The Effects of Dietary Bran on the Drug Metabolizing Enzymes
and Other Biochemical Parameters

Being a thesis presented for the award of a
Degree of Doctor of Philosophy
in the
University of Surrey

by

Michele Jeanne Sadler, Bsc. (Hons, Lon.).

June, 1983.

Dept. of Biochemistry,
University of Surrey,
Guildford,
Surrey.
To Andrew and our parents
...man knows partly but conceives beside,
Creeps ever on from fancies to the fact,
And in this striving, this converting air
Into a solid he may grasp and use,
Finds progress, Man's distinctive mark alone,
Not Gods's and not the beast's:
God is; they are;
Man partly is, and wholly hopes to be.

(G.M. Hopkins)
Acknowledgements

I would like to express my deepest gratitude to Professor D.V. Parke for his valuable advice and encouragement throughout the course of both the experimental work and the final preparation of this thesis. I would also particularly like to thank Dr Costas Ioannides for his constant support and helpful suggestions. My thanks are also extended to Mr P. Scobie-Trumper and the animal house staff for the care of my experimental animals.

I am extremely grateful to the Flour Milling and Baking Research Association for their contribution to the financial support for this project, and for the inexhaustible supply of diets and wheat bran. My sincere thanks are extended to Dr N. Fisher and Dr C. Berry for their constant encouragement and help, particularly with the vitamin D work. For the valuable contribution of housing and feeding the long-term animals, I am particularly grateful to Mr. J. Gregory and his team of expert technicians at the FMBRA.

I would like to thank my colleagues for valuable discussion and companionship throughout the course of this work, particularly David Moore for his helpful advice and encouragement.

Finally I wish to express my gratitude to Andrew for his valuable help in the collation of this thesis, particularly for
help with the graphs and contribution to the typing, and to
Jane Smith for typing the tables and help with the
photocopying.
Abstract

Experimental diets containing either 4.8 or 9.6% wheat bran, 8.5% beef suet, and a stock diet were fed to rats for long and short periods in order to determine their effects on hepatic drug metabolizing enzymes in comparison to a fibre-free control diet. The only dietary related changes observed were with the stock diet (41B), in the rats fed for either 12 wk or 18 months, which consistently induced 7-ethoxyresorufin-O-deethylase, a mixed-function oxidase enzyme known to be catalysed by cytochrome P448; at 12 wk this diet also induced cytochromes P450 (female rats only) and b₅, and biphenyl-4-hydroxylase. At this time point 7-ethoxyresorufin-0-deethylase activity was also stimulated by both the bran diets.

Intestinal drug metabolizing enzymes were found to be unaffected by three dietary levels of wheat bran (4.8, 9.6 & 28.8%), fed for 4 wk, compared to a fibre-free control diet. The effect of incorporation of safrole, an inducer of the a mixed-function oxidases and an hepatotoxic agent, into the fibre-free and 9.6% bran diets, on the levels of intestinal mixed-function oxidases was similar in both dietary groups. This indicated that at the levels of safrole and wheat bran used, bran does not adsorb safrole in vivo or prevent its biological activity.

The effect of wheat bran fed at three dietary levels, for long and short periods, on mucus synthesis was investigated, and found to be without effect. The stock diet (41B) increased mucus
synthesis in the colon and duodenum, as determined by measurement of the rate of incorporation of $^{3}H$-acetylglucosamine into mucus glycoproteins in mucosal homogenates. The effect of wheat bran, 8.5% beef suet and the stock diet on the sialic acid content of gastro-intestinal glycoproteins was without effect, indicating that the character of the mucus was not altered by these dietary regimes.

Adverse effects of wheat bran on mineral status have been reported. A study was made using well-nourished rats, of the bone mineral stores, compared to the fibre-free diet. No adverse effects of either wheat bran (4.8 or 9.6%) or of wholemeal bread incorporated into the diet at 17 or 34%, or of the stock diet, were found in comparison to the fibre-free diet. The effect of wheat bran on the circulating levels of vitamin D, determined by radioassay, was similarly without effect.

Finally, a drug-nutrient interaction, that of anti-convulsant therapy and associated folate deficiency was further investigated. Rats maintained for periods of between 13 & 18 wk on a folate deficient diet were dosed with inducing agents of the mixed-function oxidase enzyme system for periods of 4 days and 2 wk (phenobarbitone) and 7 days (3-methylcholanthrene), in order to determine whether folate deficiency prevented their inducing effects. The animals were judged to be folate deficient by determination of serum and red cell folate levels using a radioassay technique. The results conclusively showed that induction of cytochrome P450 and the mixed-function oxidase enzymes.
was not affected by folate deficiency, indicating that the long-term anti-convulsant treatment is probably required to elicit this adverse reaction.
**Contents**

<table>
<thead>
<tr>
<th>Chapter One - General Introduction.</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary Fibre: Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Historical Background</td>
<td>2</td>
</tr>
<tr>
<td>Definition</td>
<td>3</td>
</tr>
<tr>
<td>Classification</td>
<td>4</td>
</tr>
<tr>
<td>Analysis</td>
<td>8</td>
</tr>
<tr>
<td>Physical Properties</td>
<td>10</td>
</tr>
<tr>
<td>Physiological Effects</td>
<td>11</td>
</tr>
<tr>
<td>Fibre in Human Disease</td>
<td>17</td>
</tr>
</tbody>
</table>

| The Metabolism of Drugs and Environmental Chemicals: Introduction | 22 |
| Phase I Metabolism | 23 |
| Phase II Metabolism | 28 |
| Formation of Reactive Intermediates | 29 |
| Enzyme Induction | 29 |
| Multiple Enzyme Forms | 30 |
| Physiological Factors Affecting Drug Metabolism | 32 |
| Drug-nutrient Interactions | 36 |

| Aims | 39 |

| Chapter Two - The Effect of Dietary Modification the on Drug Metabolizing System in the Rat. | 40 |
Chapter One

