinse capillary tube in saline between samples and with sample before application. Incubate at room temperature in air-tight container made humid with damp tissues.
Appendix: 6.1.a.

**Methodology of Collagenase Assay**

Using human colon homogenate with DNP.

1. Samples thawed, weighted and homogenised using collagenase buffer (0.05M TRIS -0.15M NaCl - 5mM CaCl₂, pH 7.5).

2. 300 ul of homogenate + trypsin (30 ul) preincubated in glass tubes at 37°C for 15 minutes.

3. Add 30 ul of trypsin inhibitor to final volume of 360 ul.

4. Made up assay tubes (protect from sunlight).

5. Arrange (ul)

<table>
<thead>
<tr>
<th>Tube No</th>
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<th>BUFFER</th>
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<td>-</td>
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<td>0.125</td>
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<td>-</td>
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<td>0.125</td>
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<td>0.125</td>
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<td>-</td>
<td>-</td>
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<td>0.125</td>
</tr>
<tr>
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<td>-</td>
<td>0.175</td>
</tr>
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<td>0.125</td>
<td>-</td>
<td>0.175</td>
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</table>
Repeat for each homogenate sample.
Total volume = 0.3ml.

6. Mix tubes and incubate (37°C - 2 hours).

7. Add 1 ul of M. HCl to stop reaction.

8. Allow to cool - add 2 ml ethyl acetate.

9. Shake tubes vigorously to extract DNP fragments.

10. Spin tubes in centrifuge for 15 minutes (1000-1500 rpm) to break emulsion.

11. Pipette upper layer of separation and place into cuvette.
Read on spectrophotometer (365nm).
Appendix: 6.1b

Methodology of CLP Assay

- Depends on release of highly fluorescent MCA.

SUBSTRATE

(5.3 mg) – is dissolved in 211 ul DMSO and further diluted 1:15 in Tris-maleate buffer

<table>
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<tr>
<td>Tris-Maleate</td>
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<td>CaCl₂</td>
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<tr>
<td>pH 8.0</td>
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Dissolved in 500 ml distilled water

STOPPING BUFFER

1M Sodium Acetate - acetic acid added to reach pH 4.2.
STANDARD

7-amino-4-methylcoumarin. Stock solution 1mM. Diluted 1:100 in stopping buffer to give 10uM. 50 ul added to assay in place of homogenate.

Homogenate diluted 1:100 in Tris-Maleate buffer.

**Assay (ul)**

<table>
<thead>
<tr>
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<th>Blank</th>
<th>Standard</th>
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<td>50</td>
<td>50</td>
<td>50</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>Buffer</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

Incubated at 37°C - 30 minutes

Reaction stopped with 1.8 ml stopping buffer.

| Tissue Homogenate | -   | 50  | -   | - |

(ul)

Release of fluorescent MCA excited at 380nm measured at 460 nm.
Appendix : 6.1.c.

Methodology of Cathepsin B and Cathepsin H

Assays

Cathepsin B

Substrate C₆H₅-CH₂-Coo-Phe-Arg-AMC

Solutions

Buffer

Conc (mM)

KH₂PO₄ 352
Na₂HPO₄ 48
Na₂EDTA 4

pH 6.0

conductivity 57mV

Page 383
Cysteine 100 mM

**Stopping Buffer**

<table>
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<th>Conc (mM)</th>
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<tbody>
<tr>
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<td>Na Ac</td>
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<tr>
<td>Ac COOH</td>
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<tr>
<td>pH 4.3</td>
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<tr>
<td>Conductivity</td>
</tr>
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</table>

**Substrate concentration** - 10 mM, dissolved in 1ml DM50 (Dimethylsulphoxide) and diluted 1,500 with BRIJ (Polyoxyethylene lauryl ether) to give 0.02 mM.

**Standard**

7-amino-4-methylcoumarin

250 nMolar solution
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<td>Cysteine</td>
<td>80</td>
<td>80</td>
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<tr>
<td>Substrate</td>
<td>250</td>
<td>-</td>
</tr>
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Preincubate sample, buffer and cysteine - 2 minutes, 37°C. Add substrate with vigorous mixing. Incubate for 15 minutes.

Stop reaction with 1m stopping buffer.

After reaction stopped, add

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<th>Control</th>
</tr>
</thead>
<tbody>
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<tr>
<td>Total Volume</td>
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<td>1000</td>
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</table>

Measure fluorescence - exit 360, detect 460 mm.
Cathepsin H

Buffer

Conc (mM)

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<th>Concentration</th>
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<tbody>
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<td>Na$_2$EDTA</td>
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<td>pH 6.8</td>
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Assay (ul)

<table>
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<tr>
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</table>

Read prior to incubation for two minutes.

Incubate as for Cat B assay.

Add 500 ul substrate. Read as for Cat B.
Peptidase levels in normal colonic wall (W) and corresponding tumour.

Results expressed as nmol (mg protein)$^{-1}$min$^{-1}$

<table>
<thead>
<tr>
<th>CB WALL</th>
<th>CH WALL</th>
<th>CLP WALL</th>
<th>COLL WALL</th>
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<th>CLP TUM</th>
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<tr>
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### Appendix : 6.2 (Contd.)

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Results expressed as nmol (mg protein)$^{-1}$min$^{-1}$

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