CELL MEMBRANE AND DIETARY FATTY ACIDS IN COLORECTAL CANCER

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for the Degree of Doctor of Medicine

Department of Surgery, University of Leicester
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To Sarah and Emily
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SUMMARY

The development of colorectal cancer is thought to be a multi-stage process involving a variable interaction between genetic and environmental factors. There is a great deal of epidemiological and experimental evidence which suggests that dietary fat is an environmental influence with a strong aetiological role in colorectal cancer. The mechanism of the promotional effect of dietary fat is unknown, but the cell membrane is a possible target point at which fat might exert an influence. This thesis is concerned with the relationship between dietary fat and cell membrane fatty acids in colorectal cancer.

In chapter 1 the literature relating to dietary fat and colorectal cancer is reviewed. Chapters 2 and 3 summarise those facts about cell membrane structure and function and the biochemistry of fatty acids which are relevant to the subsequent work. Chapter 4 describes the methodology used in this work and includes a study of the validity of those methods.

The second section of the thesis contains the results of the human studies. Chapter 5 is a discussion of the ways in which dietary fat intake can be assessed in man. Chapters 6 describes a case-control study of erythrocyte fatty acid profiles in human colorectal cancer. Chapter 7 describes an analysis of cell membrane fatty acid profiles in human colonic mucosa and tumours.
Section three contains the results of studies using an experimental colorectal tumours model. Chapter 8 describes the development of animal models of colorectal carcinogenesis and addresses the question of their relevance to the human situation. Chapter 9 presents a study of the influence of the type and quantity of dietary fat on the development of experimental colorectal tumours. Chapter 10 concentrates on the cell membrane fatty acid profiles of erythrocytes, colonic mucosa and colorectal tumours in the experimental model.

The final section (chapter 11) consists of a summary of the findings of these studies and some concluding remarks including suggestions for further study.
Although much is known about the causes of colorectal cancer, our knowledge is far from complete. The aetiological role of dietary factors in general and dietary fat in particular have, nevertheless, been areas of some interest and the weight of the available epidemiological and experimental evidence suggests that dietary fat is an important factor. However, the mechanism by which dietary fat and colorectal cancer are linked has not been adequately established.

This chapter begins with a brief review of the epidemiology and aetiology of colorectal cancer. This is followed by a discussion of the findings of the epidemiological and experimental studies which have investigated the relationship between dietary fat and colorectal cancer. The last section of the chapter addresses the potential mechanisms by which dietary fat may promote the development of colorectal cancer.
The epidemiology of colorectal cancer

Colorectal cancer is a disease of the Western World being common in Europe, North America, Australasia and the River Plate area of South America and less common in Africa, Asia and the Andean countries of South and Central America (Waterhouse et al, 1976). In Europe the disease is commoner in the north and west than in the south and east. Similarly, in Britain the incidence of the disease is much higher in Scotland than it is in the south-east of England. Thus, with the notable exception of Japan which has only a moderate incidence of colorectal cancer, the disease is largely confined to highly developed industrialised nations. The risk of the disease is correlated with socio-economic status but this factor is more marked in countries which have a low overall risk. For example, in Columbia and Hong Kong where the incidence is low, cases occur predominantly in the upper socio-economic groups (Haenszel et al, 1975; Hill et al, 1979). Studies of migrant groups show that within one or two generations such people usually acquire a similar risk of developing colorectal cancer as that seen in the indigenous population of their new country. This was first demonstrated by Haenszel (1961) in a study of the foreign born residents of the United States but similar findings have been shown by many other studies and these have been reviewed by Kmet (1970). This evidence suggests that the national variations in the incidence of colorectal cancer are due to environmental influences rather than different racial characteristics.
The aetiology of colorectal cancer

There are several conditions which are known to predispose to the development of colorectal cancer and a number of genetic and environmental factors which are thought to have an aetiological role.

Predisposing conditions

The recognised factors which predispose to colorectal cancer are adenomas, ulcerative colitis, Crohn's disease and uretero-colic anastomosis.

Although there have been some dissenting voices, most notably Spratt and Moyer (1958) and Castleman and Krickstein (1962), there is now a large body of evidence which establishes that colorectal adenomas are pre-malignant lesions (Morson, 1978). The increased malignant potential of large, villous and dysplastic adenomas is well known.

Bargen (1928) was the first to describe patients with ulcerative colitis progressing to colorectal cancer and many studies since then have confirmed this finding (de Dombal et al, 1966). A number of risk factors are recognised: early age at onset of colitis, long duration of symptoms and extensive colonic involvement with the disease all being associated with an increased risk of malignancy. The precursor lesion of carcinogenesis in colitis is epithelial dysplasia (Morson and Pang, 1967). The magnitude of the risk varies with the factors mentioned above but for patients with extensive colitis for more than 10 years, a 370-fold increase in risk between the ages of 20 and 39 years has been
demonstrated in a follow up study at St. Mark's Hospital, London (Lennard-Jones, 1985).

It is now firmly established that patients with colorectal Crohn's disease have between a 4 and 20-fold increase in the risk of developing colorectal cancer (Greenstein et al, 1981; Wyatt et al, 1987). The risk factors are similar to those for ulcerative colitis (Shorter, 1983).

In patients who have undergone a uretero-sigmoid anastomosis for bladder diversion, there is a high risk of malignant change at the anastomotic site with a latency of up to 20 years (Stewart et al, 1982). The tumours appear to go through an adenoma-carcinoma or dysplasia-carcinoma sequence. This is, however, a rare cause of colorectal cancer because better methods of urinary diversion have been developed.

There are a number of other factors which are associated with colorectal cancer, but for which the exact relationship has not been precisely determined or remains in dispute. Patients who have undergone a polya gastrectomy for peptic ulcer disease have a two-fold increased risk of colorectal cancer after a latent period of 20 years (Caygill et al, 1984). Furthermore, it has been suggested that an unexpectedly high proportion of patients with colorectal cancer have undergone gastric surgery in the past (Bundred et al, 1985). There are also a number of studies which show an increased risk of colorectal cancer in patients who have previously had a cholecystectomy (Vernick & Kuller, 1981; Linos et al, 1982; Schottenfeld & Winawer, 1983). The cancers were found predominantly in women and predominantly on the right side of the colon. In an attractive and biologically plausible
hypothesis to explain this finding, it has been suggested that the high levels of secondary bile acids which predominate after cholecystectomy have a carcinogenic effect on colonic mucosa (Pomare & Heaton, 1973). However, two recent large population studies have failed to show any significant causal relationship between cholecystectomy and colorectal cancer (Friedman et al, 1987; Adami et al, 1987) and this area remains a controversial one.

Genetic factors

There is now a considerable body of evidence which suggests that genetic factors are important in the development of colorectal cancer. The first genetic links were made with the description of a number of rare autosomally dominant conditions which carry a very high risk of colorectal cancer. The best known example is familial polyposis coli which carries a near 100% risk of colorectal cancer if it is left untreated (Dukes, 1952 a&b; Dukes 1958; Bussey 1975). The genetic locus associated with this condition has now been identified on chromosome 5 (Bodmer et al, 1987; Leppert et al, 1987).

Less well known but equally important to the genetic argument has been the identification of 'cancer families' in which a number of cases of colorectal cancer develop in the same or successive generations of the family (Lynch & Krush, 1957; Kluge, 1964; Dunstone & Knaggs, 1972; Lovett, 1976). In these families the cancers are rather distinctive. They tend to occur at a younger age than usual, are predominantly proximal lesions, may be multiple and are not associated with multiple
colonic polyps. In female members of such families there is an increased risk of endometrial cancer. An association between hereditary bilateral brachydactyly (hypoplasia of the middle phalanges of the second, third and fifth fingers) and a familial predisposition to colorectal cancer has also been described (Macrae et al, 1981). The cancer family syndrome is thought to be determined by an autosomal dominant gene (Lynch et al, 1977).

First degree relatives of patients with colorectal cancer have an approximately three-fold increased risk of developing the disease compared to the normal population (Burdette, 1971; Lovett, 1976). Woolf et al (1955) showed that this familial predisposition was due to an inherited tendency to develop colonic adenomas and this may be determined by an autosomal recessive gene (Veale, 1965). That there is no relationship between colorectal cancer risk and ABO blood group suggests that the familial predisposition is not due to genetic factors but rather is due to some shared environmental element such as diet. Studies of the risk of the disease in the spouses of colorectal cancer patients provides contradictory evidence in the genetic argument. Jensen et al (1980) found that spouses of colorectal cancer patients had no excess risk of the disease but in another study spouses and patients were both found to have a three-fold prevalence of the disease compared to the general population (Lovett, 1976).

Although much work remains to be done, the molecular genetics of colorectal cancer are now being unravelled at a rapid rate. The first important finding was the demonstration of ras oncogene activation in adenomas and carcinomas of the colon and rectum (Bos et al, 1987). The mechanism by which the product of this gene contributes to colorectal
carcinogenesis is not known yet but it has been postulated that it is a second messenger which links external influences such as growth factors to the nucleus (Barbacid, 1987). More recently allele deletions from chromosomes 5, 17 and 18 have been noted in cases of colorectal cancer (Muleris et al, 1985; Rees et al, 1989) but their exact significance remains to be established.

In summary, there is now irrefutable evidence that certain colorectal cancers are genetically determined. More research is needed to piece together the precise sequence or sequences of genetic changes which underlie the development of colorectal adenomas and carcinomas. The genetic changes are presumably triggered by environmental carcinogens such as dietary factors and if the relationship between the environmental and genetic factors becomes fully understood, then prevention of colorectal carcinoma may become a realistic possibility.

Environmental influences

The wide geographical variation in the incidence of colorectal cancer suggests that the aetiology of the disease is closely linked to environmental factors. The results of migrant studies in which the incidence rates tend towards that of the adopted country support this hypothesis as do the well documented urban-rural and social class differences in the incidence of the disease (Hill et al, 1979).

There are two types of environmental factor, physical and cultural. Physical factors such as geography and climate are common to
all members of a particular community. In contrast, cultural factors such as diet and cigarette smoking, are those which are chosen by individuals and the evidence suggests that these are the more important.

Genetic and environmental factors are not necessarily mutually exclusive. The development of colorectal cancer is in all probability stimulated by environmental influences with genetic factors determining which members of a uniformly exposed population actually develop the disease. The environmental factor which has attracted the most interest is the diet and the role of dietary fat intake has been of especial interest.

Dietary fat and colorectal cancer

The relationship between dietary fat intake and colorectal cancer has been extensively investigated in both man and animal models of colonic carcinogenesis. The main area of interest has been the possible involvement of dietary fats as initiators and promotors of colorectal cancers. In 1982 the United States National Research Council felt that the evidence for a link between dietary fat and colorectal cancer was sufficiently strong to recommend a reduced consumption of both saturated and unsaturated fats (Committee on diet, nutrition and cancer, 1982). Nonetheless, the evidence for a direct effect of dietary fats in the aetiology of colorectal cancer remains controversial and the mechanism by which fats may initiate or promote carcinogenesis is unknown.
Human studies

Two main approaches have been used to investigate the links between diet and colorectal cancer in man, namely the comparison of populations and the comparison of cases and controls. Population studies were first stimulated by the well documented international differences in colorectal tumour incidence (Doll, 1972). In the comparison of populations, a group of countries is selected for which data on colorectal cancer incidence and dietary intake are available and the risk of the disease is then correlated with the various dietary components. Almost all international studies have demonstrated a positive correlation between fat consumption per head of population and national incidence rates of large bowel cancer (Table 1.1). A notable exception is the study of Reddy et al (1978) in which no difference in fat intake was found when studying a low risk Finnish population and a high risk New York population. Population studies have also suggested that the type as well as the quantity of dietary fat is important, the strongest correlations being found with saturated fats of animal origin (Drasar & Irving, 1973; Knox, 1977; Liu et al, 1979). Meat is an abundant source of saturated fat in Western diets and strong positive correlations exist between the levels of meat consumption and colorectal cancer incidence (Liu et al, 1979; Mckeown-Eyssen & Bright-See, 1984). Studies concentrating on fat consumption within Great Britain (Bingham et al, 1979) and the United States (Lyon & Sorenson, 1978) have not supported the strong positive correlations found in the international data (Table 1.1). However, major differences in regional dietary habits are not pronounced in these countries.
The main strengths of population studies are that a wide range of cancer incidence rates can be included and extreme diets are consumed by some populations. There are nevertheless two major weaknesses of this approach. Firstly, the populations differ in many other respects as well as their diets and these racial, climatic, economic and health care differences make it difficult to justify direct comparisons. Secondly, the data concerning cancer incidence and dietary intake is not uniformly reliable in all countries. Attempts have been made to overcome these criticisms by studying more selected populations in Scandinavia (International Agency for Research into

Table 1.1 Results of population studies relating dietary fat intake and colorectal cancer incidence

<table>
<thead>
<tr>
<th>Positive correlation demonstrated</th>
<th>No correlation demonstrated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>International studies</strong></td>
<td></td>
</tr>
<tr>
<td>Armstrong &amp; Doll (1975)</td>
<td></td>
</tr>
<tr>
<td>Knox (1977)</td>
<td></td>
</tr>
<tr>
<td>Liu et al (1979)</td>
<td></td>
</tr>
<tr>
<td>Hill et al (1979)</td>
<td></td>
</tr>
<tr>
<td>Mckeown-Eyssen &amp; Bright-See (1984)</td>
<td></td>
</tr>
<tr>
<td><strong>National Studies</strong></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>Lyon &amp; Sorenson (1978)</td>
</tr>
<tr>
<td></td>
<td>Bingham et al (1979)</td>
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</tbody>
</table>
Cancer, 1977; 1982) and Hong Kong (Hill et al, 1979). The problems of reliability of cancer incidence data were obviated by only using cancer registries approved by the International Agency for Research into Cancer. Nonetheless, the lack of reliable dietary data remains the great weakness of population studies. Most studies use Food and Agriculture Organisation data, which takes no account of food not bought in shops or of food wastage.

A further problem in the interpretation of the results of population studies is that the correlation analyses used can only demonstrate that a relationship exists between two factors but can never prove that such a relationship is causal. Thus Armstrong & Doll (1975) found a positive correlation between colonic cancer incidence and gross national product and similarly, Drasar & Irving (1973) found a significant relationship between colorectal cancer and radio ownership.

Studies of religious groups who habitually consume diets containing little or no animal fat provide conflicting evidence as to an association between colorectal cancer and fat intake. In a comparative study of Seventh Day Adventists with the general Californian population, Phillips (1975) reported lower rates of all types of cancer, including colorectal cancer, in the Seventh Day Adventist group. The lower rates of cancer in this religious group have been largely attributed to the low fat diet which is a major feature of their life-style. In a second study, Phillips (1980) compared the cancer incidence in Seventh Day adventists to a more selected control group, who had greater comparability in terms of social, economic and educational status to the high income, highly
educated Adventists. Although confirming a trend towards lower cancer incidence in the Adventists, the difference was no longer statistically significant.

Several case-control studies of dietary habits in colorectal cancer patients have been carried out. Although the majority of these demonstrate a positive correlation between colorectal cancer and dietary fat intake, there are large studies from several different countries which have failed to show an association between any dietary component and colorectal cancer (Table 1.2).

Table 1.2: Results of case-control studies relating diet to the risk of colorectal cancer

<table>
<thead>
<tr>
<th>Positive correlation demonstrated</th>
<th>No correlation demonstrated</th>
</tr>
</thead>
</table>

**Dietary fat**
- Pernu (1960)                  - Higginson (1966)
- Jain et al (1980a)            -
- Jain et al (1982)             -
- Bristol et al (1985)          -

**Meat consumption**
- Haenszel et al (1973)         - Graham & Mettlin (1979)
- Howell (1975)                 -
In case-control studies, the investigator attempts to control for all factors other than diet by matching the two groups for age, sex, race, climate, standard of living and so on. The major disadvantage of these studies is that the closely matched cases and controls tend to have very similar diets and very small differences are sought. The validity of case-control studies is therefore determined by the accuracy of the assessment of dietary intake in individuals. Most workers have used dietary recall histories as the only method of dietary assessment and have concentrated on the diet at diagnosis or for the short period before presentation. Although the dietary recall history is a well established technique, it is doubtful whether it is sufficiently accurate to discriminate between cases and controls who have only small differences in diet. It is perhaps not surprising then that the observed results of these studies are inconsistent (Table 1.2) and the results obtained from case-control studies should be interpreted cautiously.

Animal studies

The strongest available evidence of a relationship between dietary fat and colorectal carcinogenesis comes from the extensive animal studies which have been undertaken. A large number of studies in rodents have demonstrated that increasing the intake of dietary fat causes an increase in the incidence and multiplicity of bowel tumours which have been induced by a variety of chemical carcinogens (Table 1.3). At low levels of dietary fat (5%), polyunsaturated fats appear
<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>Animal</th>
<th>A</th>
<th>Diets under study</th>
<th>B</th>
<th>Effect of A against B</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOM</td>
<td>Rat (SD)</td>
<td>35% beef fat (S)</td>
<td>Normal chow</td>
<td>E</td>
<td>Nigro et al 1975</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (SD)</td>
<td>20% corn oil (PU)</td>
<td>20% lard (S)</td>
<td>N</td>
<td>Reddy et al 1974</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (SD)</td>
<td>5% corn oil (PU)</td>
<td>5% lard (S)</td>
<td>E</td>
<td>Reddy et al 1976</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (SD)</td>
<td>20% coconut oil (SV)</td>
<td>5% coconut oil (SV)</td>
<td>E</td>
<td>Reddy et al 1977a</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (SD)</td>
<td>20% safflower oil (PU)</td>
<td>20% coconut oil (SV)</td>
<td>E</td>
<td>Brodman et al 1977</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (W)</td>
<td>30% lard (S)</td>
<td>Low fat standard diet</td>
<td>E</td>
<td>Bansal et al 1978</td>
<td></td>
</tr>
<tr>
<td>DMH/MAM</td>
<td>INMU/IDMAB</td>
<td>Rat (F344)</td>
<td>20% beef fat (S)</td>
<td>E</td>
<td>Reddy et al 1977b</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (SD)</td>
<td>30% beef fat (S) (after AOM)</td>
<td>5% beef fat (S) (after AOM)</td>
<td>E</td>
<td>Reddy et al 1980</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (SD)</td>
<td>30% beef fat (S) (before AOM)</td>
<td>5% beef fat (S) (before AOM)</td>
<td>N</td>
<td>Bull et al 1979</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (F344)</td>
<td>23.52% corn oil (PU)</td>
<td>5% corn oil (PU)</td>
<td>E</td>
<td>Reddy &amp; Maesura 1984</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (F344)</td>
<td>23.52% safflower oil (PU)</td>
<td>5% safflower oil (PU)</td>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (F344)</td>
<td>23.52% olive oil (M)</td>
<td>5% olive oil (M)</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (F344)</td>
<td>a.23.52% coconut oil (SV) or b.5.88% corn oil (PU) + 17.64% MCT</td>
<td>b.5% safflower oil (PU) or c.5% olive oil (M)</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>Rat (SD)</td>
<td>20% beef fat (S)</td>
<td>20% corn oil (PU)</td>
<td>N</td>
<td>Wilson et al 1977</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (Donryu)</td>
<td>5% linoleic acid (PU)</td>
<td>4.7% stearic acid (S) + 0.3% linoleic acid</td>
<td>E</td>
<td>Sakaguchi et al 1984</td>
<td></td>
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<tr>
<td>AOM</td>
<td>Rat (F344)</td>
<td>4% Menhaden fish oil (PU) + 1% corn oil</td>
<td>5% corn oil (PU)</td>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (F344)</td>
<td>22.5% Menhaden fish oil + 1% corn oil</td>
<td>23.5% corn oil (PU)</td>
<td>I</td>
<td>Reddy &amp; Maruyama 1986</td>
<td></td>
</tr>
<tr>
<td>AOM</td>
<td>Rat (Donryu)</td>
<td>4.7% eicosapentaenoic acid + 0.3% linoleic (PU)</td>
<td>5% linoleic acid (PU)</td>
<td>I</td>
<td>Minoura et al 1988</td>
<td></td>
</tr>
</tbody>
</table>

AOM: azoxymethane; DMH: dimethylhydrazine; MAM: methylazoxymethanol; INMU: intrarectal nitrosomethylurea; IDMAB: intrarectal diethylaminobiphenyl; S: saturated fat (animal origin); PU: polyunsaturated fat; SV: saturated fat (vegetable origin); M: monounsaturated fat; MCT: medium chain triglyceride

E: enhanced; N: no effect; I: inhibition
to cause an increase in the incidence, yield and malignant
differentiation of colonic tumours when compared to the equivalent
level of saturated fats (Reddy et al, 1974; 1976; 1977b; Sakaguchi et
al, 1984). At higher levels of dietary fat intake (20%), there is no
clear cut difference between the effects of polyunsaturated and
Therefore, it is clear that the type of dietary fat as well as the
total amount is important in experimental colorectal carcinogenesis.
Bull et al (1979) found that the effect of dietary fat was present when
given after tumour induction by a chemical carcinogen but not before or
during induction, suggesting that the mechanism was one of tumour
promotion rather than initiation.

The most recent studies have focused on the effects of marine
oils on colonic tumour promotion and growth. These substances are
derived from oily fish such as mackerel or herring and contain high
levels of the long chain polyunsaturated fatty acids eicosapentaenoic
acid and docosahexaenoic acid. Reddy and Maruyama (1986) and Minoura
et al (1988) have demonstrated that these fats have an inhibitory
effect on the promotion of colorectal tumours by chemical carcinogens.
It has also been shown that fish oil has a marked suppressive effect on
the growth of colorectal tumours implanted subcutaneously in mice
(M. Sakaguchi, personal communication).

Despite the inconsistencies in the epidemiological data, after
reviewing all of the available evidence the American National Research
Council Committee on Diet, Nutrition and Cancer (1982) concluded that
of all the dietary components studied the experimental and
epidemiological evidence was most suggestive of a causal relationship
between dietary fat intake and the occurrence of cancer.
Theories linking dietary fat to colorectal cancer

Faecal bile acids

Hill and other workers have postulated that faecal bile acids and metabolites of cholesterol function as carcinogens, co-carcinogens or promotors of tumourigenesis in the large bowel (Hill et al, 1971; Nigro et al, 1973; Reddy and Wynder, 1973; Narisawa et al, 1974; Mastromarino et al, 1976). In support of this theory, several secondary bile acids have been shown to be genotoxic using in vitro autogenicity tests (Silverman & Andrews, 1977; Ferguson & Parry, 1984; Wilpart et al, 1984; Watanabe & Bernstein, 1985). High levels of dietary fat not only increase the excretion of faecal bile acids but by determining the composition of the anaerobic flora of the large bowel, cause increased degradation of this substrate to yield a higher level of carcinogens in the bowel (Hill, 1975; Wynder & Reddy, 1975).

The evidence from human studies has been conflicting. Several population studies have demonstrated a positive correlation between total faecal bile acid concentration and the risk of colorectal cancer (Reddy & Wynder, 1973; Antonis & Bersohn, 1962; Hill & Aries, 1971; Crowther et al, 1976; Hill et al, 1982; IARC working party, 1977; 1982). Observations that cholecystectomy, which increases the concentration of secondary bile acids, is associated with higher rates of right sided colorectal cancer are also consistent with the hypothesis (Linos et al, 1981; Vernick & Kuller, 1981; McMichael & Potter, 1985). Nevertheless, other studies have failed to show a difference in patients with colonic polyps both with respect to the
bacterial flora and with respect to faecal bile acid concentrations (Mudd et al, 1980; Tanida et al, 1984). The results of case-control studies have also been inconsistent, with some workers demonstrating higher faecal bile acid concentrations in patients with colonic cancer (Reddy & Wynder 1977a; Hill et al, 1975), whereas several others have not detected any differences between cases and controls (Mudd et al, 1978; Moskowitz et al, 1979; Murray et al, 1980; Kaibara et al, 1983; Hikasa et al, 1984; Stemmermann et al, 1984; Breuer et al, 1985). Indeed Hill's group were unable to confirm their original findings of increased faecal bile acid output in patients with colorectal cancer, in more recent studies (Owen et al, 1984; Owen et al, 1986). Clearly, further studies are required to clarify the situation.

Experimental evidence linking faecal bile acids and colorectal cancer is also available. In rats high fat diets cause an increase in the excretion of faecal bile acids (Reddy et al, 1977b; Reddy, 1981) and Chomchai et al (1974) have shown that an increase in the quantity of bile acids in the colonic mucosa was associated with an increase in colon tumours induced by azoxymethane. The rat, however, has an extremely complex faecal bile acid profile with over 27 different bile acids (Bull et al, 1984). Obviously this makes the rat a less than ideal model in this situation and the results of rodent based studies cannot be extrapolated to make conclusions about the human situation.
Other potential mechanisms

There are other mechanisms by which dietary fats may promote the development of colorectal cancer. Firstly, fats may have a direct effect on cellular proliferation. Bull et al (1984) showed that hydroperoxy and hydroxy derivatives of linoleic and arachidonic acids (which can occur by auto-oxidation) produced increased DNA synthesis and ornithine decarboxylase activity when instilled directly into the colon of rats. Ornithine decarboxylase is the enzyme controlling the rate limiting step in the formation of putrescine and other polyamines. These chemicals are present in all human cells and are essential for normal growth and differentiation (Williams-Ashman & Canellakis, 1979). Ornithine decarboxylase is present in very small amounts in quiescent cells but its activity increases several hundred fold during the early phases of cellular responses to such stimuli as hormones, drugs, and tissue growth factors (Williams-Ashman & Canellakis, 1979; Luk et al, 1980; Pegg & McCann, 1982; Luk et al, 1983). Further work in the field of tumour growth factors is likely to improve our knowledge of the aetiology of colorectal cancer.

Secondly, the effects of fat may be mediated by impairment of the immune system. Diets which are high in polyunsaturated fatty acids reduce T-lymphocyte responsiveness and linoleic, linolenic and arachidonic acids have all been shown to have some immunosuppressive action (Hillyard & Abraham, 1979; Cinader et al, 1983). The mechanisms by which these fatty acids produce immunosuppression include alteration in T cell membrane fluidity resulting in changes in the configuration of receptor sites, thus rendering the cells less competent (Cinader et
1983), changes in cAMP concentrations or prostaglandin synthesis thereby affecting antigen/T-lymphocyte interaction (Hillyard & Abraham, 1979) and decreased cholesterol synthesis within the cell which is known to be a pre-requisite for DNA synthesis and blastogenesis (Chen et al, 1975)

Thirdly, fatty acids can alter the membrane function of target cells rendering them more susceptible to carcinogenic stimuli. Alterations in the fatty acid composition of cell membranes can result in increased membrane fluidity and permeability (Sandemann, 1979), raised activity of specific cell enzymes (Yoo et al, 1980), increased exposure of membrane proteins (Shinitzky & Rivnay, 1977), changes in the lateral mobility of receptors (Horwitz et al, 1974) and modulate the susceptibility of cells to complement mediated lysis (Yoo et al, 1980; Schlager & Ohanian, 1980). Neoplastic proliferation may be the result of derangement of control systems which maintain the normal behaviour of the initiated cell and can be brought about by fatty acids altering the liquid crystalline phases at aqueous interfaces in cell membranes (Horton et al, 1981). Specific changes in the lipid composition of cell membranes as a result of dietary adjustments can be related to tumour growth (Fischer et al, 1981). For example cells of a particularly malignant subline of the Ehrlich-Lettre ascites tumour have lipid membranes with increased fluidity and they also have increased unsaturated fatty acid and phosphatidylinositol content (Haeffner et al, 1982). Moreover, feeding polyunsaturated fats to rats has been related to increased fluidity of the colonic mucosal cells with enhancement of specific membrane enzyme systems (Brasitus et al, 1985).
Fourthly, dietary fatty acids derived from linoleic acid may be implicated in tumour initiation, promotion and metastatic dissemination by virtue of their being the precursors of arachidonic acid and its metabolites (prostaglandins and leukotrienes). Sakaguchi et al (1984) demonstrated that a semi-synthetic diet containing 5% unsaturated fat as linoleic acid markedly altered the fatty acid composition of rat colonic mucosa and increased the arachidonic acid content of the neutral lipid in chemically induced colonic cancers. Narisawa et al (1981) showed that the prostaglandin inhibitor indomethacin reduced the development of methylnitrosourea-induced colonic tumours in rats.

In summary, although a great deal of interest has centred on the role of faecal bile acids as the link between dietary fat and colorectal cancer, there are many other potential mechanisms by which dietary fat may exert its effects.
The relationship between dietary fat intake, cell membrane fatty acids and colorectal cancer

The possible influence of dietary fat on the structure and function of cell membranes has been little studied. Only recently has there been an attempt to study the lipid membrane composition of cells in human subjects with cancer. Wood et al (1985) reported that the ratio of stearic acid to oleic acid in the cell membrane of erythrocytes from patients with a variety of solid cancers was always less than one and that this was significantly different from a reference group of healthy controls in which the ratio was always above one. A reduction in the so called 'saturation index' was also noted in a variety of animal malignancies by the same group (Habib et al, 1987a) and it was suggested that the index would be a useful marker of malignancy. The alteration in the fatty acid composition of cells from patients with malignancies was attributed to a circulating desaturation factor and a putative protein was isolated from the urine of these patients and shown to produce membrane desaturation in vitro (Habib et al, 1987b).