Introduction
Dietary Fibre

Introduction - An item of the diet which has received much attention over the last decade because of its postulated role in protecting against certain diseases, is dietary fibre. Burkitt & Trowell (1975) suggested that low intakes of dietary fibre in Western countries may contribute to the occurrence of certain diseases and that increasing the consumption of fibrous foods, particularly bran, would decrease the incidence of diverticular disease, carcinoma of the colon, cardiovascular disease, diabetes mellitus and obesity. The upsurge of interest in the field of dietary fibre research, stimulated by reports of its preventive role in the aetiology of these diseases, has led to a vast increase in knowledge of the nutritional, physiological and epidemiological functions of the fibrous components of plant foods in our diet.

Historical Background - It was known before the middle ages that part of our plant and cereal food was indigestible but it was not until the 19th century that attempts were made to measure this component. The impetus came from the developing world of animal nutrition. An estimate of the indigestible fraction was required in order to predict the nutritive value of animal feedstuff, for economical and nutritional reasons. The original method for measuring the crude fibre content was developed in Moglin, Germany, in 1806 by Einhof (Van Soest and McQueen, 1973), and involved treating the sample sequentially with petroleum ether, hot sulphuric acid, boiling water and hot alkali. The method became well established and was incorporated into various statutory
provisions, having practical value as an index of the indigestible matter in animal feeds. It was thought to measure the cellulose content of the diet.

As far as human nutrition is concerned a major advance in the field of dietary fibre research came from McCance and Lawrence (1929) who made a formal attempt to measure the carbohydrate content of the diet; it became apparent that a significant part of plant carbohydrates were not digested in the human alimentary tract. These were termed unavailable carbohydrates. From this initial concept ensuing research in this field over the past 50 years has furthered our understanding of dietary fibre. The laxative effect of bran was demonstrated (Williams & Olsted, 1935, 1936), and it was used to treat constipation, irritable colon, haemorrhoids and chronic colitis (Dimmock, 1936). Cleave (1956) related the existence of certain diseases to the excessive consumption of refined carbohydrate foods in particularly sugar. Burkitt recognised the rarity of diseases mentioned in Cleave's hypothesis among native Africans. He studied transit times and stool weights in relation to fibre intake (Burkitt et al, 1972). His dietary fibre hypothesis, attributing some of the diseases of Western civilisation to an inadequate intake of this dietary component has stimulated an abundance of research in this field (Burkitt, 1971).

**Definition** - Dietary fibre was defined in 1974 (Trowell, 1974) as 'the remnants of the plant cell wall that are not hydrolysed by the alimentary enzymes of man', and redefined in 1976 (Trowell et
al., 1976) as 'the plant polysaccharides and lignin which are resistant to hydrolysis by the digestive enzymes of man'.

**Classification** - Dietary fibre consists of a diverse group of substances of plant origin, found mostly in the plant cell wall, which vary in chemical composition and physiological effect. There is no generally accepted classification of the components of dietary fibre (Cummings, 1976). The best classification would be one based on the molecular structure of its component members but such information is not known for all the possible constituents of fibre. Much of the analysis of cell wall substances has been done on wood, cereals and grasses, with little analytical work on the common dietary constituents of fruits and vegetables. Existing classifications show two major classes of fibre - polysaccharides and lignin. This nomenclature is based on the classical schemes of fractionation, the definitions relating to the methods of isolation rather than the chemical structure.

**Polysaccharides**

**Cellulose** - is the major structural polysaccharide in the cell wall. It is a single homopolysaccharide of D-glucose residues linked in \( \beta_1-4 \) configuration in long unbranched chains and has a crystalline structure. The \( \beta \)-configuration provides resistance to the action of pancreatic amylase but cellulases are widely distributed in fungi and bacteria, so that they are present in the rumen and colon (Yost, 1972). X-Ray diffraction studies reveal areas of cellulose molecules which are disordered and non-crystalline and may represent areas where other sugars or uronic acids are incorporated into the molecule (Southgate, 1976).
Such regions are accessible to many reagents, including cellulases, which may be due to the weaker hydrogen bond formation (Shafizadeh and McGinnis, 1971). These regions may also be where the sorption of water, for which cellulose has great affinity, takes place since the X-ray diffraction pattern is unchanged when cellulose fibres are wetted.

**Hemicelluloses** - This term refers to the non-cellulosic polysaccharides and was introduced by Schulze in 1891 (Aspinall, 1959) to describe those polysaccharides extracted from the plant cell wall by dilute alkali. Together with pectin the hemicelluloses form the matrix of the plant cell wall in which are enmeshed the cellulose fibres. They are a complex series of heteroglycans based on three types of homopolymeric backbone chains - xylans, mannans and galactans, and one type of mixed chain, glucomannan. They are smaller than cellulose molecules and carry side chains containing arabinose, galactose, xylose, mannose, glucose, rhamnose, galacturonic and glucuronic acids. They are further classified into acidic or neutral forms based on the content of uronic acids (Aspinall, 1970). The uronic acid groups are derived from oxidation of the terminal -CHOH to -COOH, and are important in determining the physical properties of hemicelluloses since when they are present as glycosides they behave as simple hydrocarboxylic acids, forming metal salts, amides and alkyl and methyl esters (Danishefsky et al., 1970; Percival, 1963).