This work has stimulated many questions. It has, however, been limited in that it has concentrated specifically on the study of erythrocyte cell membranes. The fatty acid composition of cell membranes from malignant cells and the non-malignant cells from which they arise has not previously been studied in any depth. Furthermore the potentially important influence of dietary fat intake at the level of cell membrane composition requires further more detailed study.
Conclusions

Epidemiological evidence suggests that dietary fat intake is associated with the development of colorectal cancer and this finding has been supported by many experimental studies using animal models. The mechanism by which dietary fat promotes colorectal cancer has not been fully determined and although much research has concentrated on the role of faecal bile acids, there are other mechanisms by which dietary fat may exert an influence. The cell membrane provides a potential link between fat and carcinogenesis and recent studies have reported abnormalities in the fatty acid composition of erythrocyte cell membranes in certain cancers. The studies so far have been limited in that they have concentrated purely on the erythrocyte cell membrane and have not included detailed investigation of the potentially important influence of dietary fat intake.

For these reasons a study of the relationship between dietary fat intake and cell membrane fatty acid composition in colorectal cancer was undertaken. Initial investigations in man were followed by detailed studies using an experimental model of colorectal cancer in which the dietary intake of fat could be carefully controlled.
Chapter 2
CELL MEMBRANE STRUCTURE

Cell membrane structure and function are intimately related and are of central importance in all living systems. The plasma membrane regulates the traffic of ions and molecules in and out of the cell and thus controls the internal milieu. This chapter describes our current understanding of cell membrane structure and function with particular emphasis on the role of fatty acids. The modulating influence of dietary fat on cell membrane lipid composition is also discussed.

Historical background

The term plasma membrane was first used by Nägeli and Cramer (1855), to describe the formation of a protective film where the outflowing cytoplasm of an injured cell came into contact with water. Overton (1899) noted a correlation between the lipid solubility of substances and their rate of entry into cells and from this physico-chemical property predicted that the cell membrane was lipid in nature. The modern concept of cell membrane structure started when Langmuir (1917) demonstrated that when fatty acids or phospholipids are allowed to spread over the surface of pure water, the molecules orientate themselves to form a monomolecular layer. This property is easily
explained because phospholipids are amphipathic, that is they have a hydrophilic or polar head and a hydrophobic or non-polar tail. The polar heads consist of glycerol conjugated to a nitrogenous compound such as choline, ethanolamine or serine via a phosphate bridge. The non-polar tail consists of the hydrocarbon chains of the two fatty acid molecules which are covalently linked to the glycerol molecule. When amphipathic molecules are layered on water they spontaneously form a bimolecular lipid sheet or bilayer with their hydrophobic tails facing inwards sandwiched between the hydrophilic head groups.

The experiment which first suggested that cell membranes were organised in this way was performed by Gorter and Grendel (1925). They extracted the lipids from erythrocytes using acetone, spread them on water as a monolayer and measured the surface area. They found that this was almost exactly twice the value of the calculated surface area of the original erythrocytes and postulated that the cells were enclosed by a lipid layer two molecules thick. Although this conclusion has subsequently been proved to be correct, it was based on two incorrect assumptions which fortuitously compensated each other. On the one hand, acetone is an inefficient method lipid extraction and so did not extract all of the available lipid. On the other, the calculation of erythrocyte surface area was made from dried preparations and this was substantially less than the true value for wet preparations.

The next major advance was made by Danielli and Davson (1935) who found that the surface tension of artificially created lipid bilayers was much higher than that of natural membranes. This led them to propose that a layer of protein was present on either side of the lipid
bilayer. The advent of the electron microscope in the 1950's lent support to the Davson-Danielli hypothesis inasmuch as the plasma membrane was found to consist of two electron dense layers separated by a lighter central area. The total measured thickness of the membrane was 7.5 nm, a figure which had been predicted by Davson and Danielli 20 years earlier. This trilaminar appearance was found to be characteristic of membranes in the interior of the cell as well as the plasma membrane and was termed the 'unit membrane' by Robertson (1957).

The development of the method of freeze-fracturing by Steere (1957) and Moor (1966), however, led to an entirely new interpretation of membrane structure. This method produces a high fidelity replica of the surface of frozen biological material. A small block of tissue is fixed in glutaraldehyde and then frozen by immersion in partially solidified dichlorodifluoromethane (Freon) cooled at -150°C with liquid nitrogen. The specimen is then fractured in a vacuum chamber by the impact from a knife edge cooled to -196°C. A replica of the fractured surface is then made by evaporating a heavy metal such as platinum in the vacuum. This deposits metal on the surfaces of the specimen. The more stable element carbon is then deposited on the entire specimen from a separate electrode, the vacuum is broken and the coated specimen is immersed in acid or sodium hypochlorite to dissolve the tissue. The replica remaining is washed gently and examined by electron microscopy. This results in an enhanced three dimensional appearance comparable to the exaggeration of the surface contours of an object that results from oblique lighting.

It has been shown that the line of fracture follows the path of least resistance and that for membranes this is through the hydrophobic
region of the lipid bilayer, thus cleaving the membrane in half and exposing its interior (Branton, 1966). The Davson–Danielli hypothesis of membrane structure would predict that the two inner surfaces of cell membranes would be smooth and featureless. However, the freeze-fractured membranes were very different from this with the inner half (outwardly facing) membrane containing numerous randomly distributed globular particles measuring 6 to 9 nm in diameter. The outer half (inwardly facing) membrane was relatively smooth containing about one-fifth the number of particles found on the other half. It has now been established that these particles are globules of protein within the plane of the membrane.

Current ideas about membrane structure

The currently accepted model of cell membrane structure is the fluid mosaic model suggested by Singer & Nicolson (1972) (Figure 2.1). This model was proposed purely on the basis of thermodynamic principles and the known properties of proteins. According to this model, the cell membrane is a two-dimensional solution of orientated lipid and protein molecules. It was assumed that the protein molecules in membranes were amphipathic like the phospholipids molecules of the bilayer. Thermodynamic considerations would therefore dictate the position of these proteins within the membrane with any ionic amino acid residues directed outwards in the hydrophilic portion and their non-ionic portions preferentially localised in the hydrophobic interior of the membrane. In addition the oligosaccharide chains of
glycoproteins would stick out from the surface of the membrane because of their hydrophilic nature. Protein molecules with a non-polar centre and polar regions at either end would extend through the entire thickness of the membrane to be exposed at each surface.

Figure 2.1. The fluid mosaic model of cell membrane structure (from Singer SJ and Nicolson GL, Science 1972;175:720-731)
Freeze-fracture electron microscopy studies have provided compelling morphological evidence that protein particles are randomly embedded in membranes rather than forming discrete layers on either side of the lipid bilayer. Thus, the Davson-Danielli model of membrane structure has now been displaced by the fluid mosaic model of Singer and Nicolson.

The lipid component of the membrane is principally responsible for its mechanical properties and the proteins determine the dynamic functions of the membrane as an interface between the inside and outside of the cell. It is the lipid bilayer structure which gives the membrane the property of spontaneous self assembly and the ability to re-seal itself when torn. The proteins which are freely mobile and may extend through the entire thickness of the membrane, are thought to act as functional pores through which hydrophilic molecules are transported across the membrane.

Many membrane proteins and lipids are conjugated with short chain polysaccharides on the surface of the cell membrane (Fishman & Brady, 1976). This polysaccharide layer has been called the glycocalyx and although its function is obscure, it may serve as a mechanism for intercellular signalling (Hakomori, 1981). The glycocalyx layer is seen on electron micrographs as a fuzzy coat on the external surface of the cell membrane.
Membrane fluidity

A variety of techniques have been used to demonstrate the fluid nature of cell membranes. One of the most notable experiments was performed by Frye and Edidin (1970). Human and mouse cells were fused, using Senai virus as the fusing agent. Initially human and murine antigenic determinants were confined to their respective halves of the fused cell but within 40 minutes they had intermixed and were uniformly distributed. This early evidence that membrane surface proteins enjoy lateral mobility within the fluid lipid bilayer has been confirmed more recently by electron spin–resonance spectroscopy (Kornberg & McConnell, 1971). These studies demonstrate that lipid molecules exchange places with their neighbours within a monolayer up to $10^6$ times a second (Brulet & McConnell, 1975) but that migration between the two monolayers, a process called 'flip-flop', occurs less than once a fortnight for any individual lipid molecule (Rothman & Davidowiec, 1975).

The fluidity and flexibility of a membrane is dependent on its composition (Quinn & Chapman, 1980). The hydrocarbon chains of saturated fatty acids are straight whereas for unsaturated fatty acids each double bond creates a $30^\circ$ bend in the chain. These kinks caused by double bonds prevent close packing of unsaturated fatty acids and this increases the fluidity and flexibility of the membrane. Hydrocarbon chain length also has an influence in that shorter chains have a lower tendency to react with one another. Another important determinant of membrane fluidity is cholesterol which is present in cell membranes in an almost one to one ratio with phospholipids.
Cholesterol molecules are also amphipathic and their platelike steroid rings have a kinked conformation which allows them to fit in the gaps between the kinks of the unsaturated fatty acids which prevents too close packing (Quinn & Chapman, 1980).

The modulating influence of dietary fat

The influence of dietary fat intake on the lipid composition of mammalian cell membranes has received little attention until quite recently. Clandinin et al (1983) studied the fatty acid profiles of various cell membranes in rats fed semi-synthetic diets. Changes in the dietary unsaturated to saturated fatty acid ratio were found to have a marked influence on the composition of cell membranes in small intestinal mucosa. Another study has demonstrated that the alterations produced by dietary manipulation are predominantly changes in the type of unsaturated fatty acids present rather than a change in the saturated to unsaturated ratio (Gibson et al, 1984). This work also found that cell membranes of tissues which respond to changes in dietary fat intake exhibit a degree of homeostasis in that the level of unsaturation remains relatively constant despite wide variations in the experimental diets. The importance of the modulating influence of dietary fat is that changes in membrane fatty acid composition are known to alter the physico-chemical and functional properties of biological membranes (Cronan and Gelman, 1975; Innis and Clandinin, 1981).
Conclusions

Cell membranes consist of a double layer of lipid molecules in which various membrane proteins are embedded. The lipid provides the structural backbone whereas the proteins have a functional role. The lipid bilayer is fluid with individual phospholipid molecules being able to diffuse freely within each layer and sometimes between each layer. This fluidity is determined by the fatty acid and cholesterol composition of the lipid bilayer. There is experimental evidence which suggests that the fatty acid profile of certain cell membranes can be modulated by changes in dietary fat intake.
Chapter 3
THE STRUCTURE, OCCURRENCE AND BIOCHEMISTRY OF FATTY ACIDS

This chapter provides a summary of the biochemistry of the major fatty acids found in mammalian tissues. It is not a complete account of fatty acid biochemistry, but is intended rather to contain only that information which is relevant to this thesis. Thus, the details of the metabolic pathways of fatty acids have, as far as possible, been kept to a minimum. The chapter is in three parts, an explanation of fatty acid nomenclature, a section on the synthesis of fatty acids and finally a discussion of the regulation of fatty acid biochemistry with special reference to the influence of the diet.

Fatty acid nomenclature

Fatty acids are long chained aliphatic monocarboxylic acids. The common fatty acids in both plants and animals contain an even number of carbon atoms in a straight chain with a methyl group at one end and a carboxyl group at the other. They may be fully saturated, containing no double bonds or they may be unsaturated with between one and six double bonds, which are usually, but not always in the cis-configuration.

The most commonly used systematic nomenclature (the Geneva
classification) is based on naming the fatty acid after the hydrocarbon with the same number of carbon atoms and substituting the ending -oic for the terminal -e in the name of the hydrocarbon. Saturated fatty acids end in -anoic and unsaturated fatty acids in -enoic. For example the saturated fatty acid with sixteen carbon atoms and the structural formula \( \text{CH}_3(\text{CH}_2)_{14}\text{COOH} \) is correctly named hexadecanoic acid. A shorthand nomenclature also exists in order to simplify the discussion of fatty acids. This is formed by writing the number of carbon atoms followed by the number of double bonds and separating the two by a colon. Thus, hexadecanoic acid is written simply as 16:0. Finally, most fatty acids also have a trivial name which is in common usage. The trivial name of the saturated fatty acid 16:0 is palmitic acid. Table 3.1 presents a list of the systematic, trivial and shorthand nomenclatures of the common fatty acids.

The carbon atom chains of fatty acids are numbered from the carboxyl end of the molecule which is carbon number 1. The carbon atom adjacent to the carboxyl group, carbon number 2, is also known as the alpha-carbon. Carbon number 3 is the beta-carbon and the end methyl carbon is known as the omega-carbon. A number of different conventions exist to specify the position of the double bond or bonds in unsaturated fatty acids. A double bond between carbon atoms 9 and 10 of the fatty acid can be written as delta9. As an example octadecenoic acid which has the chemical structure \( \text{CH}_3(\text{CH}_2)_{7}\text{CH=CH(CH}_2)_{7}\text{COOH} \) and the trivial name oleic acid is more accurately written as delta9-octadecenoic acid or cis-9-octadecenoic acid. In shorthand nomenclature this is designated 18:1(9). The position of the double bond can also be denoted in the form \((n-x)\), where \(n\) is the carbon chain length of the
Table 3.1. The nomenclature of some common fatty acids

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Trivial Name</th>
<th>Shorthand Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ethanoic</td>
<td>acetic</td>
<td>2:0</td>
</tr>
<tr>
<td>butanoic</td>
<td>butyric</td>
<td>4:0</td>
</tr>
<tr>
<td>dodecanoic</td>
<td>lauric</td>
<td>12:0</td>
</tr>
<tr>
<td>hexadecanoic</td>
<td>palmitic</td>
<td>16:0</td>
</tr>
<tr>
<td>heptadecanoic</td>
<td>margaric</td>
<td>17:0</td>
</tr>
<tr>
<td>octadecanoic</td>
<td>stearic</td>
<td>18:0</td>
</tr>
<tr>
<td><strong>Unsaturated fatty acids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-hexadecenoic</td>
<td>palmitoleic</td>
<td>16:1 (n-7)</td>
</tr>
<tr>
<td>9-octadecenoic</td>
<td>oleic</td>
<td>18:1 (n-9)</td>
</tr>
<tr>
<td>11-octadecenoic</td>
<td>cis-vaccenic</td>
<td>18:1 (n-7)</td>
</tr>
<tr>
<td>9,12-octadecadienoic</td>
<td>linoleic</td>
<td>18:2 (n-6)</td>
</tr>
<tr>
<td>6,9,12-octadecatrienoic</td>
<td>gamma-linolenic</td>
<td>18:3 (n-6)</td>
</tr>
<tr>
<td>5,8,11,14-eicosatetraenoic</td>
<td>arachidonic</td>
<td>20:4 (n-6)</td>
</tr>
<tr>
<td>5,8,11,14,17-eicosapentaenoic</td>
<td>-</td>
<td>20:5 (n-3)</td>
</tr>
<tr>
<td>4,7,10,13,16,19-docosahexaenoic</td>
<td>-</td>
<td>22:6 (n-3)</td>
</tr>
</tbody>
</table>
fatty acid and $x$ the number of carbon atoms from the last double bond to the terminal methyl group. Oleic acid can therefore also be written as 18:1 (n-9). This last form of nomenclature has been adopted by the IUPAC-IUB Commission on Biochemical Nomenclature (1967). Using this system several series of fatty acids can be described. Thus, fatty acids based on oleic acid which have a double bond 9 carbons from the methyl end but with increasing chain length or increasing desaturation are the n-9 fatty acids. Those based on linoleic acid which has its first double bond 6 carbons from the methyl end are the n-6 fatty acids and a series based on linolenic acid which has its first double bond 3 carbons from the methyl group are the n-3 fatty acids. These series occur because in animals additional double bonds are introduced only between the existing double bond and the carboxyl carbon.

**Saturated fatty acids**

The common naturally occurring saturated fatty acids are straight chained and have 16 to 18 carbon atoms. Palmitic acid (16:0) is the commonest example, being found in virtually all animal and plant fats and oils. Stearic acid (18:0) is also relatively common and may on occasion be more abundant than palmitic acid especially in complex lipids. Longer chain saturated fatty acids occur less frequently but are seen in various waxes. Trace amounts of the odd carbon chain acids (15:0 to 19:0) are found in animal fats and occur in larger quantities in certain fish oils. Saturated fatty acids with 10 or more carbon atoms are solids at room temperature. The lack of functional groups in the saturated fatty acids renders them fairly inert chemically.
Monounsaturated (monoenoic) fatty acids

Oleic acid (18:1, n-9) is probably the most abundant fatty acid of all and like palmitic acid it is found in virtually all lipids of animal or plant origin. Various positional isomers exist, for example cis-vaccenic acid (18:1, n-7) which is the major unsaturated fatty acid in bacteria. Animal fats often contain families of monoenoic fatty acids with different chain lengths but with similar methyl terminal structure. They arise from the common precursor or parent molecule oleic acid by chain elongation or shortening (Figure 3.1). Monoenoic fatty acids of 18 carbon atoms or less all have a low melting point. The presence of a double bond in the aliphatic chain of these fatty acids renders them more susceptible to chemical attack, especially oxidation, than their saturated counterparts.

Figure 3.1. Inter-relationships between the n-9 fatty acids

```
  16:1 (n-9)
     ↑
  Chain shortening
     ↑
  Parent fatty acid
        /\       /\
    18:1 (n-9) 20:1 (n-9) 22:1 (n-9)
     ↓       /\       /\       /\      
  Oleic acid
     /\     /\     /\     /\      
  Chain elongation
```

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Polyunsaturated fatty acids

Polyunsaturated fatty acids contain two or more cis-double bonds and can also be subdivided into several families according to their derivation from single specific fatty acid precursors. Linoleic acid (18:2, n-6) is the simplest example and is commonly found in animal and plant tissues. It is an essential fatty acid in man because it is required for health but cannot be synthesised by humans and therefore must be taken in the diet. It is the precursor of the n-6 fatty acids which are produced from it by a combination of chain elongations and desaturations (Figure 3.2).

Arachidonic acid (20:4, n-6) is the most important metabolite of linoleic acid. It is a major constituent of the complex lipids and the precursor molecule of the prostaglandins. These compounds have been shown to have profound and diverse actions in man (Ramwell, 1979). Gamma-linolenic acid (18:3, n-6) only occurs in small amounts in animal systems but is nevertheless an important intermediate in the synthesis of arachidonic acid. Alpha-linolenic acid (18:3, n-3) is a major component of the photosynthetic tissues of plants but is rare in animal tissues. It is, however, the parent molecule of the important n-3 fatty acids. These are essential fatty acids for fish and the fats in certain oily fish, especially mackerel, salmon and herring, contain large quantities of n-3 fatty acids (Exler & Weihrauch, 1976). The two most prominent examples are the long chained eicosapentanoic acid (20:5, n-3) and docosahexaenoic acid (22:6, n-3). These two fatty acids are also found in complex lipids in many human and animal tissues.
Figure 3.2. Metabolic inter-relationships of the n-6 fatty acids

Parent fatty acid

\[ 18:2 \, (n-6) \]
Linoleic acid

\[ \text{Elongation} \rightarrow \text{Desaturation} \]

\[ 20:2 \, (n-6) \]
\[ 18:3 \, (n-6) \]
\[ \gamma\text{-Linolenic acid} \]

\[ \text{Desaturation} \rightarrow \text{Elongation} \]

\[ 20:3 \, (n-6) \]

\[ \text{Elongation} \]

\[ 20:4 \, (n-6) \]
Arachidonic acid

\[ \text{Elongation} \]
\[ \& \text{Desaturation} \]

\[ 22:5 \, (n-6) \]
There are two less important series of unsaturated fatty acids, the n-9 series which, as mentioned above, have oleic acid (18:1, n-9) as the parent compound and the n-7 series which are derived from palmitoleic acid (16:1, n-7). Polyunsaturated fatty acids all have very low melting points and a tendency to deterioration by oxidation. The more double bonds they contain, the greater is their susceptibility to such chemical attack.

Lipids

Lipids can be divided into two broad categories, simple lipids and complex lipids. The simple lipids are esters of fatty acids with an alcohol, which most often is glycerol. In the triacylglycerols (triglycerides) each of the three hydroxyl groups of glycerol are esterified to a fatty acid. Triacylglycerols are by far the most abundant class of lipids in man because this is the form in which lipid is stored in the adipose tissues.

The complex lipids are esters of fatty acids and an alcohol which contain other chemical groups as well. The most important class is the phospholipids which in addition to fatty acids and an alcohol, contain a phosphoric acid residue and a nitrogen containing organic base. The four major phospholipids are phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol. The other important complex lipid classes are the sphingolipids which contain the alcohol shingosine instead of glycerol and the glycolipids which contain a carbohydrate. The importance of the phospholipids and shingolipids lies in their role as major components of human cell membranes.
The biosynthesis of fatty acids

Man has a limited capacity to store polysaccharides as glycogen and so fats are the major form of energy storage. Ingested glucose in excess of that required for immediate energy requirement is converted by the glycolitic pathway into pyruvate and then acetyl coenzyme A (acetyl-CoA), from which lipids are synthesised. These are then converted to triacylglycerols and stored in the adipose tissue deposits or used to synthesise phospholipids. Clearly a balance has to maintained between the storage of fatty acids as triglycerides and the synthesis of phospholipids as essential membrane components (Figure 3.3).

Figure 3.3. Synthesis and metabolic fate of fatty acids

```
DIETARY FAT
         ↓
         ↓
DIETARY CARBOHYDRATE
         ↓ glycolysis
       Pyruvate
         ↓
       Acetyl-CoA → TCA cycle
         ↓
       FATTY ACIDS
         ↓ Phospholipids
         ↓ Triglycerides
         ↓ Cell Membranes
         ↓ Adipose tissues
```
The fatty acids required for these lipids can be derived either from the diet or synthesised de novo. Saturated and monounsaturated fatty acids can be derived from either source but polyunsaturated fatty acids can only come from the dietary intake of linoleic (9,12-18:2) and alpha-linolenic acids (9,12,15-18:3). These two fatty acids are essential components of the diet because human cells do not possess a delta12-desaturase enzyme and therefore cannot synthesise them. These fatty acids must therefore be obtained from plant sources which do possess the appropriate delta12-desaturase enzyme, thus enabling them to insert a double bond between the 12th and 13th carbon atoms of the hydrocarbon chain. Chronic deficiency of the essential fatty acids in experimental animals leads to the development of scaly skin, growth retardation and a fatty liver (Burr and Burr, 1929).

Saturated fatty acids are synthesised in the cytosol from their ultimate precursor acetyl-CoA, which is derived from either carbohydrate or amino acid sources. The first step in the process of fatty acid synthesis is the conversion of the two carbon unit acetyl-CoA to malonyl-CoA. This reaction is catalysed by the biotin containing enzyme acetyl-CoA carboxylase as follows:

\[
\text{acetyl-CoA} + \text{HCO}_3^- + \text{H}^+ + \text{ATP} \rightarrow \text{malonyl-CoA} + \text{ADP} + \text{Pi}
\]

This carboxylation reaction is the primary regulatory or rate limiting step of fatty acid synthesis. Six further steps are required to produce palmitic acid (16:0), which is the preferred end product of the synthetic pathway. Each step is catalysed by one of the six enzymes which make up a complex collectively called fatty acid synthetase. This
complex consists of six separate enzymes held together by a seventh central protein, the acyl carrier protein (ACP), which has no enzymatic activity itself. The ACP has a long side chain (the 4'-phosphopantetheine group) on the end of which there is a single sulphhydryl or -SH group. The elongating acyl chain can therefore be attached to this side chain via a thioester linkage. The phosphopantetheine side chain acts as a swinging arm which carries the elongating acyl chain from one enzyme molecule to the next (Figure 3.4).

Fig 3.4. Schematic representation of the fatty acid synthetase system
The details of the six reactions are not important but in short the molecule is elongated progressively by the addition of acetyl groups derived from 2 of the 3 carbon atoms of a malonyl-CoA molecule. The final product is palmitic acid. This the preferred end point of fatty acid synthetase and other saturated fats are produced from it by chain elongation or retroconversion (Figure 3.5).

Figure 3.5. Synthesis and fate of palmitic acid (16:0)

```
Carbohydrate
\[\rightarrow\] Glycolysis
\[\rightarrow\] Acetyl-CoA
\[\rightarrow\] Fatty acid synthetase
16:0
Palmitic acid
(saturated)
\[\rightarrow\] Chain Elongation
\[\rightarrow\] Desaturation
\[\rightarrow\] Beta-oxidation
\[\rightarrow\] Incorporation into membranes or adipose tissue
18:0 & higher
unsaturated fatty acids
```
Higher saturated fatty acids, for example stearic acid (18:0) are synthesised by elongation of palmitic acid. This is achieved by enzymes which progressively add 2-carbon units to the carboxyl end of the parent fatty acid chain. Two different enzyme systems exist, a mitochondrial system which utilises acetyl-CoA as the source of 2-carbon units and a microsomal system in which malonyl-CoA acts as the source of 2-carbon units.

The monoenoic fatty acids palmitoleic acid (16:1) and oleic acid (18:1) are synthesised by the desaturation of palmitic and stearic acids respectively. In both cases a double bond is introduced between the 9th and 10th carbons of the acyl chain by the enzyme delta^9-desaturase. The four distinct series of mammalian polyenoic acids described above are synthesised from their parent precursor fatty acids by further elongation and/or desaturation. The four parent fatty acids are palmitoleic, oleic, linoleic and linolenic acids. As mentioned previously, mammals including man do not possess a delta^12-desaturase enzyme and so two of the precursor unsaturated fatty acids, namely linoleic and alpha-linolenic acid must be obtained in the diet from plant sources. These are the essential fatty acids.

Potentially each substrate competes for desaturation, elongation and transfer into triacylglycerols. However, the rates of elongation are considerably greater than those of desaturation (Bernert & Sprecher, 1975). Furthermore, it has been shown that the preferred substrate length for delta^9-desaturation is an 18 carbon chain, that is stearic acid (Gurr et al, 1972; Jeffcoat et al, 1977). Therefore, the major fatty acids found in de novo synthesised lipids are palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1).
The biochemistry of desaturation

The delta9-desaturase enzyme is not a single molecule but is composed of three separate proteins, NADH-cytochrome b5 reductase, cytochrome b5 and the desaturase proper (Holloway, 1971; Shimakata et al, 1972). Coenzyme-A esters act as substrates for desaturation rather than free fatty acids. Desaturation of preformed fatty acids has two functions related to their physiology. Firstly, the desaturation of saturated fatty acids to produce mono-unsaturated fatty acids reduces their melting points and allows them to be further metabolised and transported by the blood stream (Jeffcoat, 1979a). Secondly, the desaturation of oleic, linoleic and alpha-linolenic acids produces the precursor fatty acids of membrane phospholipids and the prostaglandins, prostacyclin, thromboxanes and leucotrienes.

The delta6- and delta5- desaturases are the enzymes responsible for the conversion of the essential fatty acids into more complex fatty acids as a source of membrane phospholipids. Arachidonic acid (20:4) is a particularly important fatty acid produced by the desaturation of the essential fatty acid linoleic acid. It is the immediate precursor of several groups of important compounds including the prostaglandins. These compounds are abundant in animal tissues and have a wide range of profound hormone-like physiological actions. The biological half-life of most prostaglandins is only seconds long and so they are produced and consumed at the sites of their actions. The arachidonic acid from which they are synthesised is not found in free form, but rather is hydrolysed from cell membrane phospholipids by the enzyme phospholipase (Figure 3.6).
The dietary control of lipogenesis

Lipids are an important source of stored energy and one of the most important influences which controls the fate of fatty acids is the diet. Animal experiments demonstrate that the key enzymes of lipid synthesis are induced by lipogenic substrates in the diet. Thus, rats fed a diet containing high concentrations of sucrose or fructose showed elevated levels of fatty acid synthetase and acetyl-CoA carboxylase (Bruckdorfer et al, 1972). Pre-starving the animals was shown to increase the effect and to also enhance the levels of stearoyl-CoA desaturase (delta9-desaturase) (Jeffcoat & James, 1977). This enables the animal to increase the production and storage of lipids when
presented with dietary carbohydrate in excess of that required for immediate energy needs. Conversely, dietary fatty acids inhibit the enzymes fatty acid synthetase and stearoyl-CoA desaturase, the most potent inhibitor being linoleic acid (Jeffcoat & James, 1977; Flick et al, 1977). The fact that saturated and monounsaturated fatty acids are not the most effective inhibitors of fatty acid synthesis suggests that the action of linoleic acid is by a mechanism other than end-product inhibition. It is now clearly established that the nutritional control of hepatic lipid synthesis is via the action of linoleic acid on the enzyme stearoyl-CoA desaturase and not on fatty acid synthetase. This was elegantly demonstrated by Jeffcoat & James (1978). They induced high levels of fatty acid synthetase and stearoyl-CoA desaturase in rats by feeding them a high carbohydrate fat free diet. The diet was then supplemented with 5% (w/w) corn oil which contains a high proportion of linoleic acid. The activity of hepatic stearoyl-CoA decayed in less than 12 hours, that is within the diurnal feeding pattern of the rat, whilst the activity of fatty acid synthetase only decayed after 2 to 3 days, suggesting that the important control exerted by dietary linoleic acid is at the level of fatty acid desaturation and not synthesis (Oshino & Sato, 1972). Further studies using 14C-labelled acetate and stearate in isolated rat hepatocytes indicate that the rate limiting step in the formation of monounsaturated fatty acids from acetate is the desaturation of the saturated fatty acid (Jeffcoat et al, 1979b). It was also shown in this study that a linoleic acid rich diet decreased overall triacylglycerol production and stearoyl-CoA desaturase activity to a virtually identical degree, thus confirming the important role of fatty
acid desaturation in the overall synthesis of lipid by the liver. In summary then dietary carbohydrate stimulates hepatic lipogenesis, saturated fat inhibits fatty acid synthesis and linoleic acid controls the enzyme levels. Thus, dietary linoleic acid controls both the desaturation of saturated fatty acid and fatty acid synthesis. In short, linoleic acid is the active dietary component controlling lipid metabolism.

Conclusions

The major mammalian fatty acids are palmitic (16:0) and stearic acid (18:0) which are saturated, palmitoleic (16:1) and oleic acid (18:1) which are monounsaturated and linoleic (18:2), linolenic (18:3) and arachidonic acid (20:4) which are polyunsaturated. These long chained aliphatic carboxylic acids are important components of cell membrane phospholipids and provide an efficient method of energy storage as triglycerides. Arachidonic acid is the immediate precursor molecule of the prostaglandins.

Although many fatty acids are synthesised de novo, certain polyunsaturated fatty acids cannot be synthesised by man and these essential fatty acids must be taken in the diet. One of the major factors regulating overall lipid metabolism is dietary fat intake. The most important controlling influence is dietary linoleic acid and this exerts its effect by influencing fatty acid desaturation.
Chapter 4
MATERIALS AND METHODS

This chapter is divided into three sections. The first part describes the methods used to obtain cell membranes from tissue specimens and the subsequent processes used to extract the fatty acid content of those cell membranes. The second part provides a general account of the principles behind gas liquid chromatography and then a description of the specific protocol used for the analysis of fatty acids by this technique. The third section describes the results of a study of the validity of the methods used.

Cell membrane preparation and fatty acid extraction

Preparation of erythrocytes from whole blood (Human studies)

Red blood cells were washed and lysed using the method of Rose and Ocklander (1965). After collection blood samples were left to stand on ice for 90 minutes and then centrifuged (1500g for 15 minutes at 4°C) to separate the cells from the plasma. The plasma and buffy coat were discarded and 4 ml of the packed red cells were pipetted into Sterilin universal tubes. 20ml of ice cold 0.9% saline were added to each sample, the cells were then mixed gently and centrifuged (1500g for 12 minutes at 4°C). This washing was repeated twice more. Duplicate 1ml
samples of the washed erythrocytes were transferred to 30 ml glass
stoppered tubes and 1ml distilled water was added to each tube. The
samples were mixed gently and left on ice for 15 minutes.

Preparation of erythrocyte ghosts (Animal studies)

A modification of the methods of Steck and Kant (1974) and Niggli
et al (1981) was used to prepare red cell ghosts from the blood of
experimental animals. 5ml of blood was drawn into EDTA bottles and
then centrifuged (2000g for 10 minutes at 4°C) to separate the red
cells from the plasma and buffy coat which were then discarded. The
pellet of red cells was washed twice with buffer solution (130mM KCl,
20 mM Tris, pH 7.4) and centrifuged (5000g for 10 minutes at 4°C) to
recover the pellet. The erythrocytes were haemolysed with 2mM EDTA,
1mM Tris buffer (pH 8.0) and mixed for 5 minutes. The lysed cells were
then centrifuged (22,000g for 10 minutes at 4°C) and the supernatant
haemoglobin carefully aspirated to leave the erythrocyte membranes.
These red cell 'ghosts' were washed twice in the lysis buffer and twice
in 10mM Tris buffer (pH 7.4), the washing solution being removed in
each case after centrifugation (22,000g, 10 minutes, 4°C). The washed
erthrocyte ghosts were then resuspended in a small volume of 10mM Tris
buffer, frozen in liquid nitrogen, and stored at -70°C until required
for analysis.
Adipose tissue preparation

Between 0.1-0.5 g of adipose tissue was placed in 5 ml 0.15M NaCl, 10 mM Tris-HCl buffer at pH 7.8 (Lopes et al 1973). The sample was then homogenised using a Polytron homogeniser (Kinematica) at a speed setting of 5 for approximately 10 seconds. Duplicate 1 ml samples were then placed in glass stoppered tubes and 1 ml of distilled water added.

Colonic mucosa and tumour cell membrane preparation

This was carried out using an adaptation of the methods of van Blitterswijk et al (1973) and Standring and Williams (1978). All procedures were performed at 4°C. Approximately 1 g of the tissue sample was cleaned of fat, cut into small pieces, placed in 5 ml 0.15M NaCl, 10 mM Tris-HCl buffer at pH 7.8 (Lopes et al 1973) and homogenised (Polytron homogeniser, speed setting 6 for 20 seconds). The homogenate was centrifuged at 14,000 g for 15 minutes. The pellet produced which contained nuclei, mitochondria and lysosomes, was discarded. The supernatant was centrifuged at 70,000 g for 60 minutes using a fixed-angle rotor in an ultracentrifuge (Centrikon). The supernatant was discarded and the crude membrane pellet was homogenised in a further 5 ml of NaCl-Tris HCl buffer (Polytron homogeniser, speed setting 5, 10 seconds). The homogenate was then layered onto 30 ml of 33 g/100 g sucrose solution (density = 1.15 g/cm) and centrifuged at 110,000 g for 15 hours using a swing-out rotor (Centrikon ultracentrifuge). The interface produced was fractioned with a Pasteur pipette, diluted to
30ml with the NaCl-Tris HCl buffer and spun at 70,000g for 60 minutes (Centrikon ultracentrifuge with fixed angle rotor), in order to wash the membrane preparation. The pellet containing cell membranes was then taken up in 1ml distilled water, placed in a glass stoppered tube and left to stand on ice.

The success of the method used to produce cell membranes from solid tissue samples was assessed by electron microscopic examination of the final membrane pellet. Plate 1 shows that the adopted method yielded a high quality, uncontaminated preparation of pure cell membranes.
Plate 1. Electron micrograph of the final membrane pellet prepared from a sample of human colonic mucosa.
Extraction of fatty acids from prepared tissue

The method of Rose and Oklander (1965) was used. After allowing the prepared tissue (erythrocytes, adipose tissue, mucosa or tumour) to stand on ice for 15 minutes, 11ml of propan-2-ol (spectrograde, Fisons U.K.) containing the antioxidant 9mg/ml 2-6-di-t-butyl-p-cresol (butylated hydroxytoluene), was slowly added to each sample. After standing on ice with occasional mixing for one hour, 7ml spectrograde chloroform was added, the sample was mixed and left on ice for a further hour. Samples of washed erythrocytes were then centrifuged (1000g for 10 minutes, at 40°C) in order to pellet the dark red clump of cells and the clear supernatant was decanted into a glass universal bottle. Adipose tissue samples were filtered to remove any pieces of undissolved tissue and the clear filtrate was collected into a glass universal bottle. Samples of mucosa and tumour did not require any centrifugation or filtration at this stage of the procedure. If the samples were to be methylated immediately, the solvent was evaporated to dryness in a water bath at 70°C under a stream of nitrogen. As an alternative, samples could be stored at -20°C and the solvent evaporated before methylation when required.

Saponification of fatty acids

This was performed using a modification of the method of Alexander et al (1985). The prepared tissue samples were placed in a glass universal bottle and 1.5ml 10% sodium hydroxide solution added with
1.5ml methanol containing 20nmoles/ml heptadecanoic acid as a fatty acid standard (Nishida et al 1987). The tubes were flushed with nitrogen and the tops screwed on lightly. Tubes were heated in a water bath at 60°C for 30 minutes (B. Thomas, personal communication).

**Methylation of fatty acids**

The GLC machine can only quantify fatty acid methyl esters (FAMES) and so after the preparation already described, fatty acids were methylated using one of the following methods:

**Method 1 (Human erythrocytes and adipose tissue)**

The method of Christie (1972) was used. 0.3ml of spectrograde hexane was added to each sample followed by 0.1ml 2M sodium methoxide in methanol. The tubes were mixed on a roller mixer for 5 minutes. A further 2.5ml of hexane and a spatula tip of anhydrous calcium chloride were added and the samples left at room temperature for one hour. The samples were filtered into glass tubes to remove the calcium chloride and the filtrate was then evaporated to dryness under a stream of nitrogen in a water bath at 70°C. Each sample was reconstituted in 200μl hexane, the tubes flushed with nitrogen, capped and stored at -20°C.
Method 2 (Human mucosa and cancer specimens; all animal tissues)

The method of Alexander et al. (1985) was used. Samples which had been saponified as described before were allowed to cool. 1ml hydrochloric acid was added to achieve a pH of 2-3. 4ml of boron trichloride was added to each tube and the samples were flushed with nitrogen and heated in a water bath at 60°C for 15 minutes. After the tubes had cooled, 2ml saturated sodium chloride and 10ml hexane-diethyl ether were added (1:1 by volume, the diethyl ether containing butylated hydroxytoluene as an antioxidant). The tubes were mixed on a roller mixer, the two phases allowed to separate and the top organic layer removed into a separate tube. This extraction procedure was repeated with two further 10ml volumes of solvent and the extracts of each sample combined. The samples were evaporated to dryness in the Gyrovap (heated vacuum centrifuge) and then redissolved in 0.5ml hexane.

Silica preparation of fatty acid methyl esters

Silica containing Sep-Pak cartridges (Waters Associates) were used for the purification step. The cartridges were washed with hexane containing 3% diethyl ether before use. The 0.5ml sample of fatty acid methyl esters was loaded into the cartridge and once it had soaked into the silica, it was eluted with 10ml hexane containing 3% diethyl ether and the anti-oxidant butylated hydroxytoluene. The eluted sample was evaporated to dryness in the Gyrovap, redissolved in 200 μl of hexane and stored under nitrogen at -20°C.
Choice of methods

A number of different methods were available for most of the steps described above. The actual method chosen in each case was one which had previously proven to be successful and reliable when used in the laboratories of the Departments of Medicine and Surgery in the University of Leicester (Clayton, 1989).

The techniques of Forstner et al (1968) and Schmitz et al (1973) for preparing cell membranes from colonic mucosa and tumours have also been assessed but were found to be inferior to those of van Blitterswijk et al (1973) and Standring and Williams (1978). Three methods of fatty acid methylation (Christie, 1972; Christie, 1982; Alexander et al, 1985) were assessed and all were found to be equally effective. The initial method used here (Christie, 1972) was chosen because it had been used previously but was changed to the newer method of Alexander et al (1985) in the animal studies because of its greater simplicity. Similarly, silica preparation using Sep-Pak cartridges was chosen as the method of purification over thin layer chromatography and silica columns because it was a simple method which produced results which were equivalent to the other methods (Clayton, 1989).
Membrane fatty acids were separated, identified and quantified by gas liquid chromatography (GLC). This is a special form of general chromatographic technique in which the sample to be analysed is carried by a gas stream (the mobile phase) down a column which contains a liquid of low volatility held upon an inert support (the stationary phase). The components of the sample distribute between the mobile gas phase and the stationary liquid phase and are eluted from the column at different rates according to their chemical nature (length of carbon chain in the case of fatty acids). The separated components of the sample are monitored at the outlet of the column by a suitable detector.

The basic components of a gas chromatograph are: a carrier gas supply, a chromatographic column, a thermostatically controlled oven, a detector device and a recording system (Figure 4.1).

The carrier gas is delivered from a pressurised cylinder and passes at a constant rate through an injection block and along the column. Nitrogen, argon and helium are in common use as carrier gases because of their chemical inertness.

There are two broad types of GLC column, the packed column and the open tubular column. These can be made of a variety of materials including glass, plastic, copper, aluminium and stainless steel. The length of the commonly used packed column is 1 - 3 metres and the internal diameter is 2 - 6 millimetres. The exact specifications of the column are modified according to the type of analysis for which it is required. The column is packed with material prepared by
impregnating finely divided particles (100-600 μm) of an inert porous solid support with an appropriate stationary liquid phase which forms 1-20% by weight. The most popular support materials are the diatomaceous earths. These have several desirable characteristics: large surface area per unit volume, chemical inertness, thermal stability, low adsorption activity, high mechanical strength and uniformity of particle size. Adsorption of solutes onto diatomite support material can adversely affect the consistency of results in GLC but this problem is greatly reduced by acid washing and treatment with silicon compounds.
The column is enclosed in a thermostatically controlled oven which is set at a temperature which is appropriate to the type of compounds under analysis. In relatively simple analyses, in particular fatty acid studies, adequate separation can be achieved under isothermal conditions. For more complex analyses temperature programming may be needed. The temperature of the oven is programmed to increase at a specified rate and this enables better separation of fast running components at the lower temperatures and the increased temperature achieved later speeds up the elution of the slower running components.

When using a standard packed column, the sample is dissolved in a suitable solvent and a volume of 0.1-5 µl is used for analysis. The sample is injected into the GLC machine and is flash evaporated in the injection block which is heated to a temperature 30-60°C above that of the column. The vapourised components of the sample are taken through the column by the stream of carrier gas. They are resolved into different zones according to their individual chemical characteristics and are eluted from the column progressively.

The detector device senses the presence of the sample components in the effluent gas and the response of the detector to each is recorded against time to produce the chromatograph. The best and most popular non-specific detection system in GLC is the flame-ionisation detector. This works on the principle that the introduction of an organic compound into a flame increases the electrical conductivity. In the flame-ionisation detector, hydrogen is mixed with the effluent gases from the chromatographic column and is burnt in an atmosphere of air. For optimum performance, the shape and size of the flame is critical and this is determined by the relative proportions of
hydrogen, nitrogen and air in the mixture. The ions produced in the
flame yield a current when a voltage is applied between the flame,
which acts as one electrode, and a second electrode situated directly
above or around the flame. The ion current is passed through a
resistor and the voltage across the resistor is amplified and measured.
The response of the detector is recorded against time to give a
graphical representation for the sample under study. The detector
response for organic compounds is directly proportional to the carbon
content of the compound although functional side chains also have an
effect. In the separation and identification of fatty acids, molecules
with short carbon chains are eluted from the column before those with
longer carbon chains and for a given length of carbon chain, saturated
molecules are eluted before unsaturated ones. Hence, the order of
elution for the five major cell membrane fatty acids is: palmitic
(16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and arachidonic
(20:4).

The time between sample injection and the elution of a particular
fatty acid is called the retention time of that fatty acid. If GLC is
performed under standardised conditions using an appropriate column,
all the major membrane fatty acids can be separated. The component
fatty acids of a mixture can be identified by comparison of their
retention times with those of known purified fatty acid standards.

The GLC system described can also be used to quantitate fatty
acids. The total amount of any component in a separated mixture is
proportional to the detector response integrated over the time taken
for that component to pass through the detector and is obtained by
measuring the area under the relevant chromatograph peak. This value
can be obtained electronically by an integrator accessory which is interfaced with the GLC machine.

In summary, GLC provides a highly reproducible means of separating, identifying and quantitating the fatty acid composition of cell membranes. The specific details of the GLC machine and operating conditions used for the work in this thesis will now be described.

**Fatty Acid Analysis by Gas Liquid Chromatography**

The GLC machine used in the studies presented here was a Perkin-Elmer F17 fitted with an on-column injector and a flame ionisation detector. Analysis of fatty acid methyl esters (FAMES) was performed using two different columns. The first was a 7.3 mm diameter 2 metre glass column packed with 15% diethylene glycol succinate on Chromosorb W (100-120 mesh, supplied by Perkin-Elmer), heated isothermally at 190°C with nitrogen as the carrier gas. The analytical conditions for this column were as follows: carrier gas supply pressure 500kNm⁻², inlet pressure 140kNm⁻², injection port temperature 250°C. The second column was a 3.6 mm diameter stainless steel type, packed with 15% CP-Sil 84 on Chromosorb WHP (100-120 mesh, supplied by Chrompack UK). The running conditions were the same as for the first column except that the inlet pressure was set at 100kNm⁻². The GLC was interfaced with a Shimadzu integrator which was programmed to measure the area under each peak. Injection of 2 µl samples was by means of a 5µl microsyringe fitted with a silica quartz needle. After injection, the sample was allowed to run for approximately 45 minutes. Fatty acid methyl esters
were identified by comparing the retention times with those of authentic standards (Sigma, Poole). The major fatty acids detectable using this system were palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, n-9), linoleic acid (18:2, n-6) and arachidonic acid (20:4, n-6) (Figure 4.2). Several other minor fatty acids could be detected in some samples, but as their separation and quantification could not be reliably reproduced, they were not considered in the further analysis. The area under the peak of each fatty acid was expressed as a proportional value of the total area of all the major fatty acids in percentage terms.
Figure 4.2. Gas liquid chromatograph of fatty acids from human erythrocytes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Retention time (mins)</th>
<th>Area under peak (µV per second)</th>
<th>Relative % area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>5.62</td>
<td>236699</td>
<td>26.82</td>
</tr>
<tr>
<td>Stearic</td>
<td>10.31</td>
<td>167318</td>
<td>18.96</td>
</tr>
<tr>
<td>Oleic</td>
<td>11.85</td>
<td>157588</td>
<td>17.86</td>
</tr>
<tr>
<td>Linoleic</td>
<td>14.82</td>
<td>96462</td>
<td>10.93</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>34.69</td>
<td>224352</td>
<td>25.43</td>
</tr>
</tbody>
</table>
Validity study

Clearly the methods described can only be used for the quantitative assessment of membrane fatty acid profiles if they are sufficiently reproducible. A validity study was carried out with the aim of establishing the precision of the methods of membrane preparation and fatty acid extraction and of fatty acid quantitation by gas liquid chromatography itself.

Materials and methods

Blood from a healthy young volunteer, colonic mucosa from a section of sigmoid colon which had been resected because of diverticular disease and adipose tissue removed from the abdominal wall at the same laparotomy were used in this study. The samples of blood, adipose tissue and colonic mucosa were divided into ten equal aliquots and each of these was treated as an independent sample. Erythrocyte cell membranes were prepared using the method of Rose and Ocklander (1965) and colonic mucosal cell membranes were prepared using the methods of van Blitterswijk et al (1973) and Standring and Williams (1978). Fatty acids were extracted as described above and methylated using the boron trichloride method of Alexander et al (1985). Fatty acid methyl esters (FAMES) were identified and quantified using the Perkin-Elmer F17 GLC machine. The ten duplicate samples were used to assess imprecision arising from the methodology. The imprecision of the GLC quantitation was assessed by running a single sample of FAMES
from each of the three tissues under study (erythrocytes, adipose tissue and colonic mucosa) through the machine ten times. Taylor et al (1987a) have demonstrated that only five fatty acids can be measured reproducibly in erythrocyte membranes even when using the most up to date capillary GLC machinery. The five fatty acids were palmitic, stearic, oleic, linoleic and arachidonic acids and these are known to make up at least 75% of erythrocyte fatty acids (Muskiet et al, 1983). As far as erythrocyte and colonic mucosal cell membranes are concerned, these five major fatty acids were, therefore, the ones studied in this validity experiment. For adipose tissue samples the arachidonic acid peak was often difficult to identify and so this was excluded at the expense of 11-eicosanoic acid which could be qualitatively identified on all adipose tissue samples. All results were expressed as mean values together with their coefficients of variation (CV).

Results

The imprecision due to the extraction and methylation procedures was acceptable with coefficients of variation in the order of 10% or less for palmitic, stearic, oleic, linoleic and arachidonic or 11-eicosanoic in erythrocytes, adipose tissue and colonic mucosa. The measurement of oleic acid in colonic mucosa proved to show the most variation with a coefficient of variation of 11.1% (Table 4.1). The imprecision due to the gas liquid chromatographic technique was also low and in all three tissues the coefficients of variation were less than 10% for each of the fatty acids considered (Table 4.2).
Table 4.1. Imprecision due to the methods of sample preparation
(Single tissue sample divided into 10 aliquots and processed)

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Adipose tissue</th>
<th>Colonic mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>CV (%)</td>
<td>Mean</td>
</tr>
<tr>
<td>Palmitic</td>
<td>25.8</td>
<td>5.3</td>
<td>23.3</td>
</tr>
<tr>
<td>Stearic</td>
<td>17.0</td>
<td>4.8</td>
<td>5.0</td>
</tr>
<tr>
<td>Oleic</td>
<td>20.5</td>
<td>7.9</td>
<td>49.9</td>
</tr>
<tr>
<td>Linoleic</td>
<td>11.4</td>
<td>5.6</td>
<td>10.2</td>
</tr>
<tr>
<td>11-eicosanoic</td>
<td>-</td>
<td>-</td>
<td>1.9</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>24.2</td>
<td>8.0</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.2. Imprecision due to the gas liquid chromatographic method
(Single sample of FAMES run 10 times)

<table>
<thead>
<tr>
<th></th>
<th>Erythrocytes</th>
<th>Adipose tissue</th>
<th>Colonic mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>CV (%)</td>
<td>Mean</td>
</tr>
<tr>
<td>Palmitic</td>
<td>27.1</td>
<td>3.1</td>
<td>23.6</td>
</tr>
<tr>
<td>Stearic</td>
<td>18.1</td>
<td>5.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Oleic</td>
<td>18.2</td>
<td>4.7</td>
<td>48.2</td>
</tr>
<tr>
<td>Linoleic</td>
<td>13.8</td>
<td>7.1</td>
<td>9.0</td>
</tr>
<tr>
<td>11-eicosanoic</td>
<td>-</td>
<td>-</td>
<td>1.3</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>22.1</td>
<td>6.1</td>
<td>-</td>
</tr>
</tbody>
</table>
Conclusion

The results of this study suggest that the methods used yield reproducible results and serve to establish the validity of the assessment of membrane fatty acid profiles by gas liquid chromatography.
In the human studies presented in Chapter 6, the intake of various nutrients was estimated by a dietary recall method. The aims of this chapter are to describe the available methods of collecting dietary data and to critically consider their validity.

The methods of studying human dietary intake

Individual dietary intake of food and nutrients can be measured in three broad ways:

1) The collection and analysis of duplicate diets

In this method subjects collect and weigh duplicate portions of everything that they eat and drink over a specified period which is usually not less than one week. The total fluids and solids for each day are combined, homogenised and then frozen, freeze-dried and then stored in sealed containers under nitrogen, usually at -40°C. Aliquot samples may then be analysed for the dietary content of nutrients (Broadhurst et al, 1987a). For fatty acids this would involve the extraction of all fatty acids using an organic solvent, methylation and then separation and quantitation possibly by gas liquid chromatography.
2) The Use of biological markers of food consumption.

This can be any biochemical measurement which is related in a predictable way to a given dietary component. For the study of fat intake, it was thought that the measurement of faecal bile excretion might be useful (Cummings et al, 1978). Unfortunately there is a wide variation in the response of individuals to a standard fat intake (Bell et al, 1981) and so this method has not been adopted. However, the analysis of adipose tissue fatty acid composition has been shown to be a reliable indicator of habitual fatty acid consumption by groups of subjects (Beynen et al, 1980) and also for individuals (Plakke et al, 1983). Indeed because adipose tissue has such a slow turnover rate its fatty acid composition reflects that eaten in the diet over the previous three years (Beynen et al, 1980).

3) The use of food composition tables.

In this method a subject's intake of food is estimated by one or more of a combination of techniques which include weighing, food diaries, questionnaires, dietary recall and dietary histories. Nutrient intake is then calculated by using food composition tables. The standard UK food composition tables used for this purpose are those of Paul & Southgate (1978), supplemented by data from Wiles et al (1980) and unpublished data from the Dunn Nutrition Unit (93 foods, mainly brand name products). Additional data on fatty acid composition is provided by Paul, Southgate & Russell's supplement to McCance and Widdowson's 'The Composition of Foods' (1980).

As stated above there are a variety of methods for estimating the
consumption of food before reference to the food tables can be made. These methods are as follows:

a) Prospective records of food consumption with weights of foods. In these surveys the subjects are asked to weigh, describe and write down all items of food and drink that are taken in one week (Widdowson, 1936; Widdowson & McCance, 1936). This is called the individual method and important features of it are that cooked, rather than raw, food is weighed using a spring balance accurate to \( \pm 1/4 \) oz. Any edible food left over is also weighed. A refinement of this method is to weigh food which is ready for eating onto a plate. This 'weighing by addition' causes minimal disruption to the subjects routine (Marr, 1961; Marr 1965).

b) The daily (24 hour) recall of dietary intake. This method was first used by Wiehl (1942). It is a semi-quantitative dietary history of all food consumed at and between meals over a 24 hour period. Quantities are stated in ordinary units, for example, one glass of milk, two slices of bread, one medium sized potato. To assist the subject models of measured quantities of several foods can be displayed at the interview as standards of reference.

c) The diet history
The main aim of this type of study is to make an assessment of the habitual food intake. In the original description of the dietary history (Turner, 1940), three devices were used to obtain an impression of the intake of food. These were firstly a typical menu from the
subject of foods used in the morning, afternoon, evening and between meals. Secondly, a food intake list of some one hundred or so foods which was worked through with the subject to establish how often and how much particular foods were eaten and also how the recipes were prepared. Thirdly, an account of food purchases together with the costs of these was used as an indicator of amounts. This method has been improved over the years and the detailed information obtained by an experienced dietitian can be converted into nutrient intakes using food tables.

d) Questionnaires
A number of questionnaires have been developed, either for completion by subjects themselves or by lay interviewers in a short interview. Quantities are estimated by using either models (Epstein et al, 1970) or photographs (Hankin et al, 1975) to assess portion size.

The reliability of dietary data

It is conventional to assess the accuracy of data in terms of a perfect standard which is considered to be flawless. The problem with dietary data is that no gold standard is available and so validity can only be gauged by comparing the results of the different imperfect methods of dietary assessment. The correspondence among flawed indicators is more correctly called reliability rather than validity.

The collection and analysis of duplicate diets is probably the best method of dietary intake assessment. It is, however, very
expensive and only a small number of subjects can usually be studied. There are, of course, potential sources of error even with this method and in particular its accuracy is dependent on the collection of exact duplicate samples of all food and drink. With reference to fatty acids, the data obtained can also only be as accurate as the method of fatty acid analysis, for example, gas liquid chromatography.

The use of biological markers of dietary intake has many attractions. It has the potential to be more reliable and accurate than the more general dietary assessment methods because it cannot be affected by recall bias. Although it has been suggested that subcutaneous adipose tissue biopsy is no more invasive than venepuncture (Beynen, 1982), this method is too intrusive for large scale population studies. The technique can, nonetheless, be easily employed in patients who already need a surgical procedure requiring incision of the skin. A small biopsy of the order of 1 gram by weight is sufficient for analysis and this can be obtained without any increase in morbidity.

If large numbers of people are to be studied, dietary intake measurements which use food composition tables to calculate the nutrient intake are the most practical approach. Unfortunately, these methods rely on reporting by individuals and are subject to several sources of error.

All results from dietary surveys are ultimately dependent on the accuracy of food composition tables. These tables do not always contain codings for exactly the same list of foods provided by the dietary method. This problem is usually countered by pooling or averaging of the values from different foods and this is a source of
random errors. This type of error can be reduced by increasing the number of observations but this may not be practical. A further problem arises from the way in which the codings of the nutrition content of foods are estimated. These values are derived either from a review of the literature on chemical analysis of food or from ad hoc analyses. The same item of food may vary considerably in its chemical composition depending on the geographical origin (soil conditions, climate and use of fertilisers), the sampling procedure (fresh, frozen or raw) and the treatment of the sample before analysis (Paul & Southgate, 1978). These factors are well demonstrated by the considerable inconsistencies between food tables from different countries.

Food composition table data is however being standardised and improved all the time. The reliability of calculating fatty acid intake from dietary data has recently been substantially improved by Broadhurst et al (1987a). They recoded fried and roasted foods using a technique adapted from that of Fehilly et al (1984). This involved calculating the weight of fat taken up by foods in the cooking process and using this data, fried and roasted foods were given two codes, one for the food without added fat and the other an appropriate quantity for the fat itself. In addition, they analysed the fat content of 20 commonly eaten (mainly brand name) foods for which no fatty acid data were available. Where necessary manufacturers were contacted for information on the composition of the oils and margarines used in their production techniques. The fats involved were also analysed by a standard technique (Broadhurst et al, 1987b). The data obtained by these techniques were used to produce an improved database for fatty
acid composition. The standard database was compared to the updated version in a study of 11 subjects. They collected weighed duplicate diets and food diaries for 16 consecutive days. Fatty acid consumption was then calculated from the food diary data using either standard food table data alone (Paul & Southgate, 1978; Wiles et al, 1980) or these standard tables supplemented by the additional database. The results were compared with the fatty acid profiles of the duplicate diets which were measured by gas liquid chromatography. The updated method yielded improved correlation coefficients for all fatty acids except linolenic acid and the differences between the calculated and analysed total polyunsaturated fatty acids was not different. Similarly, the calculation of total monounsaturated fatty acids was improved by the new method. A further important finding in this study was that the correlation coefficients for linolenic acid were very low using any method and it seems that for this fatty acid calculation using food tables is an inadequate method of assessing intake.

The reporting of dietary intake by individuals is a particularly important source of errors. Subjects may vary in their ability to describe what they eat, depending on their age, education and background. For example people who do no cooking may be unable to estimate the type and amount of fat used, even in simple recipes. Subjects need to be well motivated and if they are not they may intentionally or inadvertently oversimplify their dietary reporting in order to avoid tedious descriptions of long recipes. Furthermore, when studying hospital patients their illness may influence their ability to recall their dietary habits. 

In recall methods of dietary assessment, there is the added
problem of subjects having to remember what they ate over some specified period. Young et al (1952a) found that the differences between the average results obtained by the 24 hour recall method and by a seven day estimated food record were less than 10%. However, other studies have suggested that the errors are greater than this. Campbell & Dodds (1967) found that 12 to 35% of daily food intake may be forgotten by institutionalised subjects and overall average energy intake was underestimated by 21% in another study, even though subjects weighed their food before hand and used a questionnaire in order to assist their memory (Acheson et al,1980). Direct questioning is however known to improve the accuracy of recall (Marr, 1971).

Methods which use the prospective recording of dietary data by subjects have a different problem. When subjects are asked to keep a record of what they eat there is always the possibility that they will alter their normal dietary habits either to simplify the task or because they become overconscious about their dietary intake. Others may alter the amount and type of food consumed in order to impress the investigators. The weighing of food as part of a dietary method certainly appears to alter intake with patients showing a tendency to reduce their intake (Trulson & McCann, 1959).