**Pectic Substances** - are present in smaller amounts than the other cell wall constituents and are biochemically less well defined. They act as inter-cell wall cementing substances. There are three groups - pectins, which have a high degree of methylation of the
carboxylic acid groups, pectinic acids, in which only some of the acidic groupings are methylated, and pectic acids, in which the polysaccharides are devoid of methyl groups. D-Galacturonic acid is the principal component and other monomers found are D-galactose, L-arabinose, D-xylose, L-rhamnose, L-fucose, 2-O-methyl-D-xylose and 2-O-methylfucose. Two important properties of pectin are its ability to form gels and its ion-binding capacity. The latter is closely related to the free uronic acid content (Branch et al., 1975).

**Gums, Mucilages and Storage Polysaccharides** - These fibre components of the diet are not cell wall constituents but are related biochemically and in their physical properties to these constituents (Aspinall, 1970). Gums are produced at the site of injury to plants. They are a complex group of branched uronic acid-containing polymers composed of glucuronic and galacturonic acids, xylose, arabinose and mannose. Examples are gum arabic and gum tragacanth. Mucilages are present in plant seeds where they prevent desiccation (Aspinall, 1970). They are mostly neutral polysaccharides. The best known example is guar gum, a galactomannan. Mucilages from cereals are characteristically highly branched with a backbone of 1-4β-D-Xylose with L-arabinose side chains. An example of a storage polysaccharide not digested by man is inulin which is found in Jerusalem artichokes.

**Non-polysaccharides**

**Lignin** - is a complex cross-linked polymer formed of substituted phenylpropane molecules derived from coumaryl, coniferyl and sinapyl alcohols, and it is a small polymer of molecular weight 1000-4500. The units are joined by C-C bonds in a variety of
linkages. Lignin is extremely inert and highly resistant to chemical degradation and enzymic digestion. It has a strengthening function in the cell wall (Pearl, 1967). Its spatial configuration is difficult to determine and it is best viewed as infiltrating around the cellulose fibrils in a 3-D system (Southgate, 1976). There is no structure for the lignin molecule and its biosynthetic pathway is thought to produce a random type of polymer (Freudenberg, 1965).

Minor Components of the Plant Cell Wall

Certain components associated with dietary fibre in the cell wall, though not themselves of a fibrous nature, are recognised to have some effect on the behaviour of dietary fibre (Cummings, 1976). Such components include protein, inorganic constituents, cutin, suberin, waxes, phytic acid, glycosides e.g. saponin, and polyhydroxyphenols e.g. tannins.

Wheat Bran - The dietary fibre used in the work reported in this thesis is wheat bran. Bran is a product of flour milling and ideally comprises the outer layers of the wheat grain i.e. the pericarp, consisting of the epidermis, cuticle, cross-cell layer, and tube-cell layer; the seed coat consisting of the testa, tegumen and pigment cells; the hyaline layer and the aleurone layer. Together these layers represent 11-16% of whole wheat. The composition of bran is - cellulose 21%, pentosan 20 - 26%, starch 7.5 - 9%, sugar 5%, protein 11 - 15%, fat 5 - 10%, ash 5 - 9% and water 14%. The fatty acids are 84% unsaturated (mostly linoleic and oleic acid) and 16% saturated. The minerals are potassium, phosphorus, and magnesium. Other substances included are phytic
acid and some vitamins.

Analysis - To date there is no one single procedure for measuring accurately and completely the dietary fibre content of mixed diets. Limitations of the crude fibre method are realised and it is gradually being replaced by more informative analytical procedures. The limitations presented by this method are due in part to its highly empirical nature, its poor reproducibility and the time-consuming procedure. A portion of the cellulose is known to be solubilised during the acid and alkali treatment and removal of some of the lignin during the preliminary solvent extraction is known to occur (Hallab and Epps, 1963). It has been estimated that the method gives recoveries of 50 - 80% of the cellulose, 10 - 50% of the lignin, and 20% of the hemicelluloses.

Since the late 1930's two main streams concerned with dietary fibre analysis have developed - the fibre school, concerned with animal nutrition and the cell wall constituent school, concerned with human nutrition. A third major area of interest, the chemistry of the plant cell wall has improved our knowledge of the structural components of the plant cell wall.

From the fibre school the methods of Van Soest have developed for acid detergent fibre (ADF), which is determined by refluxing samples with 2% cetyl trimethyl ammonium bromide in sulphuric acid, filtering, drying and weighing, and neutral detergent fibre (NDF), which is determined by refluxing samples with a 5% solution of hot detergent, filtering, drying and weighing. ADF gives an accurate
measure of the cellulose and lignin content in forage, and NDF gives a measure of the total cell wall materials (Van Soest, 1963; Van Soest, 1963a). Problems are encountered when these methods are applied to human diets since the fibre content is considerably smaller than in animal feeds and makes precision difficult. The high level of lipid in a mixed diet causes problems with foaming and filtration, and insolubility of some starch in the hot detergent, and loss of some water-soluble polysaccharides.

The method developed from the cell wall-constituent school is that of Southgate (1969, 1969a) which measures the various components of the unavailable carbohydrate fraction by a series of extraction and hydrolysis stages to separate cellulose from non-cellulosic polysaccharides, followed by splitting of the components into constituent sugars which are then determined by quantitative chromatography, ion-exchange chromatography, or gas-liquid chromatography. This gives values for the non-cellulosic polysaccharides in two fractions—those that are water-soluble and hydrolysable with dilute acid, and the others are the cellulose and associated non-cellulosic pentosans and lignins. Measurement of the component sugars gives some indications of the types of polysaccharide present.

Procedures developed from concern with plant-cell-wall chemistry are research-oriented, lengthy and not applicable to routine analysis. However, they are the most detailed methods available (Southgate, 1976).
Much current research in the field of dietary fibre has led to the general conclusion that more detailed knowledge is required of the composition of dietary fibre in order to explain its specific physiological effects. Hence as much information as possible is required at the stage of analysis, although these tend to be the more lengthy and complex procedures.

**Physical Properties**

**Water Holding Capacity** - This is determined by the chemical and physical composition of fibre and the surrounding medium. The presence of polar groups on the polysaccharides increases the hydration capacity and intermolecular bonding. Generally hemicelluloses have a greater capacity to hold water than cellulose, and lignin has even less. Particle size is an important determining factor. More moisture is retained in the interstitial spaces of large particles than small particles (Kirwan et al., 1974). The part of the plant from which the fibre material comes also influences the ability to hydrate, leaves and roots having a greater water holding capacity than seeds and tubers. Bran is one of the most hydratable materials and is effective in retaining water in physiological conditions (McConnell et al., 1974).

**Adsorption** - Some types of dietary fibre adsorb organic materials such as bile acids, bile salts and bacteria. Eastwood and Hamilton (1968) found sterol adsorption to be pH dependent and possibly to occur by hydrophobic bonding between lignin and sterol molecules. Pectins (Kay and Truswell, 1977) and guar gum have also been shown to bind bile acids. Such binding leads to increased excretion. Bile acid adsorption is dependent upon the physical and chemical
form of the fibre, the pH and osmolality of the intestinal contents, and the type of bile acid (Eastwood and Mowbray, 1976). Cellulose is less effective at adsorbing bile acids than the mucilaginous and gel-forming fibres, but lignin has the greatest capacity, particularly in acidic conditions, when conjugation of the hydroxyl groups is suppressed (Eastwood and Mowbray, 1976). Free bile acids are more readily bound than conjugates and the dihydroxy-bile acids more readily than the trihydroxy-bile acids. The presence of short chain, unsaturated fatty acids in bile acid micelles decreases their adsorption (Eastwood and Mowbray, 1976).

**Cation Exchange Properties** - Most plant fibres act as weak monofunctional ion-exchange resins, although those of cereals and tubers may behave as polyfunctional exchange resins. This action is related to the number of free carboxyl groups on the sugar residues (McConnell et al, 1974). For example, binding of calcium by dietary fibre is related to the presence of unsubstituted uronic acid groupings (Branch et al, 1975). Such in vitro studies suggest that mineral adsorption may be altered by the ingestion of dietary fibres.

**Physiological Effects of Dietary Fibre**

**Digestibility** - Although by definition dietary fibre is resistant to digestion by the alimentary enzymes in humans, it is susceptible to degradation by bacterial action in the colon. There is variation between individuals and the components of dietary fibre are affected to differing extents - 29-82% of cellulose, 56-87% of hemicellulose (Southgate et al, 1976; Southgate & Durnin, 1970; Milton-Thompson & Lewis, 1971), and 90% of pectin (Cummings et
al,1979) are lost in the gut. Lignin is highly resistant to bacterial enzymic action and is completely recovered in the stool (Southgate et al,1976; Southgate and Durnin,1970). The extent of breakdown is dependent upon the colonic transit time, the colonic bacterial flora and the composition of the fibre. Vegetable cell walls are generally more easily fermented than cereal brans which are thicker and more highly lignified. Wheat bran is one of the most resistant of the dietary components (Van Soest and Robertson,1976), with faecal recovery greater than 70%. Methane, carbon dioxide, hydrogen, water and short chain fatty acids (acetic, lactic, propionic and butyric acids) are produced as a result of fermentation. Hence in vitro actions of fibre may be eliminated as a result of degradation of fibre and the products of fermentation may be responsible for some of the effects of dietary fibre.

Transit Time - The most immediate results of a higher intake of dietary fibre are an increase in faecal weight with a decrease in transit time, which shows logarithmic correlation of transit time to faecal weight (Burkitt et al,1974). Above a certain weight, the critical faecal weight, further increases do not affect the transit time significantly (Spiller et al,1978), which may partly account for conflicting reports of increased fibre intakes and their effect on transit time (Hill,1974).

The mechanism by which fibre increases faecal weight was generally believed to be due to its water holding properties (McConnell et al,1974). However Stephen and Cummings (1979) showed the relationship between in vitro water holding and in vivo faecal bulking to be an inverse one. They found, for those fibres that
are digested in the colon, an increase in bacterial mass to cause the increase in faecal weight (Stephen, 1981).