The coding or calculating stage required in the use of food tables may be another source of error due to either mistakes or difficulties in interpretation. Whiting and Leverton (1960) cite some unpublished data of Brewer in which the nutrient composition of 21 weighed diets was calculated by 8 different nutritionists using the same food tables. There was a 10% minimum to maximum range in the energy values obtained and a 24% minimum to maximum range in the values obtained for protein.
A study of 60 dietary records obtained at interviews in Toronto showed that the coefficients of variation from errors arising purely from coding were 3% for protein, 8% for total fat and 17% for the polyunsaturated: saturated fat ratio (Beaton et al, 1979). These errors occurred despite the weights of food being known and are due to inadequate description of foods.

In the study methods which require the subject to weigh their food before eating it, the accuracy of the weighing device used obviously has a bearing on the overall accuracy. The precision of the spring balances commonly used in dietary surveys is rarely better than ±5 grams and there is therefore some measurement error with their use.

Variations in dietary intake which occur with time become important when study over a short period of time is used as a yardstick of habitual diet. Individual food intake is known to vary quite markedly from day to day. Errors arising from this can be reduced by increasing the study period but this is limited by the longest time that a subject can be expected to co-operate. It has been suggested that one week is a reasonable time period over which to study an individuals dietary intake (Widdowson & McCance, 1936) and subjects appear to be dependable over this sort of time scale (Wait & Roberts, 1932).

The dietary history appears to have an advantage over the recording or 24 hour-recall methods in that if all the information can be obtained at one interview, then all errors associated with time would be eliminated at one stroke. However, the method includes errors in the amount of food eaten, reporting error, coding errors and food tables and also, the subject has to remember how often many different
items of food are eaten. There are 13 studies which compare dietary histories with records of food intake. Seven of these have shown a positive bias (Morgan et al, 1978; Huenemann & Turner, 1942; Young et al, 1952b; Vanden Berg & Mayer, 1954; Jain et al, 1980b; Lonergan et al, 1975; Den Hartog et al, 1965), four have shown negative bias (Hart & Cox, 1967; Young et al, 1952c; Black, 1981; Rasanen, 1979) and two no difference (Trulson, 1954; Lubbe, 1968). Overall therefore dietary histories seem to overestimate consumption when compared with records of food intake. Dietitians who are experienced in the use of the dietary history recognise that patients tend to overestimate how often they eat particular foods and so can to a certain extent compensate for this. Studies of the repeatability of dietary histories have also been performed. Church et al (1954) found differences ranging from 1 to 9% in a comparison of the food intakes obtained by two or three dietitians studying three different populations. In studies which compare the results of two dietary histories obtained by the same interviewers, correlation coefficients of between 0.6 and 0.9 have been obtained (Marr et al, 1959; Rasanen, 1979; Dawber et al, 1962). There are, however, serious difficulties in interpreting these studies because the repeat interviews were conducted after an interval of up to 2 years and clearly there may have been a real change in diet during this time. Kolonel et al (1977) used evidence given by spouses to judge the reliability of dietary history data. They found substantial agreement between the dietary intake reporting of male subjects and what their wives reported that they ate. It has also been shown that habitual food items tend to correlate well on repeat questioning (Nomura et al, 1976).
The validity of the dietary history method appears to vary with the exact nutrient under study. Jain et al (1980b) obtained food records from 16 university staff in a meticulous fashion over a period of 30 days. After the records had been completed, each subject gave a dietary history, the purpose of the investigation being to validate the dietary history with the food records already obtained. Regression coefficients were significant for 7 of the 13 nutrient studied. There was good correlation between the two methods for the estimation of total fat, saturated fat, animal fat and oleic acid.

The experience and skill of the interviewer is especially important to the accuracy of the dietary history method. Great detail is required, for example the fat content per 100 grams of beef is dependent on the cut of meat, varying from 5 grams for silverside to almost 30 grams for foreribs (Paul and Southgate, 1978). This is a straightforward example given to illustrate the point but there are many subtle nuances in the taking of a dietary history and a great deal of interviewer experience is required for the method to be reliable.
Conclusions

The measurement of human dietary intake is difficult. The collection and analysis of duplicate diets is the best method but it is too expensive and complicated to be used with large numbers of subjects. The calculation of nutrient intake using dietary histories and food consumption tables is a convenient and practical method and the reliability of this method for assessing fatty acids has recently been substantially improved. The analysis of adipose tissue fatty acid content has been shown to be a reliable indicator of habitual fatty acid intake over the previous three years.
Chapter 6

ERYTHROCYTE MEMBRANE FATTY ACIDS IN HUMAN COLORECTAL CANCER

It has been previously reported that patients with various solid malignancies have an altered erythrocyte membrane fatty acid composition (Wood et al., 1985). These authors found that the ratio of stearic to oleic acid in the red cells was reduced in patients with cancer in comparison with controls. They suggested that the reduced 'saturation index' was a powerful diagnostic marker of malignancy.

This work has been criticised on the grounds that the control population was poorly matched in several ways (Soreide et al., 1987). Furthermore, the influence of the dietary fatty acid intake was not studied. The principal aim of the present study was to assess the erythrocyte membrane fatty acid profile in patients with colorectal cancer using a properly matched control group. The potentially important influence of dietary fatty acid intake on the erythrocyte membrane fatty acid composition was also studied. A secondary aim of the study was to assess the validity of the stearic to oleic acid ratio (saturation index) as a diagnostic marker.
Patients and methods

The colorectal cancer group consisted of forty-nine patients admitted for elective surgery to Leicester Royal Infirmary or Leicester General Hospital (Table 6.1). There were 30 men and 19 women with a mean age of 68.8 years (range 49-92 years). Forty-two of these were admitted for elective resection of primary tumours and the other seven patients had clinically recurrent colorectal cancer following previous resection. Of the primary tumours, 11 were Dukes stage A, 20 were Dukes B and 11 were Dukes C adenocarcinomas. Patients presenting as emergencies with obstruction, perforation or bleeding requiring blood transfusion were deliberately excluded.

The control group consisted of forty-nine patients admitted for elective surgery, for benign diseases for example varicose vein surgery or abdominal wall hernias (Table 6.2). These patients were admitted at the same time as those with cancer and were matched for age and sex. The mean age of the 30 male and 19 female control subjects was 69.7 years (range 48-90 years).

None of the patients in the study had diabetes mellitus, a lipid metabolic disorder or an acute medical condition. Those on special diets (for example a low fat diet) were specifically excluded and all of those studied were caucasian. Patients undergoing surgery for obstructive jaundice were also excluded because of the possible effects of altered hepatic metabolism upon lipid profiles.
Table 6.1: Details of patients in the malignant group

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<td>72</td>
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<td>29</td>
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<td>Varicose veins</td>
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<td>M</td>
<td>61</td>
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<td>35</td>
<td>MS</td>
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<td>F</td>
<td>71</td>
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<td>M</td>
<td>77</td>
<td>Hernia</td>
<td></td>
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<td>MF</td>
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<td>48</td>
<td>Varicose veins</td>
<td></td>
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<tr>
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<td>AJ</td>
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<td>90</td>
<td>Rectal prolapse</td>
<td></td>
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<tr>
<td>41</td>
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<td>M</td>
<td>70</td>
<td>Haemorrhoids</td>
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<tr>
<td>42</td>
<td>GT</td>
<td>M</td>
<td>76</td>
<td>Hernia</td>
<td></td>
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<tr>
<td>43</td>
<td>SB</td>
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<td>81</td>
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<td>44</td>
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<td>Hydrocele</td>
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<td>45</td>
<td>SW</td>
<td>F</td>
<td>65</td>
<td>Hernia</td>
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<td>46</td>
<td>AA</td>
<td>M</td>
<td>75</td>
<td>Hernia</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>CS</td>
<td>F</td>
<td>86</td>
<td>Rectal prolapse</td>
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<tr>
<td>48</td>
<td>AD</td>
<td>F</td>
<td>73</td>
<td>Hernia</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>LB</td>
<td>M</td>
<td>55</td>
<td>Hernia</td>
<td></td>
</tr>
</tbody>
</table>
Dietary history

A seven day dietary recall history was obtained in hospital on the day before surgery. The patients were personally interviewed for 30 to 60 minutes by one of four experienced hospital dietitians with the aid of a detailed proforma. Where necessary, a close relative who lived with the patient was interviewed in order to complete and corroborate the information obtained. The interviewer was unaware of the primary diagnosis in each case. Food items were analysed using standard food codes as previously described in chapter 5 (Paul & Southgate 1978; Paul, Southgate & Russell 1980; Wiles et al 1980). Fatty acid intake was calculated using the recommended methods of Broadhurst et al (1987a). Fried and roast foods were given two codes as previously indicated (Broadhurst et al 1987a; Fehilly et al, 1984). Information regarding 18 additional food items was provided by the AFRC Food Research Institute, Norwich, England (Broadhurst et al 1987b; S G Wharf, personal communication). Manufacturers data were used for the most popular margarines. Manufacturers were also contacted for details of the fat content of various foods for which data was not available. These included crisps, fish fingers, oven chips, margarine and salad cream. A few items for which there is no data were coded as for a similar item for which data was available. In cases where a recipe contained one or more fats that were different from standard codes, the recipe was broken down and each component coded separately.

Details of each history were entered into an Apricot XI-10 computer and analysed using the microdiet programme (University of Salford, Department of Mathematics and Computer Science).
Analysis of fatty acids

Venous blood (10 ml) was drawn into EDTA coated tubes between 7.30 a.m. and 8.00 a.m. after an overnight fast. The blood samples were then stood in ice for exactly two hours before the red cells were separated from the other blood constituents. Subcutaneous fat samples were obtained from the anterior abdominal wall at the time of surgery, immediately placed in liquid nitrogen and then stored in a refrigerator at -70°C until analysed (usually at one week).

Fatty acids were extracted from erythrocyte and adipose tissue samples using the method of Rose and Oklander (1965). After saponification (Alexander et al, 1985) and methylation (Christie, 1972), fatty acid methyl esters were identified and quantified by gas liquid chromatography (chapter 4).

In each case only the major fatty acids were studied. In erythrocyte samples these were palmitic, stearic, oleic, linoleic and arachidonic acids but for adipose tissue, 11-eicosanoic acid was measured in place of arachidonic acid. Values of individual fatty acids were expressed as a percentage of the total of the five fatty acids in each case. All samples were analysed in duplicate and the mean values were entered into a mainframe computer for analysis. The dietary components and fatty acid values of the cancer and control groups were compared using the two-tailed Mann Whitney U test. Correlation coefficients were analysed for significance by using the unpaired t-test. Significance was taken at the 5% level.
Results

Dietary analysis revealed no significant differences in consumption of major dietary components between the two groups (Table 6.3). Analysis of 25 separate dietary fatty acids revealed significant differences in only three minor ones: median intake of butyric acid (4:0) in the control group was 0.71 g/day versus 0.018 g/day in the cancer group (p < 0.0001); for lauric acid (12:0) this was 1.14 g/day versus 1.56 g/day respectively (p = 0.0472); and for arachidic acid (20:0) this was 0.0002 g/day versus 0.181 g/day respectively (p < 0.0001).

Table 6.3 : Intake of major dietary components (erythrocyte study)

<table>
<thead>
<tr>
<th>Dietary item (per 24 hours)</th>
<th>Colorectal cancer group (n = 48)</th>
<th>Control group (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (KCal)</td>
<td>1776 (766 - 3493)</td>
<td>1806 (1109 - 3022)</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>15.6 (3.9 - 34.9)</td>
<td>16.4 (5.6 - 37.7)</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>68.1 (32.3 - 111.4)</td>
<td>67.8 (42.9 - 115.2)</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>199 (99.4 - 403.1)</td>
<td>209.1 (102.8 - 360.6)</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>74.6 (22.9 - 176.2)</td>
<td>82.9 (46.0 - 172.6)</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>41.2 (5.3 - 100.2)</td>
<td>43.2 (14.3 - 105.0)</td>
</tr>
<tr>
<td>Monounsaturated fat (g)</td>
<td>36.2 (4.7 - 76.4)</td>
<td>37.8 (15.2 - 73.3)</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>6.8 (1.1 - 23.3)</td>
<td>6.5 (1.6 - 25.1)</td>
</tr>
<tr>
<td>Polyunsaturated:saturated ratio</td>
<td>0.17 (0.04 - 0.52)</td>
<td>0.16 (0.04 - 0.97)</td>
</tr>
</tbody>
</table>

Values are medians (range)
The erythrocyte fatty acid profiles of cancer patients and their controls and a statistical analysis between the two are shown Figure 6.1. A higher proportion of oleic acid was found in cancer patients when compared to controls (p=0.041). Smaller differences in stearic and arachidonic acid were also shown but these did not reach statistical significance at the 5% level.

Individual values for the stearic to oleic acid ratio are shown in Figure 6.2. There was no difference in this ratio between the cancer and the control groups. There was no correlation between the Dukes stage and the 18:0/18:1 ratio and no difference in the ratio between patients with primary cancers and those with recurrent disease.

The relationship between age and erythrocyte stearic to oleic acid ratio for cancer patients and controls is shown in Figures 6.3 and 6.4 respectively. Statistical analysis of the ranked ages and stearic: oleic acid ratios (Pearson's rank correlation method) yielded a positive r value in both the cancer and the control groups but neither value was large enough to reach statistical significance at the 5% level.

The fatty acid composition of adipose tissue in the two groups is shown in Figure 6.5. Cancer patients had a higher proportion of 11-eicosanoic acid (p=0.034) than controls, but this was the only statistically significant difference between the two groups.
Figure 6.1. Fatty acid profile of human erythrocytes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cancer group</th>
<th>Control group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>25.5 (17.3-38.9)</td>
<td>26.1 (15.9-37.6)</td>
<td>0.418</td>
</tr>
<tr>
<td>Stearic</td>
<td>18.7 (13.6-23.4)</td>
<td>17.8 (14.1-20.7)</td>
<td>0.054</td>
</tr>
<tr>
<td>Oleic</td>
<td>21.1 (15.4-26.0)</td>
<td>19.9 (14.8-24.5)</td>
<td>0.041</td>
</tr>
<tr>
<td>Linoleic</td>
<td>11.4 (8.3-16.0)</td>
<td>11.4 (8.6-18.6)</td>
<td>0.943</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>22.5 (16.5-29.5)</td>
<td>24.0 (14.9-33.9)</td>
<td>0.0734</td>
</tr>
</tbody>
</table>

Values are median relative percentages (ranges)
p values calculated by two-tailed Mann Whitney U test
Figure 6.2. Human erythrocyte stearic:oleic acid ratios in cancer patients and their controls

<table>
<thead>
<tr>
<th>Patient subgroup</th>
<th>Stearic: oleic acid ratio (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer patients</td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>Control patients</td>
<td>0.91 ± 0.08</td>
</tr>
<tr>
<td>Primary cancer</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>Recurrent cancer</td>
<td>0.88 ± 0.13</td>
</tr>
<tr>
<td>Dukes A</td>
<td>0.91 ± 0.12</td>
</tr>
<tr>
<td>Dukes B</td>
<td>0.90 ± 0.06</td>
</tr>
<tr>
<td>Dukes C</td>
<td>0.93 ± 0.11</td>
</tr>
</tbody>
</table>
Figure 6.3. Relationship between erythrocyte stearic: oleic acid ratio and age in cancer patients

Figure 6.4. Relationship between erythrocyte stearic: oleic acid ratio and age in control patients
Figure 6.5. Fatty acid profile of human adipose tissue

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cancer group</th>
<th>Control group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>25.1 (19.7-31.1)</td>
<td>26.1 (18.3-35.7)</td>
<td>0.576</td>
</tr>
<tr>
<td>Stearic</td>
<td>6.2 (3.1-9.4)</td>
<td>5.7 (1.5-8.6)</td>
<td>0.207</td>
</tr>
<tr>
<td>Oleic</td>
<td>53.9 (48.6-59.2)</td>
<td>53.0 (46.3-63.4)</td>
<td>0.769</td>
</tr>
<tr>
<td>Linoleic</td>
<td>11.2 (6.2-21.6)</td>
<td>12.7 (5.6-20.7)</td>
<td>0.404</td>
</tr>
<tr>
<td>11-eicosanoic</td>
<td>2.5 (1.1-6.3)</td>
<td>2.0 (1.4-3.4)</td>
<td>0.034</td>
</tr>
</tbody>
</table>

Values are median relative percentages (ranges)
p values calculated by two-tailed Mann Whitney U test
The overall results of the correlation analysis examining the relationships between age and dietary red cell and adipose tissue fatty acids are shown in Table 6.4. Increasing age was associated with a decreasing consumption of polyunsaturated fats in both patient groups but was most marked in the colorectal cancer group (Figures 6.6 & 6.7). Whereas dietary intake was not correlated with red cell fats in any way in the control group, a positive correlation between dietary and red cell polyunsaturated to saturated fatty acid (P:S) ratio was observed in the cancer group. There was a significant correlation of dietary and adipose tissue P:S fats in both the control group (Figure 6.8) and the cancer group (Figure 6.9).

The results of the correlation analysis examining the relationships between individual fatty acids in the diet and tissue fatty acids are summarised in Tables 6.5 to 6.7. Dietary linoleic acid was strongly correlated with linoleic acid in red cells (Figures 6.10 & 6.11) and adipose tissue (Figures 6.12 & 6.13) in the control group but not in the cancer group. Similarly, a correlation was shown between linoleic acid in red cells and adipose tissue in the control group but not in the cancer group (Figures 6.14 & 6.15).
Table 6.4: Correlation coefficients between age and dietary, erythrocyte and adipose tissue fatty acids.

<table>
<thead>
<tr>
<th></th>
<th>Colorectal cancer group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Age versus diet</td>
<td>N = 48</td>
<td>N = 49</td>
</tr>
<tr>
<td>S</td>
<td>0.241</td>
<td>NS</td>
</tr>
<tr>
<td>M</td>
<td>-0.129</td>
<td>NS</td>
</tr>
<tr>
<td>P</td>
<td>-0.359</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U</td>
<td>-0.241</td>
<td>NS</td>
</tr>
<tr>
<td>M:S</td>
<td>-0.169</td>
<td>NS</td>
</tr>
<tr>
<td>P:S</td>
<td>-0.404</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U:S</td>
<td>-0.241</td>
<td>NS</td>
</tr>
<tr>
<td>Diet versus erythrocyte</td>
<td>N = 48</td>
<td>N = 49</td>
</tr>
<tr>
<td>S</td>
<td>-0.119</td>
<td>NS</td>
</tr>
<tr>
<td>M</td>
<td>0.307</td>
<td>NS</td>
</tr>
<tr>
<td>P</td>
<td>-0.051</td>
<td>NS</td>
</tr>
<tr>
<td>U</td>
<td>-0.119</td>
<td>NS</td>
</tr>
<tr>
<td>M:S</td>
<td>-0.134</td>
<td>NS</td>
</tr>
<tr>
<td>P:S</td>
<td>0.368</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U:S</td>
<td>-0.119</td>
<td>NS</td>
</tr>
<tr>
<td>Diet versus adipose tissue</td>
<td>N = 41</td>
<td>N = 34</td>
</tr>
<tr>
<td>S</td>
<td>-0.263</td>
<td>NS</td>
</tr>
<tr>
<td>M</td>
<td>0.113</td>
<td>NS</td>
</tr>
<tr>
<td>P</td>
<td>-0.139</td>
<td>NS</td>
</tr>
<tr>
<td>U</td>
<td>0.101</td>
<td>NS</td>
</tr>
<tr>
<td>M:S</td>
<td>-0.254</td>
<td>NS</td>
</tr>
<tr>
<td>P:S</td>
<td>0.340</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>U:S</td>
<td>0.387</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used

S = saturated fatty acids
M = monounsaturated fatty acids
P = polyunsaturated fatty acids
U = unsaturated fatty acids
Figure 6.6. Correlation of dietary P:S ratio and age in cancer patients

- $r = -0.404$
- $p < 0.05$

Figure 6.7. Correlation of dietary P:S ratio and age in control patients

- $r = -0.168$
- NS
Figure 6.8. Correlation between dietary and adipose tissue P:S ratio in colorectal cancer patients

\[ r = 0.340 \]
\[ p < 0.05 \]

Adipose tissue P:S ratio

Dietary P:S ratio

Figure 6.9. Correlation between dietary and adipose tissue P:S ratios in control patients

\[ r = 0.478 \]
\[ p < 0.05 \]

Adipose tissue P:S ratio

Dietary P:S ratio
Table 6.5: Correlations of individual fatty acids in the diet and erythrocytes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cancer group (n = 48)</th>
<th>Control group (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>-0.086</td>
<td>NS</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.214</td>
<td>NS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>-0.051</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.221</td>
<td>NS</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0.073</td>
<td>NS</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used.

Table 6.6: Correlations of individual fatty acids in the diet and adipose tissue

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cancer group (n = 41)</th>
<th>Control group (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>-0.245</td>
<td>NS</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>-0.046</td>
<td>NS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>-0.139</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.272</td>
<td>NS</td>
</tr>
<tr>
<td>11-eicosanoic acid</td>
<td>0.172</td>
<td>NS</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used.
Table 6.7: Correlations of individual fatty acids common to both erythrocytes and adipose tissue

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cancer group (n = 41)</th>
<th>Control group (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>-0.136</td>
<td>NS</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.229</td>
<td>NS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>-0.035</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.226</td>
<td>NS</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used
Figure 6.10. Correlation between dietary and erythrocyte linoleic acid content in colorectal cancer patients

Erythrocyte linoleic acid content (relative %)

$\begin{array}{c}
\text{Dietary linoleic acid content (relative %)} \\
\end{array}$

$r = 0.221$
NS

Figure 6.11. Correlation between dietary and erythrocyte linoleic acid content in control patients

Erythrocyte linoleic acid content (relative %)

$\begin{array}{c}
\text{Dietary linoleic acid content (relative %)} \\
\end{array}$

$r = 0.414$
$p < 0.02$
Figure 6.12. Correlation between dietary and adipose tissue linoleic acid content in colorectal cancer patients

Adipose tissue linoleic acid content (relative %)

Dietary linoleic acid content (relative %)

$r = 0.272$

NS

Figure 6.13. Correlation between dietary and adipose tissue linoleic acid content in control patients

Adipose tissue linoleic acid content (relative %)

Dietary linoleic acid content (relative %)

$r = 0.754$

$p < 0.001$
Figure 6.14. Correlation between erythrocyte and adipose tissue linoleic acid content in cancer patients

Adipose tissue linoleic acid content (relative %) vs Erythrocyte linoleic acid content (relative %)

$r = 0.226$
NS

Figure 6.15. Correlation between erythrocyte and adipose tissue linoleic acid content in control patients

Adipose tissue linoleic acid content (relative %) vs Erythrocyte linoleic acid content (relative %)

$r = 0.534$
p < 0.002
Discussion

Abnormalities in the fatty acid composition of erythrocytes from patients with malignancies was first reported by Wood et al (1985). They found a consistently lower ratio of stearic to oleic acid in patients with a variety of solid malignancies which included cases of colorectal cancer. This group of workers referred to the stearic:oleic ratio as the saturation index and found that this was always less than 1.0 in cancer patients and always greater than 1.0 in a reference group of healthy volunteers. In a further control group, consisting of hospital patients with benign diseases of the liver, pancreas, breast, and gastro-intestinal tract, the saturation index was greater than 1.0 in 35 out of 40 cases (87.5%). In the 18 cases of colorectal cancer studied the mean saturation index was 0.66 with a range of 0.28-0.96. There was a close correlation between this ratio and the Dukes stage of colonic cancers (Habib et al, 1986) and it was also reported that after curative resection the ratio reverted to a normal value of greater than 1.0 (Wood et al, 1985). Not surprisingly, these findings were greeted with some excitement as it appeared from the initial studies that the ratio would prove to be a new marker of malignancy which could be used in the diagnosis and follow up of both primary and recurrent malignant diseases. Indeed, the original findings suggested that when used as a marker of malignancy, the saturation index of human erythrocytes had a sensitivity of 100% and a specificity of 91.7%. The positive predictive value of the test would be 100% and the negative predictive value would be 92.3%.
The findings were attributed to a circulating desaturation factor of cell membrane fatty acids in cancer patients and a putative factor was isolated from the urine of these patients (Habib et al, 1987a). The normal metabolic flow results in the conversion of the saturated stearic acid to the unsaturated oleic acid by the enzyme delta9-desaturase. The saturation index reflects the activity of the enzyme and should perhaps more logically have been named the desaturation index.

A number of criticisms can be levelled at the original study. Firstly, the authors made no attempt to match for age or sex in the cancer patients and the reference/control groups. Soreide et al (1987) examined the erythrocyte stearic: oleic acid ratio in 20 patients with various solid malignancies, a reference group of 7 healthy hospital employees and a control group of 13 patients with a variety of non-malignant diseases. They found a significant negative correlation (r = -0.62, p < 0.01) between age and stearic: oleic acid ratio in the combined reference and control groups indicating the potential importance of age as an independent factor. No correlation was found between age and the saturation index in the present human study. However, the range of ages studied here was very different from those in Soreide's series where the mean age of the reference group was only 42 years and the range was 23–90 years. Secondly, the role of hepatic metabolism of fats should have been examined as more than half of the patients in the malignant group had tumour involvement of the liver and/or biliary tree. Thirdly, treatment modalities such as blood transfusion which might affect the saturation index were not mentioned (Metcalf et al, 1985). Fourthly, no attempt was made to assess or
control for dietary fat intake. This last point is relevant since
dietary fat is known to produce rapid alterations in cell membrane
fatty acid composition in humans.

For a tumour marker to be useful, it must be consistently negative
in healthy patients, that is it must be specific to the disease state
which it marks. In the erythrocyte experiment carried out in the
present study, the specificity of a stearic: oleic acid ratio of less
than 1.0 as a marker of malignancy would have been only 12.2%. This is
not surprising as other workers have shown that common non-malignant
conditions are associated with abnormalities in the erythrocyte
membrane fatty acid profile. Thus Ollerenshaw et al (1987) found a
higher oleic and arachidonic acid content and a lower linoleic acid
content in the erythrocytes from patients with essential hypertension.
Abnormal erythrocyte lipid composition has been noted in diabetic women
(Tilvis and Niettinen, 1985) and low erythrocyte stearic: oleic acid
ratios have also been reported in patients with diabetes mellitus
(Taylor et al, 1986). The latter group of workers have suggested an
alternative explanation to that of a humoral factor causing the
desaturation of erythrocyte fatty acids (Taylor et al, 1987a). The
stearic: oleic acid ratio in plasma is low (mean 0.25) so that these
two fatty acids have an opposite relationship to that in the
erythrocyte membrane and a concentration gradient exists between the
two. Exchange of fatty acids between plasma and erythrocytes has been
shown to occur in man (Reed, 1968) and is probably governed by some
form of metabolic control. It is possible that metabolically
compromised cells (e.g. in diabetes) cannot maintain their fatty acid
composition against the concentration gradient set up by the plasma.
This might cause an increase in the erythrocyte oleic acid content relative to stearic acid thus lowering the stearic: oleic acid ratio.

The present study used cancer and control patients who were carefully matched for age and sex and excluded individuals who may have had disturbed fat metabolism. Furthermore, the influence of dietary fat intake was considered as a further variable. The results have shown that the erythrocyte stearic: oleic acid ratio is of no value as a diagnostic aid either in patients with primary colorectal cancer or in those with recurrent cancer. A number of more recent studies carried out by other groups have also failed to reproduce the findings of Wood et al (1985). Thus, patients with bronchogenic carcinoma (Taylor et al, 1987a), lymphoma or hepatoma (Lawson et al, 1987), breast carcinoma (Thomas et al, 1988) and various solid cancers (Soreide et al, 1987) were not found to have a reproducibly lower stearic to oleic acid ratio than control subjects.

The methodology used in the various studies described has varied somewhat. This may be important especially with regard to the use of fresh or frozen erythrocytes. Thomas et al (1988) found that storage of erythrocytes at -20°C led to inconsistent losses of certain fatty acids, in particular arachidonic acid. Taylor et al (1987b) found that storage of erythrocytes in EDTA at +4°C increased the relative amount of oleic acid and decreased the stearic: oleic acid ratio, thus mimicking the changes reported in cancer patients. Some loss of arachidonic acid also occurred. Differences in the storage times of cases and controls could clearly lead to artefactual results.

In addition, the method described by Wood et al (1985) made no mention of the use of antioxidants. This is another potential source
of error as the inclusion of an effective antioxidant such as butylated hydroxytoluene is essential to avoid loss of polyunsaturated fatty acids which are highly susceptible to oxidation (Dodge and Phillips, 1966).

Another possible influence on the results of different studies is the timing of sample collection in relation to meals. In the present study all samples were taken from fasting individuals in order to remove the potential effects of recent fat ingestion. Thomas et al (1988) are the only other workers who have made an effort to standardise the timing of sample collection.

In summary, the current study demonstrates that the erythrocyte stearic: oleic acid ratio is of no diagnostic value in separating cases of colorectal cancer from controls. Recent studies of other malignant diseases have produced similar findings (Taylor et al, 1987a; Lawson et al, 1987; Thomas et al, 1988; Soreide et al, 1987).