**Bacteria** - Dietary fibre can affect the metabolic activity of the gut flora by three mechanisms. The first is the effect of fibre on substrate concentration which depends on the sequestering ability, extent of gel-formation and to what extent the fibre is fermented. The second is the effect on enzyme action which relates to its effect on transit time so that when transit time is shortened, little metabolism can take place. The third is the effect of breakdown products of fibre on enzyme activity. When dietary fibre is rapidly fermented the local concentration of short chain fatty acids will increase sufficiently to cause a fall in pH. Bacterial enzymes which metabolise steroids have pH optima close to 7.

**Intestinal Absorption** - Higher intakes of dietary fibre have been shown to slow gastric filling (McCance et al., 1953; Haber et al., 1977), which may result in slower intestinal absorption; subsequent glucose and insulin responses will also be slower. Various studies have shown higher intakes of dietary fibre to prolong gastric transit time (Holt et al., 1979; Wilmshurst and Crawley, 1980). The rate and extent of small intestinal absorption is determined by the rate of passage of contents and the degree of mixing. It is only possible to use indirect methods of study in man. Measurement of hydrogen in the breath, from caecal flora metabolism, can be used as a marker of the arrival of the front of a meal at the terminal ileum (Jenkins et al., 1978). Using this technique it has been demonstrated that guar gum delays small intestinal transit time, pectin and methylcellulose have no effect, and gum tragacanth and wheat bran have an intermediate effect
In vitro studies have shown glucose, maltose and oligosaccharides to be released more slowly from cooked ground soya beans than from wholemeal bread, and lentils to have an intermediate effect (Jenkins et al., 1980). This may be partly due to different rates of enzymic action, but mostly to trapping of products in the interstices of the food. These results were consistent with blood glucose responses when fed to healthy volunteers, indicating different rates of digestion depending on the type of food. An in vitro study of the rate of hydrolysis of starch in cereal foods has shown that stoneground wholemeal flour is hydrolysed more slowly than white flour; cooking made the starch more readily available for enzyme hydrolysis and fibre exerted an inhibitory effect by forming a physical barrier to limit access of the hydrolytic enzymes to the starch (Snow and O'Dea, 1981).

An increase in the proportion of goblet cells in the duodenum and colon of rats fed 20% wheat bran has been reported (Schneeman et al., 1982), indicating a higher production of mucus. Mucus is believed to contribute to the thickness of the unstirred layer in the intestine (Nimmerfall and Rosenthaler, 1980) which in greater quantities may reduce the absorption rate, especially of lipid-soluble compounds (Thompson, 1978).

Bioavailability - Reports of decreased bioavailability of minerals resulting in negative mineral balances by increased intakes of dietary fibre are numerous. Reviews of the effect of fibre on
iron, zinc and copper balances (Kelsay, 1981), and on calcium, magnesium and phosphorus balances in humans (Kelsay, 1982) have recently been published. Important considerations when assessing the effect of fibre on mineral balance are the type and level of dietary fibre, the level of mineral intake, the time of feeding and the presence of mineral binding agents other than dietary fibre e.g. phytic acid.

Carotene appears to be bound by the plant cell wall fraction, decreasing its bioavailability in human subjects (Kelsay, 1982). Gregory (1980) found low bioavailability of vitamin $B_6$ in cereals compared to non-fat dried milk. Vitamin $B_6$ bioavailability appears to be decreased by those dietary fibres that are digested by intestinal bacteria (Cullen and Oace, 1980). A study in rats of the effect of 10% dietary pectin on plasma and red cell vitamin E concentrations, and tissue contents of vitamin E, showed that pectin reduced the bioavailability of vitamin E (De Lumen et al, 1982).

A study of the effect of wheat and rice bran supplements (fed for 26 days, 20g/day), on protein utilisation in human female adults suggested a trend towards lowered protein utilisation when bran was incorporated into white bread; this was indicated by decreased urinary and increased faecal nitrogen excretion, although other parameters were not altered (Jank et al, 1981). An animal study (Wojcik and Delorme, 1982) showed a decreased efficiency of protein utilisation in rats consuming marginal protein and high cellulose (28.6%), compared to rats fed a cellulose-free control
diet.

Hence it seems that interactions occur between all types of fibres and nutrients such as mineral salts, vitamins, and protein. In Western Society food intake is high and fibre intake comparatively low so that any potential adverse effects are not generally manifested. However these interactions could be of importance in developing countries, where traditional high fibre staple diets are associated with malnutrition, or in certain population groups of the Western countries.

Pancreatic Enzymes - Examination of the effects of dietary fibre on the upper gastro-intestinal tract has revealed that dietary fibre can alter metabolic parameters. Schneemann et al (1982) found that a 20% wheat bran diet fed to rats for 2 wk increased amylase and trypsin activities in the pancreas compared with a fibre-free diet; in the intestinal contents only lipase activity was elevated. Isaksson et al (1982), using an in vitro buffer system or human duodenal juice, determined the effects of various fibres on the pancreatic enzymes. They found that pectin, guar gum, and wheat bran caused a marked decrease in amylase and lipase activities, that pectins and wheat bran reduced trypsin activity and that pectins reduced phospholipase activity. The effects of fibre on enzyme activities were greater in the duodenal juice than in the buffer solutions. These studies are not directly comparable, since they use different species, but the results are not necessarily contradictory, as Schneeman et al (1982) measured the enzymes at their site of production and Isaksson et al (1982) were measuring enzyme activity after production.
Fibre in Human Disease

Colorectal Cancer - Epidemiological data indicates that the incidence of colon cancer is determined by environmental rather than genetic factors. The aetiology of the disease is thought to be multi-factorial and diet to be of major importance in its causation (Burkitt, 1978; Sherlock et al., 1980). Colorectal cancer has been shown to correlate positively with meat and fat consumption and negatively with dietary fibre consumption (Burkitt, 1978). However no single dietary component has been identified as causing colon cancer so that the relationship is complex.

None of the preformed carcinogens present in the diet has been shown to be related to colon cancer (Hill, 1982). Bacterial metabolism in the colon was postulated as a mechanism of production of the carcinogenic molecule (Aries et al., 1969; Hill et al., 1971). The composition of the diet would determine the amount of substrate available. Numerous substrates have been investigated (Hill, 1977) and the bile acids have been implicated to be possible precarcinogens. Their faecal concentration correlates with dietary fat (Cummings et al., 1978), and inversely with dietary cereal fibre (Cummings et al., 1976), and they are structurally related to the cyclopenta(α)phenanthrenes, a known group of carcinogens (Coombs and Croft, 1973).

The two major hypotheses of colon cancer are based on dietary fibre deficiency (Burkitt, 1971) and high dietary fat consumption
The role of dietary fibre in reducing colon cancer is related to a more rapid transit of gut contents decreasing the opportunity for bacteria to produce carcinogenic metabolites, and for any such metabolites to act on the colon wall. However the correlation between colon cancer and dietary fibre intake from epidemiological data is poor (Irving and Drasar, 1973; Hill, 1974). The dietary fat hypothesis is related to facilitated growth of intestinal bacteria (when a high fat diet is consumed), which are capable of converting dihydroxy bile acids to carcinogenic polynuclear hydrocarbons (Hill, 1975).

A decrease in colonic mucosal exposure is a necessary outcome of the dietary fibre hypothesis for protecting against colon cancer. This could occur by dilution due to chemical binding, water-holding and stool-bulking, shortened colonic transit time, modification of bile acid and of faecal flora metabolisms (Gori, 1979). The addition of 32g of dietary wheat bran per day increased the total faecal bile acid excretion, but when expressed as a concentration, bile acid was lower than in the control period (Cummings et al, 1976). Studies of the influence of dietary fibre on bile acid excretion show conflicting results (Story and Kritchevsky, 1980). However, dietary fibre can affect the bile salt pools in the colon, ileum and jejunum (Story and Kritchevsky, 1978). Ullrich et al (1981) found high fibre diets to produce no change in human neutral faecal steroids (cholesterol and coprostanol) and total steroids, with a marked increase in primary bile acids (chenodeoxycholic and cholic acids) and a decrease in secondary bile acids (lithocholic and deoxycholic acids). These results were
in agreement with suggestions of other workers (Kritchevsky et al, 1974) that binding of bile acids to fibre decreases their conversion to primary and secondary forms and increases faecal steroid excretion.

A positive correlation of colorectal cancer incidence and faecal deoxycholic acid levels has been reported (Hill, 1979; Mower et al, 1979). This secondary bile acid is produced by the action of bacterial \( \alpha \)-dehydroxylase on cholic acid and the faecal activity of this enzyme was found to be higher in colorectal cancer patients than controls (Mastromarino et al, 1976).

The influence of dietary manipulations on the faecal micro-flora shows that diet does not alter the composition of the faecal micro-flora (Finegold and Sutter, 1978). Neither fibre-free or fibre-supplemented diets were found to alter the major groups of bacteria (Bornside, 1978). However, diet has been shown to alter the metabolic activity of the faecal micro-flora.

Studies using animal models have shown that dietary fibre protects against the effects of administration of known chemical carcinogens (Freeman et al, 1978, Reddy and Mori, 1981). Citrus pulp and wheat bran have been shown to bind dimethylhydrazine (DMH; a known chemical carcinogen which requires metabolic activation) at the pH of the stomach and small intestine and to increase faecal bulk (Smith-Barbaro et al, 1981a). This decreases the amount of carcinogen coming into contact with the colonic mucosa, which may account for the decreased number of tumours observed in these
groups. These authors have also shown that certain dietary fibres can affect the levels of hepatic, small and large intestinal cytochromes P450 and $b_5$, which may partly account for the decreased number of tumours observed. Such an approach is useful for investigating mechanisms of carcinogenesis and interactions of carcinogens with dietary fibres, but their relevance to human studies is not clear.

The fundamental question of whether dietary fibre protects against human colon cancer is debatable. Much investigation of the hypothesis of Burkitt (1971) has produced greater understanding of effects of dietary fibre, but conclusive proof of the hypothesis is still awaited. However, the hypothesis has not been disproved and neither has it been superseded.

**Coronary Heart Disease** - The protective role of dietary fibre against this disease is possibly related to the effect of some types of dietary fibre in lowering serum cholesterol and blood lipid levels. Clinical studies in human subjects have shown various legumes (Luyken et al, 1962), oats (de Groot et al, 1963; Judd and Truswell, 1981), and vegetables (Grande et al, 1965) to be effective in decreasing serum cholesterol levels, whereas wheat bran has been found to be ineffective (Truswell and Kay, 1975; Kay and Truswell, 1977a). The mucilaginous and gel-forming polysaccharides appear to be the most effective in decreasing serum cholesterol levels. Jenkins et al (1975a) found guar gum and pectin to decrease serum cholesterol and wheat fibre to slightly increase it. Other studies have shown conflicting results (Durrington et al, 1976), but it is now generally agreed that bran
has no hypolipidaemic effect whereas pectin lowers serum cholesterol concentrations.