In all the previous work in the field of cell membrane fatty acids in malignant disease no attempt has been made to assess or control for dietary fat intake. Variations in diet can influence the metabolism and composition of body fats (Hirsch et al, 1960; Farquhar and Ahrens, 1963; Dayton et al, 1966; Sanders et al, 1978; Clandinin et al, 1983) and one of the main aims of the current study was therefore to make an assessment of the effect that any dietary variations might have on the erythrocyte fatty acid profile.
Fat intake was assessed using a seven day dietary recall history method (Burke 1947; Marr 1971; Gerzovitz et al, 1978) and also by adipose tissue analysis (Hirsch et al, 1960; Beynen et al, 1980; Plakke et al, 1983). The discussion presented in chapter 5 makes it clear that the interpretation of data derived by dietary history taking presents many difficulties. Nevertheless, habitual food items tend to correlate well on repeat questioning (Nomura et al, 1976). Moreover, the direct interviewing method used in this work is thought to improve the accuracy of recall (Marr, 1971). An important advantage was the availability of four dietitians who were experienced in taking accurate dietary histories. The length of interview averaged 40 minutes and close relatives were often included in order to corroborate the details given. In addition, any inaccuracies due to the coding of specific food items were minimised by following the most recent recommendations of Broadhurst et al (1987a).

Analysis of dietary intake by recall failed to reveal any significant differences between the cancer and control groups in the erythrocyte study. This finding was not unexpected and was consistent with the results of other case-control studies (Committee on Diet, Nutrition and Cancer, 1982). Because the cases and controls were very closely matched any differences are likely to be very small and the technique of dietary recall although of proven accuracy is not sufficiently sensitive to reveal such small changes. Median energy intakes and individual dietary items were all lower than those reported in two recent surveys in Britain (Bingham et al, 1981; Fehilly et al, 1984), but this probably reflects the advanced age of the patients studied here. An interesting observation in both groups was the
inverse correlation between age and the consumption of unsaturated fats relative to saturated fats.

In contrast to erythrocytes, adipose tissue fatty acids in man reflect those dietary fatty acids which have been taken habitually over a period of up to three years (Hirsch et al, 1960; Dayton et al, 1966; Beynen et al, 1980). As with the dietary recall, no major differences were found between the cancer and control groups. The relative proportions of fatty acids found here are similar to those previously reported (Hirsch et al, 1960; Sanders et al, 1978; Riemersma et al, 1986), although the P:S were higher than those in a recent study of Scottish men (Wood et al, 1984). The significant correlations which were found between dietary and adipose tissue polyunsaturated, P:S and U:S fatty acids in the control group give some validity to the dietary history assessment technique used (Plakke et al, 1983). Also in the control group there were significant correlations between dietary linoleic acid and the relative percentages in erythrocytes and adipose tissue, as might be anticipated (Farquhar and Ahrens, 1963; Wood et al, 1984). In contrast, different patterns of association between dietary, erythrocyte and adipose tissue fatty acids were observed in the cancer group. In particular dietary and erythrocyte P:S ratios correlated and dietary linoleic acid did not correlate with either erythrocyte or adipose tissue linoleic acid. It is unlikely that the different associations found between the groups are due to a less accurate dietary history acquisition in the cancer group. This view is supported by the finding of a high correlation between erythrocyte and adipose linoleic acid in the control group, but not in the cancer group.
In the context of the current level of understanding in this area it is difficult to give a clear explanation for the unusual associations between dietary, erythrocyte and adipose tissue fats in the cancer patients. What is clear is that the findings here cannot be attributed to the desaturation factor of Habib et al (1987a). Significant weight loss (greater than 10% body weight) was only evident in four of the patients so that overt cachexia is not directly linked to these observations. Alterations in host fat metabolism in cancer patients is still poorly understood. Beck and Tisdale (1987) have detected lipolytic activity in NMR 1 mice transplanted with a colon adenocarcinoma (MAC 16) which produces extensive loss of body fat whilst the tumour burden is less than 1% of host weight. It is conceivable that such lipolytic activity can account for the small differences observed in the erythrocytes in the present study. Marked differences might be minimised by overriding homeostatic mechanisms responsible for maintaining the correct balance between saturated and unsaturated fatty acids (Gibson et al, 1984), thereby preventing any significant changes in membrane fluidity (Popp-Snijders et al, 1986).

In conclusion, no significant differences were observed in dietary fat intake or adipose tissue composition. The small differences in erythrocyte fatty acids, and the unusual associations between dietary, erythrocyte and adipose tissue fatty acids, might be indicative of altered host fat metabolism. This possibility needs further investigation.
Conclusions

The erythrocyte stearic to oleic acid ratio was not different in cancer and control patients. There was no correlation between this ratio and the Dukes stage of colorectal cancers and no difference between patients with primary cancers and those with recurrent disease. The erythrocyte stearic to oleic acid ratio is of no value as a tumour marker in cases of human colorectal cancer.

In this study there were no differences in the dietary intake of major nutrients between cancer patients and their controls. Cancer and control patients, however, demonstrated different patterns of association between dietary, erythrocyte and adipose tissue fatty acids. These findings may be due to disturbed fat metabolism in cancer patients.
Chapter 7

COLONIC MUCOSA AND TUMOUR CELL MEMBRANE FATTY ACIDS
IN HUMAN COLORECTAL CANCER

Following on from the studies of erythrocytes and adipose tissue, this second human study was performed in order to characterise the cell membrane fatty acid composition of colonic mucosa from patients with and without colorectal cancers and of colorectal cancer tissue itself. The relationship between dietary fat intake and colonic mucosal fatty acid composition was also investigated.

Patients and methods

Thirty-four patients with primary colorectal cancer were studied (Table 7.1). All these patients were recruited after admission to hospital for elective resection of malignant lesions of the colon and rectum. As before, patients presenting as emergencies with complications were specifically excluded. Only patients of caucasian origin were studied and none of those included had an acute medical condition, diabetes mellitus or a disorder of lipid metabolism. The study group consisted of 16 men and 18 women with a mean age of 69.9 years (range 55-88 years). There were 6 Dukes A, 16 Dukes B and 12 Dukes C tumours in this group.
A seven day dietary recall history was obtained in the immediate pre-operative period by the method described in the previous chapter. Samples of colorectal cancer and colonic mucosa were taken from the resection specimen immediately after removal. The cancer specimen was excised from one edge of the tumour and the colonic mucosa specimen was dissected from the underlying muscle at the proximal resection margin of the specimen. Both samples were snap frozen in liquid nitrogen and then stored at -70°C until analysed.

Thirty-four control patients without colorectal cancer were also studied (Table 7.2). These patients were from two separate groups: those with diverticular disease of the colon which required elective resectional surgery (n=10) and patients undergoing elective haemorrhoidectomy (n=24). Dietary histories were taken in the pre-operative period as before. In all of the patients with diverticular disease the operation performed was a sigmoid colectomy and colonic mucosa was removed from the proximal resection margin of the operative specimen at the time of surgery. Patients undergoing haemorrhoidectomy had a low rectal biopsy performed under direct vision during the operation. This was performed by excising a small strip of rectal mucosa using dissecting scissors and repairing the defect with a catgut suture.

In the diverticular disease control group there were 4 men and 6 women with a mean age of 70.5 years (range 50-82 years). The 12 men and 12 women in the haemorrhoid group had a mean age of 43.7 years (range 25-66 years). There was no significant difference in age between patients in the diverticular control group and those in the cancer group but the patients in the haemorrhoid control group were
Table 7.1: Details of patients in the malignant group  
(human mucosa and cancer study)

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</tr>
<tr>
<td>32</td>
<td>MJ</td>
<td>F</td>
<td>47</td>
<td>Hx</td>
<td>Rectum</td>
</tr>
<tr>
<td>33</td>
<td>GS</td>
<td>M</td>
<td>34</td>
<td>Hx</td>
<td>Rectum</td>
</tr>
</tbody>
</table>

DD = diverticular disease  
Hx = haemorrhoids
significantly younger than the cancer group (p < 0.001, Mann Whitney U test). The ages of the combined control group (51.6 years) were significantly lower than those of the malignant group (69.9 years) (p < 0.001, Mann Whitney U test).

Tissue samples were processed for cell membrane separation, fatty acid extraction and quantitation by the methods previously described (chapter 4). Results were expressed as relative percentage values of those fatty acids under study.

Results

The results of the analysis of dietary intake are shown in tables 7.3 and 7.4. There were no significant differences in the intake of major nutrients by patients in the diverticular and haemorrhoid control groups. These two groups were merged to form a single combined control group. There were no significant differences in dietary intake between this combined group and the cancer group.

Analysis of the dietary intake of the five major fatty acids showed a number of differences between the cancer and control groups (figure 7.1). Again the data from control patients has been presented as separate diverticular and haemorrhoid groups as well as a combined control group. The dietary intake of palmitic, stearic and linoleic acids was different in cancer patients and the combined controls. All the differences shown were due to the influence of the haemorrhoid controls because there were no significant differences between the cancer patients and the diverticular controls. This is confirmed by a
Table 7.3. Colonic mucosa and cancer tissue study:
Intake of major dietary components

<table>
<thead>
<tr>
<th>Dietary item (per 24 hours)</th>
<th>Colorectal cancer group (n = 26)</th>
<th>Combined control group (n = 26)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (KCal)</td>
<td>1833 (884 - 4136)</td>
<td>1861 (925 - 3330)</td>
<td>NS</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>14.9 (6.4 - 24.2)</td>
<td>18.7 (4.8 - 43.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>67.7 (30.5 - 113.4)</td>
<td>64.9 (34.8 - 107.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>191.6 (79.5 - 359.0)</td>
<td>209.4 (124.2 - 408.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>81.2 (29.2 - 205.0)</td>
<td>78.6 (29.5 - 146.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>46.3 (19.0 - 105.7)</td>
<td>42.0 (14.0 - 85.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated fat (g)</td>
<td>34.5 (17.7 - 69.6)</td>
<td>38.3 (13.2 - 80.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>9.9 (3.2 - 29.6)</td>
<td>11.6 (2.7 - 33.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Unsaturated fat (g)</td>
<td>46.2 (20.9 - 88.9)</td>
<td>50.9 (15.9 - 91.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated: saturated ratio</td>
<td>0.24 (0.09 - 0.79)</td>
<td>0.28 (0.12 - 0.77)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are medians (range)
Table 7.4. Colonic mucosa and cancer tissue study: Intake of major dietary components

<table>
<thead>
<tr>
<th>Dietary item (per 24 hours)</th>
<th>Diverticular disease group (n=7)</th>
<th>Haemorrhoid group (n=19)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (KCal)</td>
<td>1861 (1118 - 3330)</td>
<td>1793 (925 - 3005)</td>
<td>NS</td>
</tr>
<tr>
<td>Fibre (g)</td>
<td>13.5 (4.8 - 34.0)</td>
<td>18.85 (8.0 - 43.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>69.6 (34.8 - 107.4)</td>
<td>64.2 (39.8 - 100.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>209.4 (124.2 - 408.1)</td>
<td>211.9 (104.3 - 349.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>73.9 (40.8 - 146.7)</td>
<td>80.3 (29.5 - 140.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated fat (g)</td>
<td>46.4 (19.6 - 78.5)</td>
<td>41.8 (14.0 - 85.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated fat (g)</td>
<td>43.5 (13.2 - 73.0)</td>
<td>37.8 (13.3 - 80.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>9.4 (2.7 - 14.1)</td>
<td>12.9 (6.1 - 33.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Unsaturated fat (g)</td>
<td>50.9 (15.9 - 84.6)</td>
<td>51.3 (24.1 - 91.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated: saturated ratio</td>
<td>0.28 (0.12 - 0.41)</td>
<td>0.30 (0.13 - 0.77)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are medians (range)
Figure 7.1. Dietary intake of the five major fatty acids in cancer patients and control groups

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Cancer group v Combined control group</th>
<th>Cancer group v Haemorrhoid group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>M:S ratio</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>U:S ratio</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>P:S ratio</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cancer group v Diverticular group</th>
<th>Diverticular group v Haemorrhoid group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>NS</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>NS</td>
</tr>
<tr>
<td>M:S ratio</td>
<td>NS</td>
</tr>
<tr>
<td>U:S ratio</td>
<td>NS</td>
</tr>
<tr>
<td>P:S ratio</td>
<td>NS</td>
</tr>
</tbody>
</table>
comparison of the individual fatty acid intake in the diverticular and haemorrhoid control groups which demonstrates differences for the same three fatty acids between the two control groups.

The influence of patient age on the dietary intake of fatty acids is summarised in table 7.5. In both the cancer and control groups there was a tendency towards consumption of more saturated and less unsaturated fat in older patients. These trends were not statistically significant in the cancer patients but were more marked in the control group which overall had a lower mean age. In the combined control group, there was a significant inverse correlation between age and the dietary intake of linoleic acid.

Table 7.5. Mucosa and cancer tissue study: Correlation analysis of age and dietary fat intake

<table>
<thead>
<tr>
<th></th>
<th>Cancer group (n=26)</th>
<th>Combined control group (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>0.100</td>
<td>NS</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>-0.084</td>
<td>NS</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.333</td>
<td>NS</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>-0.320</td>
<td>NS</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td>Saturated</td>
<td>-0.001</td>
<td>NS</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>0.295</td>
<td>NS</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>-0.042</td>
<td>NS</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>-0.001</td>
<td>NS</td>
</tr>
<tr>
<td>M:S ratio</td>
<td>0.072</td>
<td>NS</td>
</tr>
<tr>
<td>U:S ratio</td>
<td>-0.001</td>
<td>NS</td>
</tr>
<tr>
<td>P:S ratio</td>
<td>0.339</td>
<td>NS</td>
</tr>
</tbody>
</table>
The fatty acid profiles of colonic mucosa from both diverticular and haemorrhoid control groups in comparison to non-malignant colonic mucosa removed from the resection specimens of patients with colorectal cancer is shown in figure 7.2. This demonstrates a similar profile of fatty acids in the two types of control mucosa. However, colonic mucosa from patients with diverticular disease was found to contain a significantly higher level of palmitic acid than rectal mucosa from haemorrhoid patients. This difference was reflected in a lower proportion of polyunsaturated fatty acids and a lower polyunsaturated to saturated fatty acid ratio in the diverticular mucosa. The level of palmitic acid was also higher in the diverticular mucosa when compared to non-malignant colonic mucosa from cancer patients. There were no differences between rectal mucosa from haemorrhoid patients and non-malignant mucosa from cancer patients and examination of figure 7.2 shows that the overall fatty acid profiles of the three groups under consideration was similar.

A comparison of the fatty acid compositions of cell membranes from cancer tissue, non-malignant colonic mucosa from the same patients and colonic mucosa from the combined control groups is shown in figure 7.3. As expected from the previous analysis, there were no differences between non-malignant colonic mucosa from cancer patients and colonic mucosa from the combined control groups. Malignant cancer tissue was, however, found to have a different profile of fatty acids in its cell membranes than the adjacent non-malignant mucosa removed from the edge of the same resection specimens. The cancer tissue membranes contained a higher proportion of palmitic acid and lower linoleic acid than non-malignant colonic mucosa from either cancer patients or control
Figure 7.2. Fatty acid composition of benign control mucosa and non-malignant colonic mucosa from cancer patients

Fatty acid composition of benign control mucosa and non-malignant colonic mucosa from cancer patients

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diverticular mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemorrhoid mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-malignant mucosa from cancer patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean + SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

<table>
<thead>
<tr>
<th></th>
<th>Hx mucosa v DD mucosa</th>
<th>DD mucosa v Ca mucosa</th>
<th>Hx mucosa v Ca mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>p&lt;0.01</td>
<td>p&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>18:0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>20:4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sat</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mono</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Poly</td>
<td>p&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Unsat</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>M:S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>U:S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>P:S</td>
<td>p&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Ca mucosa** = non-malignant colonic mucosa from cancer patients  
**Hx mucosa** = rectal mucosa from haemorrhoid patients  
**DD mucosa** = colonic mucosa from diverticular disease patients

16:0 palmitic acid  
18:0 stearic acid  
18:1 oleic acid  
18:2 linoleic acid  
20:4 arachidonic acid  

Sat = saturated fatty acids  
Mono = monounsaturated fatty acids  
Poly = polyunsaturated fatty acids  
Unsat = unsaturated fatty acids  

M:S = ratio of monounsaturated and saturated fatty acids  
U:S = ratio of unsaturated and saturated fatty acids  
P:S = ratio polyunsaturated and saturated fatty acids

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Figure 7.3. Fatty acid composition of cancer tissue, non-malignant mucosa from cancer patients and benign control mucosa

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-malignant mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean + SEM

Statistical analysis

<table>
<thead>
<tr>
<th>Control mucosa</th>
<th>Control mucosa</th>
<th>Cancer tissue</th>
<th>Cancer tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>v Cancer mucosa</td>
<td>v Cancer tissue</td>
<td>v Cancer mucosa</td>
<td>v Cancer mucosa</td>
</tr>
<tr>
<td>16:0</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>18:0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:2</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>20:4</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Sat</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Mono</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Poly</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Unsat</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>M:S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>U:S</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>P:S</td>
<td>NS</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Cancer tissue = malignant mucosa from cancer patients
Cancer mucosa = non-neoplastic colonic mucosa from the same cancer patients
patients. This was reflected by differences in the proportions of saturated and unsaturated fatty acids with a higher P:S and U:S ratio in malignant tissue.

An analysis of the mucosal membranes from the two control groups compared separately to malignant membranes is shown in figure 7.4. There were no differences between the diverticular mucosa and cancer tissue and it is clear that the differences between cancer tissue and the combined control mucosa group were due purely to higher levels of palmitic acid and lower linoleic acid in the rectal mucosa from haemorrhoid patients.

The relationships between dietary and tissue fatty acid content for control patients and cancer patients are shown in tables 7.6 and 7.7 respectively. In the control patients the only significant correlations between dietary and tissue fatty acids were for oleic acid and the monounsaturated to saturated ratio in the haemorrhoid group. Both these correlations were negative. Similarly, in the cancer patients there were very few significant correlations. Arachidonic acid in the diet was negatively correlated with the level in malignant tissue cell membranes. The P:S ratio between non-malignant colonic mucosal membranes from cancer patients and the dietary P:S ratio was positively correlated.

The stearic: oleic acid ratio was calculated for all tissue membrane samples. There were no significant differences for this ratio between the cancer tissue and the non-malignant colonic mucosa from the same patients or between the cancer tissue and the two types of control mucosa.
Figure 7.4. Fatty acid content of cancer tissue and benign control mucosa

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Cancer tissue</th>
<th>Diverticular mucosa</th>
<th>Haemorrhoid mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>50</td>
<td>40</td>
<td>30</td>
</tr>
<tr>
<td>Stearic</td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Oleic</td>
<td>30</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Linoleic</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>

Statistical analysis

<table>
<thead>
<tr>
<th></th>
<th>Hx mucosa v DD mucosa</th>
<th>DD mucosa v Cancer tissue</th>
<th>Hx mucosa v Cancer tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>p&lt;0.01</td>
<td>NS</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>18:0</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>18:2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>20:4</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Sat</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Mono</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Poly</td>
<td>p&lt;0.05</td>
<td>NS</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Unsat</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>M:S</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>U:S</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>P:S</td>
<td>NS</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
</tr>
</tbody>
</table>

p values from the two-tailed Mann Whitney U test
Table 7.6. Correlation analysis between dietary and tissue fatty acid content (diverticular mucosa, haemorrhoid mucosa and the combined control mucosa group)

<table>
<thead>
<tr>
<th></th>
<th>Diverticular mucosa</th>
<th>Haemorrhoid mucosa</th>
<th>Combined control mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>16:0</td>
<td>0.772</td>
<td>NS</td>
<td>-0.071</td>
</tr>
<tr>
<td>18:0</td>
<td>-0.643</td>
<td>NS</td>
<td>-0.274</td>
</tr>
<tr>
<td>18:1</td>
<td>-0.264</td>
<td>NS</td>
<td>0.437</td>
</tr>
<tr>
<td>18:2</td>
<td>-0.318</td>
<td>NS</td>
<td>-0.026</td>
</tr>
<tr>
<td>20:4</td>
<td>0.161</td>
<td>NS</td>
<td>-0.056</td>
</tr>
<tr>
<td>Sat</td>
<td>-0.359</td>
<td>NS</td>
<td>-0.293</td>
</tr>
<tr>
<td>Poly</td>
<td>0.642</td>
<td>NS</td>
<td>-0.109</td>
</tr>
<tr>
<td>Unsat</td>
<td>-0.399</td>
<td>NS</td>
<td>-0.351</td>
</tr>
<tr>
<td>M:S</td>
<td>-0.355</td>
<td>NS</td>
<td>-0.589</td>
</tr>
<tr>
<td>U:S</td>
<td>-0.249</td>
<td>NS</td>
<td>-0.315</td>
</tr>
<tr>
<td>P:S</td>
<td>0.439</td>
<td>NS</td>
<td>0.105</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used.

Abbreviations for fatty acids:

Sat = saturated; Mono = monounsaturated; Poly = polyunsaturated; Unsat = unsaturated
Table 7.7. Correlation analysis between dietary and tissue fatty acid content (cancer tissue, cancer mucosa and combined control mucosa)

<table>
<thead>
<tr>
<th></th>
<th>Cancer tissue</th>
<th>Cancer mucosa</th>
<th>Combined control mucosa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>16:0</td>
<td>0.102</td>
<td>NS</td>
<td>0.389</td>
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<tr>
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<td>-0.075</td>
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<td>0.259</td>
</tr>
<tr>
<td>18:1</td>
<td>-0.320</td>
<td>NS</td>
<td>0.135</td>
</tr>
<tr>
<td>18:2</td>
<td>-0.190</td>
<td>NS</td>
<td>0.065</td>
</tr>
<tr>
<td>20:4</td>
<td>-0.618</td>
<td>p&lt;0.002</td>
<td>-0.060</td>
</tr>
<tr>
<td>Sat</td>
<td>-0.391</td>
<td>p&lt;0.05</td>
<td>0.243</td>
</tr>
<tr>
<td>Poly</td>
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<td>NS</td>
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</tr>
<tr>
<td>Unsat</td>
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<td>NS</td>
<td>0.023</td>
</tr>
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<tr>
<td>U:S</td>
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<td>NS</td>
<td>-0.004</td>
</tr>
<tr>
<td>P:S</td>
<td>-0.013</td>
<td>NS</td>
<td>0.462</td>
</tr>
</tbody>
</table>

Relative percentages and ratios of relative percentages were used.

Abbreviations for fatty acids:

Sat = saturated; Mono = monounsaturated;
Poly = polyunsaturated; Unsat = unsaturated
Discussion

The acquisition of control colonic mucosa was a major requirement of this study. Patients with either diverticular disease or haemorrhoids were chosen because these conditions are common and the collection of specimens of colonic or rectal mucosa was relatively simple in both cases. These two conditions involve pathological abnormalities of the colon and anorectum respectively and it could, therefore, be argued that in neither case was the colonic mucosa obtained entirely normal. However, it would have been extremely difficult to study colonic mucosa from a large number of healthy human subjects. The diverticular and haemorrhoid groups were used as a compromise in the knowledge that the colonic mucosa was entirely non-malignant and as such could be used to make a valid comparison with specimens from patients with colorectal cancer. Most surgery related to diverticular disease is performed as an emergency to treat complications and the need for elective colonic resections is now relatively rare. Thus, the majority of the control specimens were taken from patients undergoing haemorrhoidectomy.

A problem related to the source of control mucosa was that the patients in the cancer and control groups were not matched for age. The mean age of the patients in the diverticular disease group was comparable with that of the cancer group. In contrast, the patients in the haemorrhoid group were considerably younger than the cancer patients and so the mean age of the combined control group (51.6 years) was significantly less than that of the cancer group (69.9 years). Some of the findings in the erythrocyte study suggested that age could
be an important factor and so in each analysis in the mucosa study the two control groups were treated both independently and as a combined control group.

Despite the lack of age matching, there were no significant differences in the dietary intake of major nutrients between either of the control groups and the malignant group. This was consistent with the findings of the erythrocyte study. Analysis of the dietary intake of individual fatty acids did, nevertheless, reveal significant differences between the combined control patients and the cancer group. These differences were due entirely to the younger haemorrhoid patients, the most likely explanation being that age was exerting an important influence. Throughout the human studies there was a tendency towards the consumption of more saturated and less unsaturated fatty acids with increasing age. Nevertheless, the broad similarity of dietary fat intake between the cancer and control groups in this work was an important factor tending to make it very difficult to show differences which are due purely to diet. This was of course one of the reasons for performing the animal experiments (chapters 9 and 10).

The fatty acid profiles of the two types of control mucosa were found to be quite similar suggesting that it was valid to consider the two together as a combined control group for comparison against specimens from patients with colorectal cancer. The absence of differences in fatty composition between the combined control group and the non-malignant colonic mucosa from cancer patients suggested that there was not a field change in the composition of mucosal cell membranes throughout the colons of subjects who were susceptible to the development of colorectal cancers.
Cell membranes from malignant tissue were found to have a different fatty acid profile from the surrounding non-malignant mucosa in the same part of the colon and from control rectal mucosa from the haemorrhoid patients. The main changes were a lower linoleic acid content and a higher palmitic acid content in malignant tissue. Although there was a higher arachidonic acid content in the malignant tissue compared to non-malignant and control colonic mucosa, the differences were not large enough to be considered as statistically significant. The normal metabolic flow is from linoleic to arachidonic acid and it may be that the lower linoleic acid content in malignant tissue is a reflection of a higher rate of conversion to arachidonic acid and its important prostaglandin metabolites.

In contrast to the haemorrhoid group, there were no differences between the control mucosa from diverticular patients and malignant tissue. The diverticular group were well matched for age with the cancer group, whereas the haemorrhoid group were not suggesting that this could be due to an age related phenomenon. The observed differences between the malignant and non-malignant colonic tissue removed from the same operative specimens would, however, not support this explanation. It is more likely that the absence of differences is a function of the small number in the diverticular group, with only ten specimens being available to show what are only small overall differences between groups.

The stearic to oleic acid ratio was not different in malignant cell membranes when compared to non-malignant colonic membranes or control tissues. If the desaturation of erythrocyte cell membranes shown by Wood et al (1985) were due to a factor released by tumours
into the circulation as proposed by Habib et al (1987a), then it might be expected that the cell membranes of the malignant tissues themselves might also show an abnormal saturation index. The results of the mucosa study therefore cast additional doubt on the correctness of Wood's original work.

There were very few significant correlations between dietary and colonic mucosal cell membrane fatty acid levels in the human study, which was perhaps not surprising. The smaller numbers of dietary histories available in this part of the human work would mean that much higher r values (Pearson ranked correlation test) would be needed for results to attain a significant level. Furthermore, colonic mucosa has a very high rate of turnover which is very different to the situation regarding adipose tissue which has a relatively constant fat content over long periods because of its slow turnover rate. One further point is that the dietary recall technique is designed particularly to give a reflection of long term habitual food consumption and this is less likely to be reflected in tissues with high rates of cell turnover. The most notable relationship found was a strong negative correlation between the arachidonic acid content of the diet and that in the cancer tissue. The significance of this finding is unknown at the present time.

The observed differences between malignant and non-malignant cell membranes in cases of colorectal cancer were the most important findings of the human studies because they imply altered fatty acid metabolism in the malignant state. Whether this is a cause or an effect of the carcinogenic process has yet to be determined. Although the data presented exclude a field change hypothesis, it is still
possible that the changes in cell membrane fatty acid composition could have occurred in a localised area of colonic mucosa from which the cancer subsequently arose. The theory that the changes in cell membrane fatty acid profile precede the development of overt malignant change is still an attractive one. The mechanism may be one of an alteration in membrane fluidity consequent upon the altered fatty acid profile. It is, however, impossible to link the changes in fatty acid profile directly to the dietary intake of fat because the non-malignant colonic mucosa from cancer patients did not show any abnormalities of fatty acid content. The alternative explanation is that the cell membrane fatty acid abnormalities in colorectal cancer cells are secondary to the carcinogenic or malignant processes. This would explain the fact that the changes are so localised in the colon.

The regulation of membrane fluidity, or its corollary membrane rigidity, is essential for homeostasis (Cooper, 1977) and the metabolic rates of many essential cell enzymes depend on it (Sandemann, 1979). In general, decreased membrane rigidity leads to increase cell metabolism and also higher division rates which are characteristic features of malignant cells. Direct physical measurements using fluorescent probes and magnetic resonance studies have provided evidence for a decreased membrane rigidity in leukaemic cells (Petitou et al, 1978; Mountford et al 1986). The altered membrane lipid profiles of the malignant cells in the present study may form the basis for similar changes in colorectal cancer. Further work is required to verify this hypothesis.
Conclusions

There were no differences in the fatty acid composition of cell membranes from the non-neoplastic colonic mucosa of patients with colorectal cancer and colonic mucosa from patients with either diverticular disease or haemorrhoids. Colorectal cancer tissue was, however, found to have a different profile of fatty acids in its cell membranes than the adjacent non-malignant mucosa removed from the edge of the same resection specimens. The mechanism underlying these changes is unknown.
The discovery of the first useful animal model of colorectal cancer by Laqueur in 1963 was serendipitous. Whilst investigating the unusually high incidence of amyotrophic lateral sclerosis in the inhabitants of the Pacific Island of Guam, Laqueur noted that the staple diet of the islanders was the cycad nut. He fed cycad nuts to rats in the hope of producing an experimental model of amyotrophic lateral sclerosis. He failed to achieve this aim but noticed instead that the experimental animals developed malignant tumours at various sites, one of which was the large intestine (Laqueur et al, 1963). This discovery was significant because of the great rarity of spontaneous epithelial colorectal tumours in these experimental animals (Dunn, 1965; Pozharisski, 1973). Subsequently, the active component of the cycad nut was identified by Laqueur who named it cycasin (Laqueur, 1965). Further studies demonstrated that the active moiety of cycasin was the beta-glucoside of methylazoxymethanol (Matsumoto, 1979).

Chemical carcinogens: The hydrazine derivatives

The two most commonly used chemical colorectal carcinogens are dimethylhydrazine (DMH) and azoxymethane (AOM). Attempts to chemically synthesise methylazoxymethanol (MAM) met with great difficulties and this prompted Miller to suggest that a possible biochemical precursor,
azoxymethane (AOM), might also exhibit carcinogenic activity. Using similar logic, Druckrey (1967) went on to show that the structurely related 1,2 dimethylhydrazine (DMH) was an even more potent colorectal carcinogen than cycasin and proposed that DMH, AOM and MAM were linked by a metabolic pathway (Figure 8.1). The accuracy of this putative metabolic pathway was subsequently confirmed in another laboratory (Fiala, 1977).

Figure 8.1. Metabolic pathway linking the chemical carcinogens derived from dimethylhydrazine

1,2 dimethylhydrazine
\[ \text{CH}_3-\text{NH-NH-CH}_3 \]

Azomethane
\[ \text{CH}_3-\text{N=N-CH}_3 \]

Azoxymethane
\[ \text{CH}_3-\text{N=N-CH}_3 \rightarrow \text{DMH} \]

Methyldiazonium
\[ \text{CH}_3-\text{N}^+\text{N} \]

Methyl carbonium ion
\[ \text{CH}_3^+ + \text{N}_2 \]
In early studies, DMH was administered at low doses given by subcutaneous injection over a relatively lengthy period of up to 15 weeks (Druckrey, 1973). Later studies demonstrated that fewer doses were required and that by increasing the dosage level, the tumour yield increased and the latent period to the time of appearance of tumours decreased (Deschner et al, 1979; McConnell et al, 1980). Moreover, it was found that a single high dose of DMH (35 to 200 mg/Kg) given subcutaneously or orally could also induce colon cancer (Schiller et al, 1980).

Chemical oxidation of DMH in the laboratory produces azomethane, a poisonous and explosive gas, and further oxidation yields azoxymethane (Horisberger and Matsumoto, 1968). After the administration of DMH to rats, large amounts of azomethane are exhaled in the breath and azoxymethane and methylazoxymethanol are excreted in the urine. Weisburger (1971) proposed a pathway in which DMH would be metabolised in the liver to MAM, which would then be conjugated with glucuronic acid. This stable glucuronide would reach the lumen of the bowel in the bile and here would be deconjugated by bacterial beta-glucuronidase thus releasing the unstable carcinogen MAM. However, elegant transposition experiments involving the surgical interchange of segments of descending colon and small intestine have shown that DMH, AOM and MAM preferentially induce tumours in the colonic segment (Gennaro et al, 1973; Celik et al, 1981; Rubio & Nylander, 1981). Furthermore, parenteral administration of these chemical carcinogens produce tumours in colonic segments which have been isolated from the faecal stream by colostomy (Campbell et al, 1975; Matsubara et al, 1978). In addition, it has been shown that 1% or less of a dose of DMH
appears as biliary metabolites (Hawks & Magee, 1974; Fiala, 1977). These studies indicate that the carcinogens reach the colon primarily by the blood stream and that Weisburger's biliary transport pathway appears untenable.

Although the exact mechanism of action of these chemical carcinogens is not known, a number of changes have been noted in the colonic mucosa after administration. These include inhibition of DNA synthesis, cellular cytotoxicity and degeneration, followed by regeneration, hyperplasia, the migration of cells up the colonic crypts and subsequent shedding (Barkla & Tutton, 1977; Chang et al, 1979; Chang, 1981; Richards, 1981; Fisher, 1981; Sunter et al, 1981a; Sunter et al, 1981b). The ultimate form in which these carcinogens act at the cellular level is also unclear, but a putative methyl carbonium ion that can react with cellular macromolecules including DNA has been suggested (Fiala, 1981).

Azoxymethane is a more potent carcinogen than DMH because it is closer to the ultimate carcinogens in the metabolic pathway (Nigro et al, 1973) and whereas a sizeable portion of a dose of DMH is exhaled as azomethane or excreted in the urine as AOM, an analogous process does not occur with AOM (Fiala, 1977). Azoxymethane has the same organ specificity as the parent compound DMH but is less readily available and more expensive. Nonetheless, it is often the preferred agent because the effective dose is lower than with DMH and it is a water soluble, chemically stable compound (Druckery, 1972). Azoxymethane can be given by multiple oral, subcutaneous or intravenous routes and it requires hepatic metabolism for successful action; the current view being that the early metabolism of DMH and AOM occurs in the liver and
that the MAM produced is carried to the target organs via the blood. These compounds are capable of producing hepatomegaly and are toxic to the liver and kidney. Like DMH, a single high subcutaneous dose of AOM can induce colorectal carcinoma (Ward, 1975). The overall dose of AOM has been shown to affect the position of the resultant tumours in the colon and rectum, with high doses yielding tumours in the descending colon and low doses, tumours in the caecum and ascending colon (Ward et al. 1973).

The proximate carcinogen derived from both DMH and AOM is MAM. This compound itself has not been used as a chemical carcinogen because it is unstable in aqueous solution and in vivo. A stable acetate ester, methylazoxymethylacetate, is available commercially but has no special advantages over AOM.

The chemical carcinogens considered here do not specifically promote colorectal tumours alone. They are also known to initiate adenocarcinomas of the small intestine, squamous cell carcinomas of the anus and squamous cell carcinomas of the external auditory canal (Ward, 1974; Sunter et al, 1978; LaMont & O'Gorman, 1978).

Are these experimental models relevant to the human disease?

In order to answer this question, the pathology of human colorectal tumours must be reviewed. Human large bowel neoplasms can be divided at the simplest level into benign neoplastic polyps or adenomas and malignant carcinomas. A benign neoplastic polyp is characterised by abnormalities in both the cytological features of
individual epithelial cells and in the normal architectural arrangement of those cells. The cytological abnormalities of adenomas are caused by the process of dysplasia occurring in the colonic epithelium. The epithelial cells demonstrate nuclear pleomorphism, an increase in the nuclear to cytoplasmic ratio, abnormal mitotic activity, enlargement of their nucleoli and loss of nuclear polarity.

The architectural abnormalities of colorectal adenomas produce three main histological patterns: tubular adenomas, villous adenomas and tubulovillous adenomas. Tubular adenomas are almost always pedunculated polypoid lesions, the adenoma proper being connected to the mucosa by a narrow stalk which consists of connective tissue covered by muscularis mucosa and normal non-neoplastic colonic mucosa. The adenoma consists of compactly arranged tubular glands which result from excessive branching. A villous adenoma is usually sessile rather than pedunculated and with its broad base this type of lesion has a distinctive papillary appearance on both macroscopic and microscopic inspection. It is composed of narrow frond like outgrowths arising from a delicate connective tissue stroma and unlike the tubular type, the entire lesion is neoplastic. In a tubulovillous adenoma the morphological features are intermediate between tubular and villous types, so that with the naked eye they appear much the same as tubular adenomas, but histologically have a villous element.

The cytological features of carcinomas are the same as those already described for adenomas. The important distinction between the two is that in malignant neoplasms the abnormal cells invade through the muscularis mucosa into the submucosa. Although it has caused some controversy in the past, it is now generally accepted that the vast
majority of human colorectal carcinomas arise in neoplastic polyps by the so called polyp-cancer sequence. The incidence of malignant change is less in tubular adenomas than tubulovillous adenomas and highest in the pure villous adenoma. The size of adenomas is also correlated with the incidence of malignant change. Most lesions are 1 cm or less in diameter and these have a prevalence of malignant foci of the order of 1% only (Muto et al, 1975). In the same study, it was calculated that neoplastic polyps that are more than 2 cm in diameter have a 34.7% potential for malignancy.

Carcinomas of the colon and rectum are almost exclusively adenocarcinomas showing a variable degree of glandular differentiation. Two broad morphological types are recognised, the flat infiltrative type and the fungating exophytic type. The flat and infiltrative variety are more common in the descending colon and rectum. They infiltrate the bowel wall in a circumferential fashion eventually producing an annular stenotic lesion often with an ulcerated surface. These lesions often present as large bowel obstruction because of the narrowing of the lumen and the solidity of the faeces on the left side of the colon. The fungating exophytic type of carcinoma is more common in the caecum and ascending colon, where it protrudes as a cauliflower like mass into the lumen of the bowel. These lesions tend to cause recurrent occult bleeding and may present as an iron deficiency anaemia. They are not usually associated with obstruction because of the large size of the caecum and the fluidity of the faeces in the right colon. Left sided colonic lesions are more common in man than right sided ones. However, a number of studies have shown a trend towards an increase in the percentage of right sided lesions and a
proportionate decrease in left sided ones during the last 30 to 40 years (Rhodes et al, 1977; Snyder et al, 1977). Thus Snyder et al (1977) found that the incidence of carcinomas in the right colon increased from 13.4% in the period 1940–1944 to 21.8% during 1970–1973. During the same period the proportion of rectal cancers decreased from 45.5% to 34.5%.

Microscopically a variety of sub-types of adenocarcinoma are seen in the large bowel. The majority are well differentiated adenocarcinomas characterised by well formed glands. The less differentiated types demonstrate a lesser tendency to reproduce glandular structures and the most poorly differentiated consist entirely of small round cells (carcinoma simplex). Some tumours are characterised by excessive mucus production. If this is an intracellular accumulation of mucus, the nucleus is pushed to one side giving the cells a 'signet ring' appearance. Large quantities of extracellular mucus may also accumulate so that the epithelial cells are surrounded by lakes of mucus and this type is called a colloid carcinoma. Both these types of mucinous carcinoma are poorly differentiated and tend to exhibit more invasion than other types.

Colorectal carcinomas in man generally spread quite slowly so that the tumour is confined to the bowel wall for a long time. Direct spread occurs through the submucosa, muscular layers, serosa, pericolic fat and eventually to surrounding organs. Lymphatic invasion leads to metastases in the regional lymph nodes and then spread to the more central pre- and para-aortic lymph nodes. Colorectal cancer also spreads via veins which may first be permeated in the submucosa. As the large bowel drains through the portal system this mode of spread
often causes liver metastases. Transcoelomic spread may also occur when the primary tumour invades locally through the serosa of the bowel wall.

The pathological stage of a colorectal carcinoma is the most important factor determining prognosis. The Dukes classification (Dukes, 1940) is based on the degree of invasion through the bowel wall and the presence or absence of lymph node metastases. Dukes A carcinomas are confined to the wall of the bowel, not extending beyond the muscular layers and without lymph node metastases. Dukes B carcinomas have invaded through the serosa of the bowel wall and into the pericolic fat but again without lymph node involvement. Dukes C carcinomas are those associated with lymph node involvement irrespective of the extent of local invasion. Cuthbert Dukes originally described this classification for rectal carcinomas and only used the three categories described. The classification is also commonly used to describe the stage of colonic carcinomas as well and a fourth 'Dukes D' stage has been added to include carcinomas with liver and peritoneal metastases. In more recent times several more complex classifications for the stage of colorectal carcinomas have been introduced but all are based on the original Dukes method which remains the most used even after nearly 50 years.

A number of studies have addressed the area of the comparative pathology of human and chemically induced experimental colorectal neoplasms. There has been general agreement that there is a striking similarity in the overall naked eye and microscopic characteristics between the two groups (Ward et al, 1973; Ward, 1974; LaMont &
O'Gorman, 1978; Sunter et al, 1978; Pour, 1978; Teague et al, 1981). Although a huge array of classifications of experimental tumours have been proposed (Teague, 1983), only a few macroscopically different types have been described. These are pedunculated and sessile polyps, mucosal nodules and plaques and fungating tumours. The mucosal nodules and plaques do not have identical human counterparts but histologically are most often well differentiated adenocarcinomas (Teague et al, 1981). Light microscopic appearances are even more strikingly similar to the human situation with tubular, tubulovillous and villous adenomas, adenocarcinomas of varying degrees of differentiation, signet ring carcinomas and colloid carcinomas all being represented in experimental models. In particular it has been noted that papillary adenocarcinomas with superficial invasion into the submucosa in experimental models are virtually identical to many exophytic proximal colonic human neoplasms (Ross, 1982). Furthermore, the electron microscopic appearances of the human and experimental adenocarcinomas has been deemed similar in many respects (Spjut & Smith, 1967).

The site of origin of invasive colorectal carcinomas in experimental animals is variable and as aluded to above depends in part on the dose as well as the specific type of chemical carcinogen used. In a study comparing 377 experimental tumours induced in rats by DMH (total dose 120-300mg/Kg), AOM (90-160 mg/Kg) or MAM (225-300 mg/Kg), with 210 human colorectal tumours, Ross (1982) found that 43% of the experimental tumours arose in the caecum and ascending colon compared to 33% in the human group. This finding is in accordance with other work which has shown not only a higher incidence of right sided lesions but also a more uniform distribution of tumours throughout the colon in
experimental models (Ward, 1974; Pozharrisski, 1975; Maskens, 1976). Nonetheless, the animal models are sufficiently flexible to allow a certain degree of manipulation of the site of induced tumours. For example, more distal lesions can be produced either by using low dose azoxymethane (Ward et al, 1973) or by administering the chemical carcinogen by intrarectal instillation (Narisawa et al, 1971; Narisawa et al, 1976).

The pattern of invasion is also generally similar in human and experimental colorectal tumours. One of the main reasons for the poor overall survival associated with human colorectal cancers is the tendency for tumours to be of a relatively advanced stage at the time of presentation. In a recent series only 15% of resected human colorectal tumours were of Dukes stage A (Vellacott et al, 1987). In contrast, the majority of experimental tumours show only early invasion, with between 67 and 88% being equivalent to a Dukes A stage (Teague, 1983). In both groups there is a tendency towards deeper invasion of the bowel wall with the more poorly differentiated tumour types. Thus, experimentally induced mucinous adenocarcinomas have been shown to have greater invasiveness than non-mucinous ones (Sunter et al, 1978) and in man the increased malignancy of mucinous adenocarcinoma is reflected in the poorer survival figures for patients with this tumour type (Symonds & Vickery, 1976).

A difference between the metastatic potential of human and experimental colorectal cancers is apparent. In human colorectal cancer, 40–50% have evidence of lymphatic metastases at the time of presentation (Buckwalter & Kent, 1938; Coller, 1940; Grinnell, 1950) and haematogenous spread to the liver is also not uncommon (Dionne,
1965). Most studies of experimental colorectal tumours show a low incidence of metastatic disease in general and a difference in pattern when metastases do occur. It appears that it is the mucinous carcinomas of the proximal colon which are almost exclusively responsible for metastases in the animal models, distal colonic adenocarcinomas being virtually incapable of metastasis (Haase et al, 1973; Maskens, 1976; Sunter et al, 1978; Morson, 1978; Chang, 1978; Pozharriski et al, 1979). The orderly progression of lymphatic spread from pericolic to regional lymph nodes, characteristically seen in the human disease, is not commonly encountered in animals. Here although metastases have been noted in regional lymphatics, they are much more common on peritoneal surfaces especially of the liver and diaphragms. A low incidence of true hepatic metastases has also been noted in most studies using animal models (Cole, 1971; Enker and Jacobitz, 1976; LaMont and O'Gorman, 1978; Ross, 1982). One study which was not in line with the general paucity of metastases was that carried out by Teague (1983) who found that 23% of rats treated with DMH developed metastases, most of these being peritoneal. Nevertheless, the overall evidence implies that the chemical carcinogen model is not a good one for the study of colorectal metastases.

Although there has been a good deal of controversy over the existence of the polyp–cancer sequence, it is now generally, although not universally, accepted that most human colorectal arise in benign adenomatous polyps (Fenoglio & Lane, 1974; Morson, 1974a; Muto et al, 1975). The evidence which suggests that a polyp to cancer transition occurs in man has been reviewed by Morson (1978). The key points in the argument are as follows: The distribution of adenomas and
carcinomas in the large bowel is similar, with the majority occurring in the sigmoid colon and rectum (Berge et al, 1973; Ekelund & Lindstrom, 1974). Patients with polyps have a much higher incidence of subsequently developing colorectal cancer than patients without polyps (Rider et al, 1954, Rider et al, 1959). The most striking example of this is seen in patients with familial polyposis coli where the risk of colorectal cancer is extremely high (Dukes, 1958; Bussey, 1975). When the development of benign polyps is periodically observed, they sometimes show evidence of focal cancer invading their stalks, the incidence of malignant change increasing with the size of the polyp. There is a residual benign component to many malignant colorectal tumours. About 57% of early malignant tumours confined to the submucosa contain benign neoplastic tissue, whereas this figure is only 8% for the more advanced cancers with extramural spread. Also large colorectal cancers more rarely contain residual adenomatous tissue than smaller cancers. There are synchronous benign adenomas in 20% of specimens removed for cancer. Finally a study performed by Gilbertson and Helms (1978) suggested that the removal of polyps significantly reduces the subsequent risk of colorectal cancer. There is of course an alternative hypothesis for the histogenesis of colorectal cancers which holds that these tumours arise directly from flat colonic mucosa without going through a polyp stage (Castleman & Crickstein, 1967; Spratt and Moyer, 1958; Spratt and Ackerman, 1962). There have been fewer proponents of this hypothesis and the cited evidence remains less than convincing.

Experimental carcinomas have, however, clearly been shown to develop in flat non-polypoid mucosa (Shamsuddin et al, 1980, Maskens &
Dujardin-Loits, 1981; Shamsuddin et al, 1982). Indeed, a number of authors have contended that DMH-induced rat colon adenocarcinomas can only arise de novo from flat mucosa and not from benign polyps (Maskens, 1976; Ward, 1974), suggesting a serious discrepancy between animal models and human cancers. Both these studies used very high doses of DMH (20–26.6 mg/Kg) for a long period (20 weeks) and this may be a factor in explaining their findings. Others have clearly shown that adenomas as well as carcinomas are produced in DMH treated rats and that malignant change does occur in the adenomatous polyps (Wiebecke et al, 1973; Sunter et al, 1978). Thus, it appears that the truth lies in the middle ground with carcinomas in DMH-treated rats arising either de novo from flat mucosa or via a benign adenomatous polyp.

In the human colon, the development of multiple synchronous tumours is uncommon, with reported figures ranging from 2.1 to 6.1% (Ekelund & Pihl, 1974). This is clearly a point of difference with the animal models where multiple malignant tumours are extremely common occurring in over 50% of animals in some series (Ross, 1982). The number and size of experimentally induced tumours is a function of the dose of carcinogen and the duration of the experiment. It is therefore theoretically possible to reduce the number of tumours per animal to render the model more typical of the human situation. That this has not been done is a reflection of the fact that large tumour numbers per animal is an advantageous feature to those working in colorectal cancer research.

It has already been mentioned that the action of the hydrazine derivative chemical carcinogens is not specific to the colon and the
development of synchronous non-colorectal tumours is well documented (Ward, 1974; Sunter et al, 1978; LaMont & O'Gorman, 1978). The other types of tumour which are commonly encountered after chemical carcinogenesis are small intestinal adenocarcinomas and squamous cell carcinomas of the anus and auditory canal. Angiosarcomas of the liver have been seen only rarely. Although these tumours represent a difference between animal models and humans, the localisation of the small intestinal adenocarcinomas to the duodenum and proximal jejunum, with relative sparing of the distal ileum, is similar to that found in the human situation.

In experimental colorectal tumours an association with submucosal lymphoid tissue has been described. In one study of rats treated with DMH, 65% of the colorectal cancers which developed were found on the surface of, or invading into, submucosal lymphoid tissues (Ward, 1974). Although the mechanism by which this occurs is unknown, possible explanations are increased non-specific mucosal injury caused by luminal protrusion of the lymphoid tissue, increased concentration of carcinogen in the lymphoid tissue, increased susceptibility to carcinogen of the overlying mucosa as a consequence of rapid cell turnover or altered immune surveillance in the vicinity of the lymphoid tissue. A parallel tendency for the association of colorectal cancers with submucosal lymphoid tissue has not been shown in man.
Conclusions

Despite several important differences in the comparative pathology of experimental and human colorectal neoplasms, a comparison of the two is dominated by quite striking overall macroscopic and histological similarities. Experimental colorectal tumours induced by the hydrazine derivatives appear to be a good, but not ideal, model of the human situation and they provide a valuable format for colorectal cancer research.
Chapter 9

THE INFLUENCE OF DIETARY FAT ON EXPERIMENTAL COLORECTAL CARCINOGENESIS

Although the animal work in this thesis was performed as a single study containing different experimental groups, it is presented as two separate chapters for clarity. The experiments described in the present chapter examined the effects of high and low levels of saturated and unsaturated dietary fat on an animal model of colorectal carcinogenesis.

Animals and Methods

Animal diets

The animal diets were made by Special Diet Services (SDS) Limited, Witham, Essex. Four experimental diets were made by adding different quantities and types of fat to a basal diet which was virtually fat free. The detailed composition of the basal diet is shown in Table 9.1. Saturated fat diets (5% and 20% v/v) were prepared in Essex by SDS and transported to Leicester in ready-made form. Beef suet was used as the source of fat in these two diets. Unsaturated fat diets (5% and 20% v/v) were prepared in the Biomedical Services Unit in Leicester. These diets were freshly made up every other day by adding 5% (v/v) or 20% (v/v) pure corn oil (CPK UK Limited, Esher, Surrey) to
Unsaturated fat diets were stored at 4°C in air-tight polystyrene containers and never used more than 2 days after preparation. These precautions were taken in order to minimise the possibility of peroxidation of polyunsaturated fatty acids. The relative proportions of the major fatty acids in the four experimental diets are shown in Figures 9.1 and 9.2.

**Animals and treatment groups**

Weanling male Wistar rats weighing 50-75 g were purchased from Harlan OLAC Limited, Oxford. After a one week period of acclimatisation, the 5 week old rats were divided into four experimental groups according to the type of diet they would receive as follows:

1. 5% saturated fat diet (beef suet) 25 rats
2. 20% saturated fat diet (beef suet) 25 rats
3. 5% unsaturated fat diet (corn oil) 25 rats
4. 20% unsaturated fat diet (corn oil) 25 rats

Animals were housed in the Biomedical Services Unit, Leicester University, four to a cage with diets and water being given ad libitum. Each rat was examined daily and weighed weekly. Food consumption was assessed 16 weeks into the experimental protocol. Four carcinogen
Table 9.1. Composition of the basal diet used in the animal studies

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>100.000g</td>
</tr>
<tr>
<td>Moisture</td>
<td>6.363g</td>
</tr>
<tr>
<td>Crude fat</td>
<td>0.300g</td>
</tr>
<tr>
<td>Crude protein</td>
<td>12.979g</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>10.095g</td>
</tr>
<tr>
<td>Ash</td>
<td>5.973g</td>
</tr>
<tr>
<td>Total dietary fibre</td>
<td>11.420g</td>
</tr>
<tr>
<td>Starch</td>
<td>30.045g</td>
</tr>
<tr>
<td>Sugar</td>
<td>32.101g</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.828mg</td>
</tr>
<tr>
<td>Phosphate</td>
<td>0.639mg</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.320mg</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.428mg</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.170mg</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.977mg</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.780mg</td>
</tr>
<tr>
<td>Iron</td>
<td>0.080mg</td>
</tr>
<tr>
<td>Copper</td>
<td>0.016mg</td>
</tr>
<tr>
<td>Manganese</td>
<td>0.055mg</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.047mg</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.527mg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.068mg</td>
</tr>
<tr>
<td>Retinol</td>
<td>1.527mg</td>
</tr>
<tr>
<td>Calciferol</td>
<td>0.026mg</td>
</tr>
<tr>
<td>Tocopherol</td>
<td>0.047mg</td>
</tr>
<tr>
<td>B1</td>
<td>9.800mg</td>
</tr>
<tr>
<td>B2</td>
<td>11.000mg</td>
</tr>
<tr>
<td>B6</td>
<td>6.900mg</td>
</tr>
<tr>
<td>B12</td>
<td>5.650mg</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.060mg</td>
</tr>
<tr>
<td>Niacin</td>
<td>33.500mg</td>
</tr>
<tr>
<td>Pantethenic acid</td>
<td>17.750mg</td>
</tr>
<tr>
<td>Choline</td>
<td>0.405mg</td>
</tr>
<tr>
<td>Inositol</td>
<td>0.450mg</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.125mg</td>
</tr>
</tbody>
</table>
Figure 9.1. Fatty acid composition of saturated fat diets

![Graph showing fatty acid composition of saturated fats](image1)

- Palmitic
- Stearic
- Oleic
- Linoleic
- Arachidonic

Fatty acid content (relative %)

- 5% saturated fat
- 20% saturated fat

Figure 9.2. Fatty acid composition of unsaturated fat diets

![Graph showing fatty acid composition of unsaturated fats](image2)

- Palmitic
- Stearic
- Oleic
- Linoleic
- Arachidonic

Fatty acid content (relative %)

- 5% unsaturated fat
- 20% unsaturated fat
treated rats and four control rats from each dietary group were isolated in metabolic cages designed to minimise coprophagia and food waste. After a four day period of adjustment, food intake was measured daily for a further four days. Fifteen animals in each dietary group were treated with the chemical carcinogen azoxymethane, which was injected intraperitoneally at a dose of 15 mg/Kg once a week for 6 weeks. The remaining ten animals in each group were injected with carrier solution only (0.9% saline) to act as controls. Fifteen weeks after the last injection of carcinogen or saline all animals were killed and examined for the development of colorectal tumours.

Each animal was submitted to detailed autopsy, with particular attention being paid to the development of tumours in the colon, duodenum, small bowel and liver. The entire colon and rectum from the ileo-caecal junction the the anus was removed, this procedure being facilitated by division of the pubic symphysis to allow excision of the whole rectum. The large bowel was opened along its length, washed clean with water and carefully pinned out in a straight line onto a piece of cork. The total large bowel length was measured in millimetres and the position of each lesion was measured and recorded as a percentage distance along the bowel taking the most proximal part of the caecum as 0% and the anorectal junction as 100%. The macroscopic appearance of each lesion was classified as one of the following types: sessile lesion, pedunculated polyp, ulcerated tumour or stenosing tumour. The size of each lesion was measured to the nearest millimetre using calipers. The small bowel from the first part of the duodenum to the ileocaecal junction was also excised, opened along its length, washed with water and examined thoroughly with the
naked eye. The number of small bowel tumours seen was recorded for each animal. Evidence of intra-abdominal metastasis was sought and these lesions were biopsied for histology. The liver was dissected out and each lobe was examined for the presence of metastases by removing serial 2mm slices. Any suspicious lesion was excised and processed for histological examination.

The colon and rectum from each animal was fixed in 10% formalin solution overnight. Tumours were excised and individually processed for histology. Specimens were initially serially dehydrated through graded alcohol solutions, cleared in xylene and then embedded in paraffin wax. Sections were cut to a thickness of 4 microns and stained with haematoxylin and eosin. All the histological material generated by the study was examined by one consultant pathologist (Dr I.C. Talbot), who was unaware of the dietary group from which individual specimens were taken. The presence of adenomatous and carcinomatous tissue was recorded systematically. Adenomas were classified as tubular, villous or of mixed tubulo-villous type and the degree of dysplasia was assessed as mild, moderate or severe. Carcinomas were staged on their histological appearances as follows: A1: not invading deeper than the submucosa, A2: invading the muscularis layers but confined to the bowel wall and B: invading through the bowel wall. The histological grade of each malignant tumour was defined as good (well differentiated), moderate (moderately well differentiated) or poor (poorly differentiated).
Statistical analysis

All statistical analyses were performed on the University of Leicester mainframe computer using the Minitab computer package. The frequency of discrete variables in this experiment were analysed using the Chi-squared test.

Results

Food consumption and body weight data

Food consumption data and their statistical analysis are shown in figure 9.3. In general, each group of rats ate approximately the same amount of diet. The statistical analysis demonstrates, however, that for the 5% saturated fat diet, control rats consumed significantly more food than carcinogen treated rats. Furthermore, taking the control rats as a whole, animals fed the 5% saturated fat diet consumed significantly more food than those fed either the 20% saturated fat diet or the 20% unsaturated fat diet.

The results of the weekly measurements of body weight for animals fed on the saturated and unsaturated types of fat are shown in figures 9.4 and 9.5 respectively. The mean weight gain in the four groups fed unsaturated fat was similar. On the saturated fat diet, however, control animals gained more weight than carcinogen treated animals and this finding was particularly marked with the 20% saturated fat diet.
Figure 9.3. Food consumption by animals fed four different experimental diets

**Statistical analysis**

The statistical comparison between carcinogen and control treated animals is shown above the respective columns in the figure above.

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Carcinogen groups</th>
<th>Control groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% sat vs 20% sat</td>
<td>NS</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>5% unsat vs 20% unsat</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>5% sat vs 5% unsat</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>20% sat vs 20% unsat</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>5% sat vs 20% unsat</td>
<td>NS</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>20% sat vs 5% unsat</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 9.4. Mean body weights of animals fed saturated fat diets plotted against time

Mean body weight (g)

Time (weeks)

- 5% fat (carcinogen)
- 5% fat (control)
- 20% fat (carcinogen)
- 20% fat (control)

Figure 9.5. Mean body weights of animals fed unsaturated fat diets plotted against time

Mean body weight (g)

Time (weeks)

- 5% fat (carcinogen)
- 5% fat (control)
- 20% fat (carcinogen)
- 20% fat (control)
Tumour analysis

Naked eye examination of rat colons revealed tumours with macroscopic features closely resembling the human situation. Thus there were sessile polypoid lesions, pedunculated polypoid lesions, tumours with ulcerated surfaces and tumours stenosing the lumen of the bowel (Plate 2).

Microscopic examination revealed that all three types of colonic adenoma which are recognised in man were seen in this series of animal experiments. The vast majority of adenomas were of the tubular type, several of which were microadenomas with neoplastic changes only involving a small number of colonic crypts. Furthermore, single crypt adenomas were noted as incidental findings on a number of histological sections. Very few tubulovillous adenomas were identified and only a single purely villous adenoma was recorded. An example of a rat tubulovillous adenoma is shown in plate 3.