The major mechanism thought to be responsible for the hypocholesterolaemic effect of some dietary fibres is the binding of bile acids in the intestine, with interruption of their enterohepatic cycle leading to increased faecal excretion. The adsorption of bile salts and acids by dietary fibres may result in decreased micellar formation, thus affecting cholesterol absorption. An increase in bile acid excretion would cause increased synthesis to make up this loss. Both events would deplete cholesterol pools (Story and Kritchevsky, 1976).

**Diverticular Disease** - This concerns the presence of saccular outpouchings of the sigmoid colonic wall often with a thickened corrugated bowel wall. Uncomplicated diverticulosis may be symptomless, but when inflammatory reactions develop this results in abdominal pain and vomiting. Epidemiological data correlates low-fibre diets with an increased prevalence of the disease, although its aetiology is unknown. Prolonged transit times, low stool weights and increased colonic pressures are thought to be the relevant associated factors. Painter and Burkitt (1971) suggested that fibre supplements in the form of bran should alleviate the symptoms, rather than low fibre diets which were the treatment at that time. Some studies have shown this to be the case (Kelsay, 1978) although other studies have reported no effect on transit time (Brodribb and Humphreys, 1976). Results of studies aimed at demonstrating a causal relationship between disturbances
of colonic motility and the development of diverticulosis are inconclusive (Eastwood et al, 1978). As treatment of the disease with high fibre diets has progressed the number of operations for uncomplicated diverticular disease has markedly decreased (Anon., 1978).

**Diabetes** - The effects of delayed gastric emptying and slowed intestinal absorption manifested by some types of fibre have been demonstrated to be effective treatments in the management of some diabetics (Jenkins et al, 1976).

**Obesity** - Heaton (1973) has suggested that high fibre intakes may be helpful in the management of obesity by decreasing food intake of the absorption of nutrients. Titcomb (1976) found a reducing diet containing large amounts of high fibre bread to promote satiety and to facilitate weight loss. The extent to which fibre may decrease energy availability is unclear. A supplementation of 38g/day of bran in the form of biscuits was found to increase the daily faecal energy loss by 20-95 calories per day (Southgate et al, 1976).

---

**The Metabolism of Drugs and Environmental Chemicals**

**Introduction** - The effect of foreign compounds on the body and on the defence mechanisms of an organism is also the subject of much scientific research with a view to establishing the causation of cancer and other diseases. Organisms have lived for millions of years in a hostile environment. In our present industrial society hostility is increased by exposure to a vast array of man-made
chemical pollutants such as pesticides, food additives, drugs, detergents and plastics which may become distributed into the air, water and soil, may damage plant and animal life, and may have direct effects on the health of man. Foreign compounds are neither utilised for energy production or maintenance of tissues or body functions and are potentially dangerous to the body, which has a biochemical defence mechanism to protect against their harmful effects. The metabolism of such compounds is generally directed towards detoxication and excretion which is achieved by two series of reactions (Williams, 1971). Phase I reactions (biotransformations) are asynthetic and introduce or unmask functional groups. Phase II reactions (conjugations) are energy requiring and synthetic, coupling the existing or newly introduced functional groups to polar molecules and forming more polar, easily excretable compounds.

Phase I Metabolism - Phase I reactions are catalysed primarily by the mixed-function oxidase enzyme system located in the endoplasmic reticulum of certain tissues. The liver is the major site and exhibits high activity; lung, skin, gastro-intestinal mucosa, colonic mucosa, kidneys and adrenals are the other sites of activity. The enzyme system is multi-component, comprising a phospholipid - phosphatidylcholine, a flavoprotein reductase - NADPH cytochrome P450 reductase, and a haemoprotein - cytochrome P450 (Lu and Coon, 1968). The occurrence of cytochrome P450, a pigment producing a Soret peak at 450nm when complexed in reduced form with carbon monoxide, was reported independently by Klingberg (1958), and Garfinkel (1958). The studies of Omura and Sato
(1964, 1964a) revealed its haemoprotein nature and its basic properties. Its role as the site of oxygen activation and as the terminal oxidase for steroid hydroxylation (Estabrook et al., 1968) and drug oxidation (Cooper et al., 1965) was soon determined. The unknown significance of NADPH cytochrome C reductase, discovered in 1950 (Hogeboom and Schneider, 1950) was elucidated when steroid and drug oxidations were shown to be NADPH dependent. Its role in reduction of cytochrome P450 was finally shown after purification of the enzyme system (Lu and Coon, 1968).

Cytochrome P450 is widespread throughout the plant and animal kingdom. In addition to its role in xenobiotic and steroid metabolism it hydroxylates many endogenous substrates in intermediary metabolism: biosynthesis of prostaglandins, thromboxanes and prostacyclins, synthesis of cholesterol and its degradation to bile acids, corticosteroid and sex hormone metabolism, \( \omega \)-hydroxylation of fatty acids, and regulation of carbohydrate metabolism. Cytochrome P450 has a molecular weight of 45-60K and belongs to the \( \text{b} \) class of cytochromes containing protohaem, the iron complex of protoporphyrin, as its prosthetic group (Omura and Sato, 1964). This lies centrally in the porphyrin nucleus with the nitrogen atoms occupying four of the co-ordination valencies in a plane and separating the fifth and sixth ligands which are available for binding two axial ligands of either the apoprotein or of other molecules e.g. oxygen or carbon monoxide. The fifth ligand is currently thought to be a form of sulphur, possibly a cysteine residue from the apoprotein (Mason et al., 1965; Jefcoate and Gaylor, 1969; Ojaki et al., 1978). The nature of the
sixth ligand and co-ordination site where molecular oxygen binds and is activated is uncertain.