The histological features of the rat carcinomas found were also very similar to those of human cancers, ranging from early malignant change in adenomas, consisting of malignant cells breaching the muscularis mucosa and invading the submucosa, to large cancers of advanced grade and stage. A number of different types of malignant lesion were seen including mucinous carcinomas and signet ring cell carcinomas. An example of a rat adenocarcinoma is shown in plate 4.
Plate 2. Macroscopic appearance of experimental colorectal tumours initiated by azoxymethane
Plate 3. Photomicrograph of a tubulovillous adenoma from the colon of a rat 27 weeks after commencing a course of azoxymethane (Haematoxylin & eosin)
Plate 4. Photomicrograph of a colonic adenocarcinoma from a rat 27 weeks after commencing a course of azoxymethane (Haematoxylin & eosin)
Experimental adenomas

A total of 99 adenomas were found in the four groups of carcinogen treated animals (n=59) submitted to histological examination. Eighty-eight (89%) of these were of the tubular type, 10 were tubulovillous adenomas and there was only one villous adenoma (Table 9.2).

Adenomas ranged in size from 1mm to 13mm and the details of adenoma size by dietary group are shown in Table 9.3. The maximal diameter of adenomas was highest in the 20% unsaturated fat group (4.1 ± 0.3, Mean ± SEM) and this was significantly larger than adenomas in the 20% saturated fat group (3.04 ± 0.33, p=0.0176, Mann Whitney U test). There were, however, no other significant size differences between the other dietary groups.

The degree of dysplasia in colorectal adenomas was classified as either mild, moderate or severe (Table 9.4). Animals fed a 5% saturated fat diet developed the most adenomas with severe dysplastic changes, the frequency of this finding being significantly higher than in both the 20% saturated fat diet (Χ² = 9.717, df = 1, p<0.01) and the 5% unsaturated fat diet groups (Χ² = 8.376, df = 1, p<0.01; Table 9.4). A comparison of the two 20% fat diets showed no differences in the frequency of severe dysplasia (Χ² = 0.368, df = 1, N.S., Table 9.4).
Table 9.2: Distribution of rat colonic adenomas according to histological type

<table>
<thead>
<tr>
<th>Type of adenoma</th>
<th>Saturated fat 5%</th>
<th>Saturated fat 20%</th>
<th>Unsaturated fat 5%</th>
<th>Unsaturated fat 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular</td>
<td>23</td>
<td>27</td>
<td>13</td>
<td>25</td>
</tr>
<tr>
<td>Tubulovillous</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Villous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 9.3. Size of colorectal adenomas in the four dietary groups

<table>
<thead>
<tr>
<th>Adenoma size (mm)</th>
<th>Saturated fat 5%</th>
<th>Saturated fat 20%</th>
<th>Unsaturated fat 5%</th>
<th>Unsaturated fat 20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean±SEM</td>
<td>3.5 ± 0.5</td>
<td>3.0 ± 0.3</td>
<td>3.4 ± 0.4</td>
<td>4.1 ± 0.3a</td>
</tr>
<tr>
<td>Range</td>
<td>1 - 13</td>
<td>1 - 8</td>
<td>1 - 8</td>
<td>2 - 6</td>
</tr>
</tbody>
</table>

a significantly higher than 20% saturated fat group, p=0.0176, Mann Whitney U test.
Table 9.4. Degree of dysplasia in rat colorectal adenomas

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Degree of dysplasia</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild or moderate</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>5% saturated fat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20% saturated fat</td>
<td></td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$X^2 = 9.717, df = 1, p &lt; 0.01$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                     |                      | 15 |  1 |
| 5% unsaturated fat  |                      |    |    |
| 20% unsaturated fat |                      | 26 |  5 |
|                     |                      |    |    |
| $X^2 = 0.925, df = 1, N.S.$ |
The site of each adenoma in the four dietary groups is shown in Figure 9.6. The majority of lesions developed in the distal half of the colon (Table 9.5), but in the 20% saturated fat group, 36% of the adenomas were found to be in the proximal half of the colon. This was significantly more than in the 20% unsaturated fat animals ($X^2 = 4.23$, $df = 1$, $p < 0.05$). There were no statistically significant differences between any of the other dietary groups. There was no correlation between the size (distance along the colon) and size of colorectal adenomas ($r = 0.05$, $p = 0.625$).

**Experimental carcinomas**

Four types of carcinoma were encountered: straightforward adenocarcinomas, malignant change seen in adenomatous polyps, signet ring cell carcinomas and mucinous adenocarcinomas. The macroscopic appearances were similar to the human situation with polypoid, sessile, ulcerating and stenosing varieties.

The position of each colorectal carcinoma is shown in Figure 9.7. As with the adenomatous lesions, the majority of the malignant lesions were situated in the distal half of the colon (Table 9.6). Rats fed a 20% saturated fat diet had a larger number of malignant lesions (36%) in the proximal colon than any of the other dietary groups. However, there were no statistically significant differences between the four dietary groups in this respect. The sizes of colorectal carcinomas are shown in Table 9.7. There were no statistically significant differences between carcinoma sizes in any of the four dietary groups.
Figure 9.6. Position of individual colorectal adenomas in rats fed four different diets

Anorectal junction
Relative distance along the colon (%)
Proximal caecum

Each dot represents a single adenoma

Type of dietary fat
5% saturated 20% saturated 5% unsaturated 20% unsaturated

Figure 9.7. Position of individual colorectal carcinomas in rats fed four different diets

Anorectal junction
Relative distance along the colon (%)
Proximal caecum

Each cross represents a single carcinoma

Type of dietary fat
5% saturated 20% saturated 5% unsaturated 20% unsaturated
Table 9.5. Site of rat colorectal adenomas

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Proximal half of colon</th>
<th>Distal half of colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated fat</td>
<td>3 (13%)</td>
<td>21 (87%)</td>
</tr>
<tr>
<td>20% saturated fat</td>
<td>10 (36%)</td>
<td>18 (64%)</td>
</tr>
<tr>
<td>5% unsaturated fat</td>
<td>0</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>20% unsaturated fat</td>
<td>4 (13%)</td>
<td>27 (87%)</td>
</tr>
</tbody>
</table>

*a significantly lower than 20% saturated fat group: $X^2 = 4.23$, df = 1, $p < 0.05$

Table 9.6. Site of rat colorectal carcinomas

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Proximal half of colon</th>
<th>Distal half of colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated fat</td>
<td>3 (25%)</td>
<td>9 (75%)</td>
</tr>
<tr>
<td>20% saturated fat</td>
<td>10 (36%)</td>
<td>18 (64%)</td>
</tr>
<tr>
<td>5% unsaturated fat</td>
<td>0</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>20% unsaturated fat</td>
<td>0</td>
<td>2 (100%)</td>
</tr>
</tbody>
</table>
Table 9.7. Size of colorectal carcinomas in the four dietary groups

<table>
<thead>
<tr>
<th>Carcinoma size (mm)</th>
<th>Dietary group</th>
<th>Saturated fat</th>
<th>Unsaturated fat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>20%</td>
<td>5%</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>4.3 ± 0.5</td>
<td>4.4 ± 0.3</td>
<td>3.0*</td>
</tr>
<tr>
<td>Range</td>
<td>2 - 8</td>
<td>2 - 8</td>
<td>-</td>
</tr>
</tbody>
</table>

* Single carcinoma in this group

Details of the histological grade and stage of the rat colorectal carcinomas is shown in Tables 9.8 and 9.9. The majority of carcinomas were well differentiated or moderately well differentiated. The 20% saturated fat diet group contained the most poorly differentiated carcinomas but there were no statistically significant differences between the four dietary groups.

Most carcinomas in this series were of early type invading into the submucosa and less frequently into the muscle layers of the colon. Only 2 out of the total of 43 cancers were grade B with invasion through the bowel wall and into the pericolic structures. Peritoneal carcinomatosis was seen in one rat from the 5% saturated fat group and one rat from the 20% saturated fat group. Hepatic metastases were found in a single animal which had received the 20% saturated fat diet.
Table 9.8. Histological differentiation (grade) of rat colorectal carcinomas

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>Total number of carcinomas</th>
<th>Histological grade - numbers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Good</td>
</tr>
<tr>
<td>5% saturated</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>20% saturated</td>
<td>28</td>
<td>3 (11%)</td>
</tr>
<tr>
<td>5% unsaturated</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>20% unsaturated</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 9.9. Histological stage of rat colorectal carcinomas

<table>
<thead>
<tr>
<th>Dietary fat</th>
<th>Total number of carcinomas</th>
<th>Histological stage - numbers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>5% saturated</td>
<td>12</td>
<td>9 (75%)</td>
</tr>
<tr>
<td>20% saturated</td>
<td>28</td>
<td>25 (89%)</td>
</tr>
<tr>
<td>5% unsaturated</td>
<td>1</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>20% unsaturated</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
</tbody>
</table>

A1 = invasion into the submucosa  
A2 = invasion into the muscular layers  
but confined to the bowel wall  
B = invasion through the bowel wall
Tumour incidence

No colorectal tumours developed in any of the 40 saline control animals. In the 20% saturated fat diet group, only 14 rats were examined and submitted to histological examination because one animal died in the fourteen week of the experimental protocol (autopsy of this animal did not reveal the cause of death). Table 9.10 shows the incidence of benign colorectal adenomas in the four histology groups of animals treated with azoxymethane. There were no differences in the incidence of adenoma development between the 5% or 20% saturated fat diet groups ($X^2 = 0.015, \text{N.S.}$) or between the two unsaturated fat diet groups ($X^2 = 0.253, \text{N.S.}$). A comparison of adenoma incidence rate between the two 5% fat diet groups ($X^2 = 0.12, \text{df} = 1, \text{N.S.}$) and the two 20% fat diet groups ($X^2 = 0.001, \text{df} = 1, \text{N.S.}$) again showed no differences.

Incidence data for carcinomas is shown in Table 9.11. The incidence of carcinomas in the groups of rats fed on either a 5% or 20% saturated fat diet was not different ($X^2 = 0.236, \text{df} = 1, \text{N.S.}$) and there was also no difference between the 5% and 20% unsaturated fat diets ($X^2 = 0.303, \text{df} = 1, \text{N.S.}$). However, there were significantly more carcinomas in rats fed a 5% saturated fat diet compared to a 5% unsaturated fat diet ($X^2 = 4.327, \text{df} = 1, p<0.05$). Similarly, more carcinomas developed in rats receiving a 20% fat diet containing saturated fat rather than unsaturated fat ($X^2 = 4.298, \text{df} = 1, p<0.05$). Although there were more carcinomas in the 5% saturated fat group when compared with the 20% unsaturated group, the differences were only of marginal statistical significance ($X^2 = 2.762, \text{df} = 1, 0.05 > p < 0.10$).
Table 9.10. Incidence of colorectal adenomas in the four dietary groups

<table>
<thead>
<tr>
<th>Dietary fat content</th>
<th>No. of rats examined</th>
<th>No. of rats with adenomas</th>
<th>% adenoma incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated</td>
<td>15</td>
<td>11</td>
<td>73.3</td>
</tr>
<tr>
<td>20% saturated</td>
<td>14</td>
<td>11</td>
<td>78.6</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.082, \text{ df} = 1, \text{ N.S.} \]

| 5% unsaturated      | 15                   | 9                        | 60.0               |
| 20% unsaturated     | 15                   | 12                       | 80.0               |

\[ \chi^2 = 0.253, \text{ df} = 1, \text{ N.S.} \]

Table 9.11. Incidence of colorectal carcinomas in the four dietary groups

<table>
<thead>
<tr>
<th>Dietary fat content</th>
<th>No. of rats examined</th>
<th>No. of rats with carcinomas</th>
<th>% carcinoma incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated</td>
<td>15</td>
<td>8</td>
<td>53.3</td>
</tr>
<tr>
<td>20% saturated</td>
<td>14</td>
<td>10</td>
<td>71.4</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.236, \text{ df} = 1, \text{ N.S.} \]

| 5% unsaturated fat  | 15                   | 1                          | 6.7                   |
| 20% unsaturated fat | 15                   | 2                          | 13.3                  |

\[ \chi^2 = 0.303, \text{ df} = 1, \text{ N.S.} \]
The type or level of fat in the experimental diets had no effect on the incidence rates of colorectal tumours as a whole in the four dietary groups studied (Table 9.12).

Table 9.12. Incidence of colorectal tumours as a whole (adenomas or carcinomas) in the four dietary groups

<table>
<thead>
<tr>
<th>Dietary fat content</th>
<th>No. of rats examined</th>
<th>No. of rats with tumours</th>
<th>% tumour incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated</td>
<td>15</td>
<td>14</td>
<td>93.3</td>
</tr>
<tr>
<td>20% saturated</td>
<td>14</td>
<td>12</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td><em>X2 = 0.025, df = 1, N.S.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% unsaturated</td>
<td>15</td>
<td>9</td>
<td>60.0</td>
</tr>
<tr>
<td>20% unsaturated</td>
<td>15</td>
<td>12</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td><em>X2 = 0.253, df = 1, N.S.</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Tumour yield

In the section that follows, exact statistical p values are quoted because a number of the comparisons made gave results which were close to, but not lower than, the 5% level of significance. There were no significant differences in the number of adenomas per rat between any of the four dietary groups (Figure 9.8). The number of carcinomas per animal is shown in Figure 9.9. There were more carcinomas in rats fed the 20% saturated fat diet than the other groups, the difference being less marked with the 5% saturated fat diet \( (p = 0.0736, \text{Mann Whitney U test}) \) than either the 5% unsaturated \( (p = 0.0018, \text{Mann Whitney U test}) \) or 20% unsaturated fat diets \( (p = 0.0028) \). The 5% saturated fat diet promoted a greater number of carcinomas per rat than both the 5% \( (p = 0.0279) \) and 20% \( (p = 0.0538) \) unsaturated fat diets.

The number of colorectal tumours as a whole was also highest in rats fed a 20% saturated fat diet (Figure 9.10), with more tumours per rat in this group than in animals fed on 5% saturated fat \( (p = 0.0972, \text{Mann Whitney U test}) \), 5% unsaturated fat \( (p = 0.0032) \) and 20% unsaturated fat \( (p = 0.0847) \). The tumour promoting effect of the 5% saturated fat diet was higher than that of the 5% unsaturated fat diet \( (p = 0.0202) \), but no different from a 20% unsaturated fat diet.
Figure 9.8. Yield of experimental colorectal adenomas in the four dietary groups

Number of adenomas per animal (mean ± SEM)

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Number of adenomas per animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated fat (n=15)</td>
<td>1</td>
</tr>
<tr>
<td>20% saturated fat (n=14)</td>
<td>2</td>
</tr>
<tr>
<td>5% unsaturated fat (n=15)</td>
<td>3</td>
</tr>
<tr>
<td>20% unsaturated fat (n=15)</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 9.9. Yield of experimental colorectal carcinomas in the four dietary groups

Number of carcinomas per animal (mean ± SEM)

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Number of carcinomas per animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% saturated fat (n=15)</td>
<td>1</td>
</tr>
<tr>
<td>20% saturated fat (n=14)</td>
<td>2</td>
</tr>
<tr>
<td>5% unsaturated fat (n=15)</td>
<td>3</td>
</tr>
<tr>
<td>20% unsaturated fat (n=15)</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 9.10. Yield of experimental colorectal tumours (benign and malignant) in the four dietary groups

Number of tumours per animal (mean ± SEM)

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>5% saturated fat (n=15)</th>
<th>20% saturated fat (n=14)</th>
<th>5% unsaturated fat (n=15)</th>
<th>20% unsaturated fat (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.5 ± 0.5</td>
<td>4.5 ± 0.5</td>
<td>1.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
</tr>
</tbody>
</table>

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Discussion

The macroscopic features of the tumours produced by the animal model set up in this study were similar to those seen in human colorectal cancer. Microscopic examination of the experimentally induced tumours demonstrated not only that a wide range of benign and malignant lesions had developed but also that the specific histological features of each type of lesion bore a striking resemblance to human counterparts. In this respect the findings of the present study are consistent with previous work in which hydrazine derivative carcinogens were used (Ward et al, 1973; Ward, 1974; Lamont and O'Gorman, 1978; Sunter et al, 1978; Pour, 1978; Teague et al, 1981).

Although the overall pattern of invasion of the malignant experimental tumours in this series was the same as that seen in man, the degree of invasion was less marked in the animal model. For example, 95% of the malignant experimental tumours in this study were equivalent to Dukes stage A, which is in stark contrast to the situation in man where only 15% are Dukes stage A (Vellacott et al, 1987). This feature of the hydrazine derivative experimental model of colorectal cancer has been noted by others (Teague et al, 1981; Ross, 1982). The metastatic potential of experimental and human colorectal cancers also appears to be different. Lymph node, peritoneal and hepatic metastases are all commonly seen in the human disease but were encountered only rarely in the experimental tumours in this study. This again is consistent with previous studies (Sunter et al, 1978; Chang, 1978; Lamont, 1978).

There has been a good deal of controversy relating to the role of
the polyp-cancer sequence in animal models of colorectal tumours. Some workers have seen malignant change in experimentally induced adenomatous polyps (Wiebecke et al, 1973; Sunter et al, 1978), whereas other groups have suggested that malignant lesions only arise from flat colonic mucosa (Ward, 1974; Maskens, 1976). The results of the present study demonstrate that azoxymethane induced carcinomas may arise both de novo from flat colonic mucosa and from an adenomatous polyp.

The differences between human colorectal cancers and the chemically induced cancers of animal models, suggest that the experimental tumours described are not good models for the study of colorectal metastasis. The many animal studies which have investigated the relationship between dietary fat intake and colorectal cancer do, however, suggest that the animal model is a useful one for this specific purpose. The overall features of the animal model in this study were similar to those found in other comparable studies and this validates its use in the present investigation.

Analysis of the tumour yield data shows a clear trend towards high saturated fat diets being associated with greater numbers of colorectal tumours than low saturated fat diets. This finding serves to confirm the work of other groups who have shown that diets containing high levels of a number of different types of fat promote experimental tumours which have been induced by chemical carcinogens (Nigro et al, 1975; Reddy et al, 1975; Reddy et al, 1976; Broitman et al, 1977; Bansal et al, 1978; Bull et al, 1979; Reddy and Maeura, 1984).

The tumour yield data for malignant lesions and total tumours also showed that saturated fat was more effective in azoxymethane induced colorectal carcinogenesis than unsaturated fat. Indeed, the number of
cancers in the unsaturated fat diet groups were so low that it is suggested that these diets were exerting a protective effect against the development of colorectal cancers induced by this chemical means. This finding is in conflict with the work of Reddy et al (1974; 1977a), who found that a 5% corn oil based unsaturated fat diet enhanced colorectal tumour induction when compared to a 5% lard based saturated fat diet and Sakaguchi et al (1984) who found that a semipurified diet containing 5% unsaturated fat as linoleic acid promoted more tumours than a 5% saturated fat diet made up largely with stearic acid. Reddy et al (1974; 1977a) also investigated the effect of 20% saturated and unsaturated fat diets, but found no differences in tumour promotion at this level of fat intake.

The experiments carried out by these other groups are not exactly comparable with the present study. Reddy et al (1974; 1977a) used dimethylhydrazine as the chemical carcinogen and a different strain of rats from the Wistar strain used in the present study. The experimental design of the animal work in this thesis was, however, similar to that of Sakaguchi et al (1984). In fact, the latter group used a different administration protocol for azoxymethane, but despite this the total dose used was 81.4 mg which compares very favourably with the 90 mg total dose of the present study. It would, therefore, be difficult to attribute the major differences between tumour incidences found in the work of Sakaguchi et al and the present study to such minor differences in experimental protocol. It is more likely that although a diet containing 5% unsaturated fat was used in both studies, the specific composition of the fat was very different in each case. Sakaguchi et al (1984) used a semipurified diet supplying the
unsaturated fat as linoleic acid ethyl ester. The corn oil used in the work described in chapter 8 contains a high proportion of non-esterified linoleic acid and has a more complex overall fatty acid composition.

The four diets used in the present study were specially devised so that the level and type of fat was the only major difference between them. The levels of all other nutrients were balanced in order to effectively eliminate any influence that they may have had on the development of colorectal tumours. Differences in total food intake between the four dietary groups could, however, have led to discrepancies in the absolute levels of consumption of different nutrients. In order to take account of this, an attempt was made to assess food consumption in the different experimental groups. In general terms there was a similar level of food consumption in the different dietary groups. There was, nevertheless, a tendency for animals fed diets containing a 20% level of fat to eat less than those fed a diet containing a 5% level of fat and this finding has previously been noted by other workers (Reddy and Maeura, 1984). However, the difference in food consumption between groups was never greater than 12% and it is unlikely that such small differences would, per se, have an important effect on tumour development. Furthermore, animal groups fed the 20% saturated fat diet gained more weight than those fed the 5% saturated fat despite the fact that the food consumption data suggested that the latter were eating more.

Although it is possible that the markedly lower number of tumours in the unsaturated fat groups in the present study were due to an error in the administration of the chemical carcinogen, this is an unlikely
explanation. All the carcinogen injections were given by two experienced workers, using the same batch of azoxymethane. Furthermore, because the carcinogen was administered in weekly aliquots administered over a six week period, any error occurring at individual injections would have a smaller impact on the final total dose given than if this was administered in one aliquot.

Thus, the large differences in tumour incidence between the dietary groups must be regarded as a real phenomenon. The animal experiments were designed with the aim of using four uniform groups of rats in which the type and quantity of fat intake was the major variable. The differences in tumour incidence between the groups are, therefore, assumed to be due to the differences in dietary fat intake.

In this study, there were some notable differences in tumour distribution between the dietary groups. Every tumour in the animals fed unsaturated fat diet developed in the distal half of the colon. In contrast, rats fed saturated fat developed proximal tumours as well as distal ones, with a higher proportion of proximal lesions in the 20% group than the 5% group. It is known that the total dose of carcinogen influences the distribution of lesions, with more distal tumours being produced by the administration of lower doses of azoxymethane (Ward, 1975). The differences in tumour distribution in this study might suggest that the animals fed unsaturated fat diets were given a lower dose of carcinogen than those fed saturated fat. For this to happen the rats in the unsaturated fat group would have had to receive a reduction in total dose of carcinogen in the order of 50% and in view of the careful techniques used, this seems a most unlikely explanation.
Conclusions

Animals fed a high or low saturated fat diet developed significantly more carcinomas per rat than those fed a high or low unsaturated fat diet. There were, however, no differences in the yield of adenomas between the four dietary groups. These results suggest that saturated fat diets promote colorectal carcinomas which have been initiated by a chemical carcinogen. In contrast, unsaturated fat diets, whilst not affecting the development of colorectal adenomas, appeared to exert a marked inhibitory effect on the development of colorectal carcinomas.
Alterations in cell membrane structure and function is one possible mechanism by which dietary fat and colorectal cancer are linked. This chapter examines the relationship between dietary fat intake and cell membrane fatty acid profiles in an experimental model of colorectal cancer.

Animals and methods

The same animal model described in chapter 9 was used. Five week old rats were divided into four dietary groups as before:

1. 5% saturated fat diet 42 rats
2. 20% saturated fat diet 42 rats
3. 5% unsaturated fat diet 42 rats
4. 20% unsaturated fat diet 42 rats

In each dietary group, 25 rats were treated with azoxymethane at a dose of 15 mg/Kg i.p. once a week for 6 weeks. The remaining 17 rats in each group acted as controls and were injected with an equal volume of carrier solution (0.9% saline) weekly for 6 weeks. The 10 control rats in each dietary group were the same animals used as controls in the tumour study (chapter 9).
Diet and water were given ad libitum and the rats were observed daily and weighed weekly. The 42 animals in each dietary group were sacrificed at two separate times to provide two points of analysis as follows:

a) To study the influence of azoxymethane

In this experiment, 7 carcinogen treated rats were killed one week after the last injection of azoxymethane and 7 control rats were killed one week after the last injection of saline. Samples of blood, intra-abdominal adipose tissue and colonic mucosa were taken from each animal for fatty acid analysis. Colonic mucosa was separated from the underlying muscle layers by drawing a clean glass microscope slide along the length of the colon under pressure. Microscopic examination of the samples produced by this technique confirmed that perfect separation of mucosa from muscle was achieved.

b) To study the influence of colorectal tumours

Eighteen azoxymethane treated rats and 10 saline treated control rats from each dietary group were killed 20 weeks after the last injection of carcinogen. Samples of blood, adipose tissue, colonic mucosa and colonic tumour were removed for fatty acid analysis. The whole colon and rectum was removed, opened along its length and faecal material washed away with water. Macroscopic tumours were carefully removed and all the tumours from each colon were pooled for analysis. The colonic mucosa remaining was then removed using the glass slide technique.
Membrane fatty acid analysis

Cell membrane preparation, fatty acid extraction, methylation, identification and quantitation in erythrocytes, adipose tissue, mucosa and tumours was performed using the methods described in chapter 4.

Statistical analysis

All statistical analyses were performed on the University of Leicester mainframe computer using the Minitab computer package. Fatty acid data was analysed using two separate methods:

1) The levels of individual fatty acids between groups were compared using the non-parametric Mann Whitney U test.

2) Fatty acids profiles were analysed by multivariate analysis of variance according to the method suggested by Aitchison (1982). Because the data were in the form of proportions, they were subject to the constraint:

\[ X_1 + X_2 + X_3 + X_4 + X_5 = 100\% \]

(where \(X_1\)–\(X_5\) were the individual fatty acids in the profile).

This means that although in each case there appeared to be five readings, there were effectively only four, because when four were known, the fifth was automatically determined. In order to produce approximate normality the data were transformed into log ratios as
follows:

\[
\frac{Y_1}{X_1} = \log \frac{X_2}{X_1} \quad \frac{Y_2}{X_1} = \log \frac{X_3}{X_1} \quad \frac{Y_3}{X_1} = \log \frac{X_4}{X_1} \quad \frac{Y_4}{X_1} = \log \frac{X_5}{X_1}
\]

Y1 - Y4 were then analysed by the multivariate method using the following four statistical tests: Wilks' criterion, Pillai's trace, the Hotelling-Lawley trace and Roy's maximum root criterion. These tests all yielded an F value, the statistical significance of which was then determined by reference to statistical tables (Documenta-Geigy). The results of the four tests were consistent in all cases and for simplicity only the F values from the Wilks' criterion will be quoted for each analysis. This method of analysis was used to determine how the fatty acid profile as a whole was affected by a particular influence.

Results

The influence of azoxymethane

The influence of the chemical carcinogen was tested in rats that were killed one week after the last injection of azoxymethane i.e. early in the latent period before tumour development. The results have been summarised in bar charts showing the tissue fatty acid profiles of carcinogen and control animal results plotted side by side for comparison. The level of individual fatty acids in carcinogen and control tissues were compared by the Mann Whitney U test and the p values of significant results are given above the relevant fatty acid bars.
Erythrocytes

Erythrocyte fatty acid profiles for these animals are shown in figures 10.1 - 10.4. For all four diets differences were recorded between the carcinogen and control groups. In particular, the proportion of arachidonic acid was always lower in the carcinogen treated animals when compared to controls fed on the same diet, although this difference did not reach statistical significance in either of the unsaturated fat groups. The type of diet also had an effect, with fewer differences between carcinogen and control animals found in rats fed unsaturated fat than in those fed saturated fat.

Adipose tissue

Adipose tissue fatty acid profiles are shown in figures 10.5 - 10.8. Azoxymethane treated animals demonstrated different adipose tissue fatty acid profiles from their controls. As with the erythrocytes, the pattern of differences seen varied with diet but there were no consistent features.

Colonic mucosa

Colonic mucosa fatty acid profiles are shown in figures 10.9 - 10.12. Once again differences were apparent between carcinogen and control animals for all four dietary groups. In contrast to the erythrocyte findings, differences were less marked in the rats fed the saturated fat diet.
Figure 10.1. Influence of azoxymethane: Erythrocyte fatty acid profile in rats fed 5% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic</td>
<td>p&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td>p&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.2. Influence of azoxymethane: Erythrocyte fatty acid profile in rats fed 20% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic</td>
<td>p&lt;0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td>p&lt;0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10.3. Influence of azoxymethane: Erythrocyte fatty acid profile in rats fed 5% unsaturated fat diet

- Palmitic
- Stearic
- Oleic
- Linoleic
- Arachidonic

Fatty acid content (relative %)

Carcinogen
Control
Mean ± SEM

Figure 10.4. Influence of azoxymethane: Erythrocyte fatty acid profile in rats fed 20% unsaturated fat diet

- Palmitic
- Stearic
- Oleic
- Linoleic
- Arachidonic

Fatty acid content (relative %)

Carcinogen
Control
Mean ± SEM

194
Figure 10.5. Influence of azoxymethane: Adipose tissue fatty acid profile in rats fed 5% saturated fat diet

![Fatty acid content (relative %) for Palmitic, Stearic, Oleic, Linoleic, and Arachidonic fatty acids.](image1)

- Carcinogen
- Control
- Mean ± SEM

Figure 10.6. Influence of azoxymethane: Adipose tissue fatty acid profile in rats fed 20% saturated fat diet

![Fatty acid content (relative %) for Palmitic, Stearic, Oleic, Linoleic, and Arachidonic fatty acids.](image2)

- Carcinogen
- Control
- Mean ± SEM
Figure 10.7. Influence of azoxymethane: Adipose tissue fatty acid profile in rats fed 5% unsaturated fat diet

Figure 10.8. Influence of azoxymethane: Adipose tissue fatty acid profile in rats fed 20% unsaturated fat diet
Figure 10.9. Influence of azoxymethane: Colonic mucosa fatty acid profile in rats fed 5% saturated fat diet

Fatty acid content (relative %)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Carcinogen</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>p &lt; 0.02</td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.10. Influence of azoxymethane: Colonic mucosa fatty acid profile in rats fed 20% saturated fat diet

Fatty acid content (relative %)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Carcinogen</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>p &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>p &lt; 0.02</td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10.11. Influence of azoxymethane: Colonic mucosa fatty acid profile in rats fed 5% unsaturated fat diet

Figure 10.12. Influence of azoxymethane: Colonic mucosa fatty acid profile in rats fed 20% unsaturated fat diet
Multivariate analysis of azoxymethane control groups
(animals sacrificed 7 weeks into the experimental protocol)

The influence of a number of different factors on the overall profile of tissue fatty acids were analysed by multivariate analysis of variance (Aitchison, 1982) and the results are summarised in Table 10.1. This statistical technique can be used to assess the influence of both individual factors and combinations of factors on the fatty acid profile. In this part of the animal study the influence of azoxymethane was the single factor of interest.

Analysis A shows that both the type and level of dietary fat have a significant influence on membrane fatty acid profiles and that a combination of these two factors has an effect which is more complex than a simple summation of the individual effects of either. Thus all four diets have different effects on membrane fatty composition.

Analysis B demonstrates that the fatty acid profiles of the three tissues are different and that the administration of azoxymethane does have a significant influence on the fatty acid profiles of these tissues.

Analysis C shows that the treatment (carcinogen or saline) and tissue effects depend on the type and quantity of fat in the diet. In addition, the treatment effect depends on the tissue under consideration.
Table 10.1. Factors influencing membrane fatty acid profiles in animals sacrificed 7 weeks into the experimental protocol

<table>
<thead>
<tr>
<th>Variable</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Type of fat (sat/unsat)</td>
<td>50.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Level of fat (5/20%)</td>
<td>12.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Type × Level</td>
<td>16.0</td>
<td>0.0001</td>
</tr>
<tr>
<td>B) Tissue (rbc/fat/muc)</td>
<td>93.5</td>
<td>0.00001</td>
</tr>
<tr>
<td>Treatment (aom/control)</td>
<td>68.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>C) Tissue × Type of fat</td>
<td>35.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tissue × Level of fat</td>
<td>6.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tissue × Type × Level</td>
<td>4.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment × Type of fat</td>
<td>6.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment × Level of fat</td>
<td>6.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Treatment × Type × Level</td>
<td>3.7</td>
<td>0.008</td>
</tr>
<tr>
<td>Treatment × Tissue</td>
<td>7.2</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

All F values quoted were calculated by Wilk's criterion × denotes interaction between the factors listed

aom azoxymethane
rbc erythrocytes
fat adipose tissue
muc colonic mucosa
The influence of colorectal tumours

Erythrocytes

The fatty acid profiles of erythrocytes from rats fed the four experimental diets are shown in figures 10.13 - 10.16. Rats fed a 5% saturated fat diet had a similar overall profile of fatty acids to those fed a 20% saturated fat diet and the same can be seen in animals fed unsaturated fat. Only in the 20% saturated fat group were there significant differences between carcinogen and control animals.

Erythrocyte stearic: oleic acid ratio

The erythrocyte stearic: oleic acid ratios for the different dietary groups are shown in figure 10.17. In the 5% saturated fat group, the ratio was found to be significantly lower in carcinogen treated tumour bearing animals compared to controls. The stearic: oleic ratio was also lower in carcinogen treated animals fed with 20% saturated or 20% unsaturated fat when compared to their appropriate controls but the differences in these groups did not reach statistical significance. In rats fed a 5% unsaturated fat diet, the mean stearic: oleic acid ratio was slightly higher for animals with tumours compared to controls, but again the differences were not statistically significant. Figure 10.17 also shows that the mean values of the stearic: oleic acid ratio varied considerably between the four dietary groups. Animals fed either type of unsaturated fat demonstrated ratios close to one but considerably higher values were found in the animals fed high or low saturated fat diets.
Figure 10.13. Erythrocyte fatty acid profile in rats fed 5% saturated fat diet

Fatty acid content (relative %)

Fatty acids

Figure 10.14. Erythrocyte fatty acid profile in rats fed 20% saturated fat diet

Fatty acid content (relative %)

Fatty acids
Figure 10.15. Erythrocyte fatty acid profile in rats fed 5% unsaturated fat diet

Fatty acid content (relative %)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.16. Erythrocyte fatty acid profile in rats fed 20% unsaturated fat diet

Fatty acid content (relative %)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Palmitic</th>
<th>Stearic</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Arachidonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinogen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10.17. Erythrocyte stearic: oleic acid ratios in rats fed four different experimental diets
Adipose tissue

The fatty acid composition of adipose tissue from cancer and control animals is shown in figures 10.18 - 10.21. Similarities in overall fatty acid profiles for rats fed either saturated or unsaturated fats can be seen by naked eye examination of the bar charts. There were few differences between the profiles of cancer and control animals except for those rats fed 20% unsaturated fat. The unsaturated fat diet contained a high proportion of linoleic acid (see figure 9.2) and this is reflected in the adipose tissue composition of animals fed this type of fat. The proportion of linoleic acid in adipose tissue for rats fed the 5% and 20% unsaturated fat diet was approximately 40% and 50% respectively. The adipose tissue level of linoleic acid in rats fed the saturated type of fat was considerably lower (10% - 30%) and in these animals a higher level of oleic acid was seen in the adipose tissue. Comparison between figures 9.1 - 9.2 and 10.18 - 10.21 suggest that these differences are a direct reflection of the dietary level of these individual fatty acids.

Colonic mucosa

The results of the analysis of colonic mucosa fatty acid composition are shown in figures 10.22 - 10.25. For each of the four dietary groups, the overall fatty acid profiles of colonic mucosa and adipose tissue are similar. The proportion of linoleic acid in colonic cell membranes was higher in the animals fed unsaturated fat than those fed saturated fat.
Figure 10.18. Adipose tissue fatty acid profile in rats fed 5% saturated fat diet

![Graph showing fatty acid profile](image-url)

Figure 10.19. Adipose tissue fatty acid profile in rats fed 20% saturated fat diet

![Graph showing fatty acid profile](image-url)
Figure 10.20. Adipose tissue fatty acid profile in rats fed 5% unsaturated fat diet

Figure 10.21. Adipose tissue fatty acid profile in rats fed 20% unsaturated fat diet
Figure 10.22. Colonic mucosa fatty acid profile in rats fed 5% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Carcinogen</th>
<th>Control</th>
<th>Mean + SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.23. Colonic mucosa fatty acid profile in rats fed 20% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Carcinogen</th>
<th>Control</th>
<th>Mean + SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10.24. Colonic mucosa fatty acid profile in rats fed 5% unsaturated fat diet

Figure 10.25. Colonic mucosa fatty acid profile in rats fed 20% unsaturated fat diet
Multivariate analysis of tumour bearing animals and their controls (animals sacrificed 27 weeks into the experimental protocol)

Tissue fatty acid profiles were analysed using the multivariate model in order to demonstrate the influence of both individual factors and combinations of factors. The results are summarised in Table 10.2. Analysis A shows that both the type and level of dietary fat intake have a significant influence on the tissue fatty acid profiles in both the carcinogen and control animals. There is a significant interaction between the type and level of fat in influencing the fatty acid profiles. In other words, the effects of these two factors are not simply additive. Thus, all four diets have different effects on membrane fatty acid profile.

Analysis B confirms that the fatty acid profiles of the three tissues are different. In contrast to the study of tissues from rats killed 7 weeks into the experimental protocol, by 27 weeks the treatment with either azoxymethane or saline does not have a significant effect on membrane fatty acid profiles.

Analysis C demonstrates that the tissue effect is different for each of the four different diets, with the type and level of fat and the interaction of the two all having significant influences.
Table 10.2. Factors influencing the membrane fatty acid profiles of erythrocytes, adipose tissue and colonic mucosa in animals killed 27 weeks into the experimental protocol

<table>
<thead>
<tr>
<th>Variable</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Type of fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(sat/unsat)</td>
<td>265.8</td>
<td>0.000001</td>
</tr>
<tr>
<td>Level of fat (5/20%)</td>
<td>71.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Type * Level</td>
<td>62.5</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>B) Tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rbc/fat/muc)</td>
<td>352.2</td>
<td>0.000001</td>
</tr>
<tr>
<td>Treatment (aom/saline)</td>
<td>1.3</td>
<td>0.29 (NS)</td>
</tr>
<tr>
<td><strong>C) Tissue * Type of fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue * Level of fat</td>
<td>33.9</td>
<td>0.000001</td>
</tr>
<tr>
<td>Tissue * Type * Level</td>
<td>17.8</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

All F values quoted were calculated by Wilk's criterion
\* denotes interaction between the factors listed

aom azoxymethane
rbc erythrocytes
fat adipose tissue
muc colonic mucosa
Colonic tumour membrane fatty acid profiles

Figures 10.26 - 10.29 compare the fatty acid content of non-malignant colonic mucosa and colonic tumour tissues from carcinogen treated animals in each of the dietary groups. In all four groups there was a significantly higher proportion of arachidonic acid in tumour cell membranes when compared to cell membranes isolated from the surrounding non-malignant colonic mucosa. In rats fed saturated fat the higher tumour arachidonic acid was balanced by lower proportions of palmitic acid (5% group; Figure 10.26) and oleic acid (20% group; Figure 10.27). On the unsaturated fat diets, the higher tumour arachidonic acid was balanced mainly by a lower proportion of linoleic acid, although other more minor differences were seen between tumour tissue and colonic mucosa (Figures 10.28 and 10.29).

The results of the multivariate analysis of the tumour and colonic mucosa fatty acid profiles overall are summarised in Table 10.3. Analysis A shows that the type and level of dietary fat each had a significant effect on fatty acid profiles. However, the F value for the interaction between the two is also significant thus demonstrating that the four diets all have a different effect.

Analysis B confirms that the mucosa and tumour cell membranes were significantly different over the whole experiment. Analysis C shows that the difference between mucosa and tumour varies with both the type and level of dietary fat. That the analysis of the interaction between these two factors yields a non-significant F value shows that the influences of type and level of fat interact in a simple additive way and not by a more complex mechanism. Thus, the effect of the 20% level...
Figure 10.26. Fatty acid profiles of colonic mucosa and tumour tissue in rats fed 5% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Colonic mucosa</th>
<th>Tumour tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>Mean ± SEM</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 10.27. Fatty acid profiles of colonic mucosa and tumours in rats fed 20% saturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Colonic mucosa</th>
<th>Tumour tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>Mean ± SEM</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Stearic</td>
<td></td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 10.28. Fatty acid profile of colonic mucosa and tumour tissue from rats fed 5% unsaturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Colonic mucosa</th>
<th>Tumour tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td>p&lt;0.01</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM

Figure 10.29. Fatty acid profile of colonic mucosa and tumours in rats fed 20% unsaturated fat diet

<table>
<thead>
<tr>
<th>Fatty acid content (relative %)</th>
<th>Colonic mucosa</th>
<th>Tumour tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Stearic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>p&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td>p&lt;0.0001</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SEM
and the saturated type of fat can be predicted by adding together the individual effects of each. With saturated fat the difference between the 5% and 20% level is minimal, that is the proportion of arachidonic acid is higher in the tumours but the other fatty acids are about the same (Figures 10.26 and 10.27). With the unsaturated fats there is an increase in stearic acid and arachidonic acid and a decrease in linoleic acid (Figures 10.28 and 10.29).

Table 10.3. Factors influencing the membrane fatty acid profiles of colonic mucosa and colonic tumours in animals killed 27 weeks into the experimental protocol

<table>
<thead>
<tr>
<th>Variable</th>
<th>F value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Type of fat (sat/unsat)</td>
<td>87.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Level of fat (5/20%)</td>
<td>17.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>Type × Level</td>
<td>82.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>B) Tissue (mucosa/tumour)</td>
<td>48.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>C) Tissue × Type of fat</td>
<td>19.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tissue × Level of fat</td>
<td>7.5</td>
<td>0.0001</td>
</tr>
<tr>
<td>Tissue × Type × Level</td>
<td>1.1</td>
<td>0.37 (NS)</td>
</tr>
</tbody>
</table>

All F values quoted were calculated by Wilk's criterion ×denotes interaction between the factors listed.
The influence of dietary linoleic acid

To assess the influence of dietary linoleic acid, the content of linoleic acid in the adipose tissue from rats fed both 5% and 20% unsaturated fat was correlated with the levels of other fatty acids in various tissues. The results are summarised in Tables 10.4 and 10.5. It can be seen that there is a significant negative correlation between adipose tissue linoleic acid and oleic acid in colonic mucosa and tumours in carcinogen treated animals (Figures 10.30 and 10.31). For the control colonic mucosa a similar trend was seen but the relationship did not quite reach statistical significance. Similarly the r values derived by correlating adipose tissue linoleic acid and colonic mucosa or tumour arachidonic acid were always negative suggesting an inverse relationship between the two. However, in this case the r value was only high enough to produce a significant result in colonic tumour tissue.
Table 10.4. Correlations between adipose tissue linoleic acid content in rats fed unsaturated fat and the levels of oleic acid in colonic mucosa and tumours

<table>
<thead>
<tr>
<th></th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonic mucosa from saline treated controls</td>
<td>-0.403</td>
<td>NS</td>
</tr>
<tr>
<td>Colonic mucosa from azoxymethane treated animals</td>
<td>-0.818</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Colonic tumours</td>
<td>-0.614</td>
<td>p &lt; 0.002</td>
</tr>
</tbody>
</table>

Table 10.5. Correlations between adipose tissue linoleic acid content in rats fed unsaturated fat and the levels of arachidonic acid in colonic mucosa and tumours

<table>
<thead>
<tr>
<th></th>
<th>r value</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonic mucosa from saline treated controls</td>
<td>-0.021</td>
<td>NS</td>
</tr>
<tr>
<td>Colonic mucosa from azoxymethane treated animals</td>
<td>-0.094</td>
<td>NS</td>
</tr>
<tr>
<td>Colonic tumours</td>
<td>-0.528</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 10.30. Relationship between adipose tissue linoleic acid content and colonic mucosal oleic acid content in carcinogen treated rats

Adipose tissue linoleic acid content (relative %)

Colonic mucosal oleic acid content (relative %)

$r = -0.818$
$p < 0.001$

Figure 10.31. Relationship between adipose tissue linoleic acid content and colonic tumour oleic acid content in carcinogen treated rats

Adipose tissue linoleic acid content (relative %)

Colonic tumour oleic acid content (relative %)

$r = -0.614$
$p < 0.002$
Discussion

Multivariate analysis demonstrated that both the type and quantity of dietary fat influenced the fatty acid profiles of the adipose tissue and the cell membranes in erythrocytes, colonic mucosa and colorectal tumours. That the fatty acid composition of the adipose tissue is closely related to the dietary intake of fat has previously been noted in several human studies (Insull and Bartsch, 1967; Sanders et al, 1978; Beynon et al, 1980; Riemersma et al, 1986). This finding is not unexpected in view of the very low turnover rate of adipose tissue, which is a reflection of its primary role as a long term energy store (Beynon et al, 1980).

The fatty acid profiles of adipose tissues and colonic mucosa within individual dietary groups were often similar and the multivariate analysis confirmed that the cell membrane structure of colonic mucosal cells is heavily influenced by the type and quantity of dietary fat. This new finding is potentially of great significance because the colonocyte cell membrane is likely to be an important site at which carcinogenic stimuli exert their effects. It is clear from chapter 2 that the functional characteristics of cell membranes, such as fluidity and permeability, are largely determined by their fatty acid profiles and control mechanisms which are thought to maintain a relatively constant ratio of saturated and unsaturated fatty acids have been described both in man (Popp-Snijders et al, 1986) and the rat (Gibson et al, 1984). This regulation of cell membrane fluidity is essential for homeostasis and there is evidence of increased membrane fluidity in certain malignant cells (Petitou et al, 1978; Mountford et
Increased membrane fluidity is associated with higher rates of cell division (Sandemann, 1979) which is a characteristic feature of malignant cells. This is, therefore, one mechanism by which dietary fat and colorectal cancer could be linked.

The multivariate analysis used in this work also indicated that azoxymethane per se had an influence on cell membrane fatty acid composition in the immediate period after its administration but that this effect had disappeared 20 weeks later. Many studies which have used chemical carcinogens have not included appropriate animal groups to control for these substances and this is clearly a requirement before the results can be interpreted.

The most striking finding in the analysis of tumour cell membrane fatty acid composition was the consistently high levels of arachidonic acid in colorectal tumour tissue when compared to the surrounding non-malignant colonic mucosa. As discussed previously, the proportional nature of the data dictates that changes in individual fatty acids within the profile must be interpreted with caution. However, the proportionate level of arachidonic acid was found to be high in tumours independent of the type and level of dietary fat intake. Furthermore, the multivariate analysis confirms the presence of excess arachidonic acid in tumours which was suggested by the simpler form of analysis. An excess of arachidonic acid in the neutral lipid and phospholipid fractions of experimental colorectal tumours has previously been reported (Sakaguchi et al, 1984; Minoura et al, 1988), but this is the first demonstration of increased arachidonic acid when using purified cell membrane preparations. The excess of arachidonic acid was found to be of a slightly lower magnitude in animals fed unsaturated rather
than saturated fat and this may be relevant in view of the much lower yield of malignant tumours in the unsaturated groups. The main importance of arachidonic acid is that it is the immediate precursor of the prostaglandins. These substances have a very short half-life and are therefore synthesised locally from arachidonic acid, which has been released from cell membrane phospholipids by the phospholipase enzymes.

There has been an accumulation of evidence which suggests that prostaglandins are involved in certain types of carcinogenesis (Lynch et al 1978; Pelus and Bockman, 1979; Pollard and Luckert, 1981; Kollmorgen et al, 1983). An especially relevant study is the work of Minoura et al (1988), which demonstrated increased levels of prostaglandin E2 in experimental colorectal tumours relative to the surrounding non-malignant colonic mucosa. Other indirect evidence is also available. The phorbol diesters, which are potent tumour promoters, have been shown to stimulate the conversion of cell membrane arachidonic acid into prostaglandins (Levine et al, 1977). It has also been shown that phospholipase A2 inhibitors, which block the release of membrane arachidonic acid, are potent inhibitors of skin tumour promotion (Fischer et al, 1982), suggesting that the metabolic products of arachidonic acid metabolism are essential for tumour growth. Furthermore, Narisawa et al (1981) showed that the cyclo-oxygenase inhibitor indomethacin reduces the development of methylnitrosourea-induced colon tumours in rats. Finally, prostaglandins have been shown to be co-carcinogens in mouse cutaneous carcinogenesis (Lupulescu, 1980).

Thus, the main implication of the study of tumour fatty acid profiles is that arachidonic acid or its prostaglandin metabolites are
possible promotors or co-carcinogens in the development of colorectal tumours, at least in chemically induced experimental models.

As discussed in chapter 9, the tumour yield data suggest that unsaturated fat may exert a protective effect against the development of colorectal tumours which have been induced experimentally using azoxymethane. Pure corn oil, which contains a very high proportion of linoleic acid was used as the source of unsaturated fat. Dietary linoleic acid is known to be the single most important factor in the control of fatty acid synthesis. In particular, linoleic acid suppresses the delta9-desaturase enzyme which controls the desaturation of stearic to oleic acid (Jeffcoat and James, 1978). Adipose tissue linoleic acid content has been shown to reflect the dietary intake of this fatty acid (Riemersma et al, 1986) and it was assumed that this relationship held true in the present study. The adipose tissue linoleic acid was therefore correlated with the oleic acid and arachidonic acid in colonic mucosa and tumours. This revealed strong negative correlations between adipose (dietary) linoleic acid and colonic mucosa and tumour oleic acid which is consistent with the inhibitory action of dietary linoleic acid on the delta9-desaturase system. What was more interesting, however, was the strong negative correlation between adipose (dietary) linoleic acid and tumour arachidonic acid in the malignant group. This finding suggests that high dietary linoleic acid in some way inhibits the accumulation of arachidonic acid in tumour cell membranes and this is a possible explanation of the apparent inhibitory effect of the unsaturated fat diets on colorectal carcinogenesis in the animal model. There is no immediately clear biochemical explanation for this tentative mechanism.
Conclusions

The data presented demonstrates that the fatty acid composition of the diet exerts an important influence on the profile of fatty acids in the cell membranes of colonocytes. The modulation of colonic mucosal fatty acid composition by dietary fat may increase the susceptibility of the colon to carcinogens. The presence of very high levels of arachidonic acid in experimental colorectal tumours suggests that this fatty acid or its prostaglandin metabolites are possible promoters or co-carcinogens in colorectal cancer.
Chapter 11
SUMMARY AND CONCLUSIONS

The available evidence indicates that the development of colorectal cancer is a multi-stage process with a variable interaction between genetic and environmental factors at each stage. Epidemiological studies suggest that dietary fat intake plays a significant role in the aetiology of colorectal cancer and this finding has been supported by experimental evidence using rodent models of colorectal cancer.

Although the mechanism of the promotional effect of dietary fat is unknown, the cell membrane is a likely point at which dietary fat might exert an influence and this has been the focus of the work presented in the preceding chapters. Recent interest has centred on the erythrocyte membrane fatty acid composition in a variety of human and experimental malignancies. The present study is, however, the first to investigate the relationship between dietary fat intake and the fatty acid composition of malignant colorectal tissue at the cell membrane level.

The data presented in chapters 6 and 10 do not support the finding of a consistently low erythrocyte stearic: oleic acid ratio in both human (Wood et al, 1985) and animal malignancies (Habib et al, 1987b). Work from several other centres which have sought to investigate the erythrocyte fatty acid profile in a number of different tumours has also shown that the erythrocyte saturation index cannot be used to separate cancer patients from controls with any accuracy (Taylor et al, 1987b; Lawson et al, 1987; Thomas et al, 1988; Soreide et al, 1987).
The erythrocyte stearic: oleic acid ratio cannot, therefore, be used as a marker for any of the tumours studied so far. It is difficult to explain the discrepancy between the results of the original work in this area (Wood et al, 1985) and those subsequent studies mentioned above. Nonetheless, it is clear that methodology in general, and the handling of erythrocytes in particular, is important and has not been standardised between studies.

The case-control study of erythrocyte fatty acid profiles described in chapter 6 included an analysis of dietary fat intake. Although the assessment of dietary nutrient intake in man is difficult, an attempt was made to use a modern method and to standardise this very carefully. The positive correlations between certain parameters of fat intake in the diet and the same parameters in the adipose tissue lends some support to the claim that the seven day dietary recall method provides an accurate assessment of habitual fat intake. It was not, however, surprising to find that the dietary analysis failed to demonstrate any significant differences between the cancer and control groups in both the human studies. This finding is consistent with the results of several other case-control studies as reviewed in chapter 1. An interesting observation from the dietary analysis was the inverse correlation between age and the consumption of unsaturated fats relative to saturated fats.

The small differences in erythrocyte fatty acids, and the unusual associations between dietary, erythrocyte and adipose tissue fatty acids, cannot be fully explained at the present time but might be indicative of altered host fat metabolism. However, it was not a specific aim of this work to investigate this area which remains a
poorly understood one.

The second human study described in chapter 7 demonstrated that the colonic mucosal cell membrane fatty acid profiles of cancer patients and their non-malignant controls in either the haemorrhoid or diverticular groups were not statistically different. In contrast, cell membranes from malignant tissue were found to have a different fatty acid profile from the surrounding non-malignant mucosa in the same part of the colon. This finding implies altered fatty acid metabolism in the malignant tissue, but whether this is a cause or an effect of the carcinogenic process has yet to be determined. Although there was a slightly higher arachidonic acid content in the human malignant tissue compared to non-malignant and control colonic mucosa, the differences were not statistically significant. Thus, with specific regard to tumour arachidonic acid levels, there was a discrepancy between the human and animal work. The stearic to oleic acid ratio was not different in malignant cell membranes when compared to non-malignant colonic membranes or control tissues. It is difficult to reconcile this finding with the suggestion that tumours release a desaturation factor into the circulation (Habib et al, 1987a).

The evidence presented in chapter 2 demonstrates the existence of an intimate relationship between fatty acid composition and functional characteristics in cell membranes. Decreased membrane rigidity has been demonstrated in certain malignant cells and the altered membrane lipid profiles of the malignant cells demonstrated by the present study are likely to form the basis for similar changes in colorectal cancer. This suggests a biologically plausible mechanism by which dietary fat intake and colorectal cancer may be linked. However, the effects of
specific changes in colonic cell membrane fatty acid composition on their functional characteristics has not been investigated in this work and further study is required to verify this hypothesis.

It was not possible to link the changes in human colonic cell membrane fatty acid profile directly to the dietary intake of fat in the human study. This may have been due to the limitations imposed by the dietary method used to assess fat intake and was one of the reasons for carrying out the animal work.

The most important finding of the animal work presented in chapter 9, was the inhibitory effect of unsaturated fats on experimental colorectal carcinogenesis. The unsaturated fat diets did not inhibit the development of adenomatous lesions but did appear to prevent their progression to frankly carcinomatous lesions. The unsaturated fat was provided as corn oil, which contains linoleic acid as the major component and it is likely that this essential fatty acid is the important factor. The results of the animal work prompt two questions. Firstly, does unsaturated fat have an inhibitory effect on colorectal carcinogenesis in man? and secondly what is the mechanism of action? These important questions can only be answered by further study.

Cell membrane fatty acid analysis of various tissues in the animal model (chapter 10) demonstrated several important findings. Dietary fatty acid composition was found to be an independently significant influence on the fatty acid profiles of cell membranes. Azoxymethane per se was found to exert an influence on the cell membrane fatty acid content of tissues in the period immediately after its administration, but this influence had disappeared twenty weeks later, suggesting that azoxymethane initially exerts a toxic effect. Colorectal tumours were
characterised by a markedly increased content of cell membrane arachidonic acid when compared to that of the surrounding non-malignant colonic mucosal membranes.

An increased availability of arachidonic acid in tumours has been found in other experimental studies of colorectal cancer and there is both human and experimental evidence which suggests that the prostaglandins derived from arachidonic acid are primary agents of co-carcinogenesis. The fatty acid analysis in the present study suggested an inverse relationship between dietary linoleic acid and tumour arachidonic acid and this indicates a possible mechanism by which dietary unsaturated fat with its high linoleic acid content may exert an inhibitory effect on colorectal carcinogenesis.

The work described in this thesis suggests several areas of future research. The mechanism by which unsaturated fat exerts an inhibitory effect on colorectal carcinogenesis requires elucidation. The possible role of prostaglandins has already been an area of some interest. The influence of various cyclo-oxygenase inhibitors, such as indomethacin and aspirin, has already received some attention in animal models but could be studied further in conjunction with unsaturated fats. In addition, the relationship between dietary fat intake and the functional properties of cell membranes such as fluidity and permeability to carcinogens and tumour growth factors should be investigated in the model used here.

There are many mechanisms by which dietary fats may exert an influence on colonic carcinogenesis and one factor which needs further investigation is colonic cell turnover rate. The influence of dietary fat intake on colonic cellular proliferation can be studied in vitro.
both in experimental models of colorectal cancer and in the human disease.

A logical progression of the work presented here would take it into prospective studies designed with the aim of establishing the influence of dietary fat intake in human colorectal cancer. The potential effects of dietary fat on both the treatment and the prevention of colorectal cancer should be addressed separately. Thus, investigation of the influence of dietary fats as adjunctive therapy in patients under treatment for colorectal cancer and investigation of the influence of dietary fat on the development of adenomatous colorectal polyps in susceptible individuals might be of benefit. Unsaturated fats and the marine oils, which have generated interest recently, would be the obvious types of fat to investigate.

The ultimate aim of all research into colorectal cancer must be to find ways in which this common disease can either be prevented or treated. The cause of colorectal cancer is multifactorial and so progress will necessitate advances on a number of different fronts. Nonetheless, it is conceivable that there will be a time when relatively simple and practical dietary manipulation will be a useful adjunct in the prevention and treatment of colorectal cancer.
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CELL MEMBRANE AND DIETARY FATTY ACIDS IN COLORECTAL CANCER

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ABSTRACT

There is a great deal of epidemiological and experimental evidence which suggests that dietary fat has an aetiological role in colorectal cancer. Although the mechanism of the promotional effect of dietary fat is unknown, the cell membrane is a possible target point at which fat might exert an influence. This thesis addresses the relationship between dietary fat and cell membrane fatty acids in human and experimental colorectal cancer.

In a case-control study of erythrocyte fatty acid profiles in human colorectal malignancies, cancer patients were found to demonstrate small differences in erythrocyte fatty acid profiles and unusual associations between dietary, erythrocyte and adipose tissue fatty acids. An analysis of cell membrane fatty acid profiles in the human colon demonstrated that tumour cell membranes contain a different fatty acid profile when compared to non-neoplastic colonic mucosa from either cancer or control patients.

An animal model of colorectal carcinogenesis was established in order to study the influence of the type and quantity of dietary fat on the development of experimental colorectal tumours. Both high and low levels of dietary unsaturated fat were found to exert an inhibitory effect on the development of experimental colorectal cancers but had no such effect on the development of colorectal adenomas. The type and quantity of dietary fat were found to influence the fatty acid profiles of erythrocytes, colonic mucosa and colonic tumours. Cell membranes from experimental colorectal tumours were found to contain an excess of the prostaglandin precursor arachidonic acid.

These results suggest that dietary and cell membrane fatty acids are intimately related factors in colorectal carcinogenesis. It is conceivable that modification of dietary fat intake, may in the future, have a valuable role to play in the prevention of colorectal cancer.