In its oxidised form in vitro the haemoprotein binds with substrates to give two different types of spectra. Many lipophilic compounds produce a spectral change with a peak at 385nm and a trough at 420nm (type I spectra). Several lipophilic nitrogen-containing species e.g. aniline, produce a spectra with a peak at 430nm and a trough at 595nm (type II spectra). Reverse type I spectra have a peak at 415-420nm and a trough at 390nm. These spectral changes indicate different interactions between substrate and haemoprotein, and modification of the spin state of the haem iron. Cytochromes exist in either a low spin or a high spin state, determined by the position of the five 3d electrons in the ferric ion. Maximal pairing gives a low-spin state and maximal separation a high-spin state. In the microsomal membrane about 50% of cytochrome P450 exists in the high spin form. Type I spectral changes are produced by a hydrophobic interaction with a hydrocarbon residue of the apoprotein (Yoshida and Kumaoka, 1975), with a change from a low spin state of the haem iron with loss of the endogenous sixth ligand. Type II compounds interact directly with the haem iron at the sixth ligand (Yoshida and Kumaoka, 1975) and cause conversion from the high to the low spin state of the haem iron. The interaction is by hydrophobic forces and requires provision of an atom with a free electron pair for the sixth co-ordination site so that it remains in a low spin state.

The enzyme cycle of cytochrome P450 is shown in Fig.1. The
Fig. 1. Mechanism of Cytochrome P-450 Enzyme Systems: Liver Microsomal Electron Transport for Drug Oxidation and Reduction.

NADPH
Cytochrome
C-Reductase

NADP⁺

NADPH

FP

Cytochrome P-450

fp

Cytochrome P-450

0₂

Cytochrome P-450

Drug-H

Drug (Substrate)

O₂

Drug-OH

(product)

Cytochrome P450

Drug-H

Cytochrome P450

0₂

Drug-H

Cytochrome b₅?

NADH - flavoprotein?

(After Zannoni, 1977; Gillette and Jollow, 1974)
substrate sterically binds to the oxidised form of cytochrome P450 and the change in spin state and associated increase in redox potential facilitates the following reduction of the haem iron by an electron from NADPH cytochrome P450 reductase. Dioxygen combines with this complex and a second electron is inserted, resulting in an unstable intermediate which decomposes and loses water and allows insertion of active oxygen into the substrate resulting in the monoxygenated product and oxidised low-spin ferric haemoprotein.

There are two theories of the mechanism of oxygen insertion. It may be via an oxene intermediate, which directly inserts oxygen into the C-C bond, or via an acylated ferrous-oxy intermediate such that the ultimate hydroxylating species is a peracid. The reducing equivalents are both provided by reduced pyridine nucleotides (NADPH and NADH). Flavoproteins transduce the electron flow so that one electron at a time is inserted. NADPH is the preferred source of reducing equivalents (Sato et al., 1965). Addition of NADH to a drug oxidation system supported by NADPH often results in "NADH synergism" i.e. a more than additive increase. NADH-Cytochrome b5 reductase and cytochrome b5 are thought to be involved in transfer of the second electron (West et al., 1974; Cohen and Estabrook, 1971; Cohen and Estabrook, 1971a).

The first electron reduction step is rate-limiting in the overall cycle for most substrates (Holtzman et al., 1968; Davies et al., 1969). The operation of the cycle is generally tightly coupled to oxygenation of the substrate, although a small degree of
uncoupling occurs with some relatively good substrates (Hildebrandt et al., 1975).

This enzyme system is the most versatile known as it can accept as substrates organic chemicals of exogenous and endogenous origin and of widely differing molecular weight and chemical structure. The reactions catalysed vary considerably and include aromatic and aliphatic hydroxylation, oxidative deamination, N-, S- and O-dealkylation, arene oxide formation, desulphuration, N-hydroxylation, N- and S-oxidation and dechlorination. Compounds thus metabolized may be excreted in urine or bile, or further metabolized.

**Phase II Metabolism** - Foreign compounds with polar groups and products of phase I metabolism may be conjugated with glutathione, glycine, acetate (acetyl CoA), glucuronic acid (uridine diphosphoglucuronic acid), sulphate (3'-phosphoadenosine 5'-phosphosulphate) and methyl (5'-adenosyl-methionine). The endogenous component is usually activated but in some cases the foreign compound/phase I metabolite may possess the energy. Each compound with a polar group may be conjugated with one or more of the endogenous substances. This occurs by the action of transferase enzymes located in the microsomes, mitochondria or soluble fraction of the cell. There is often a multiplicity of transferases for each endogenous substance, each with its own substrate specificity. A diversity of metabolites for a given compound is thus possible.
Formation of Reactive Intermediates - In addition to detoxication reactions both phase I and phase II reactions participate in the formation of reactive intermediates which can covalently bind to the tissue macromolecules and lead to toxicity, mutagenicity and/or carcinogenicity. Biotransformations which result in activated metabolites generally involve hydroxylation at sterically hindered positions (bay regions) resulting in unacceptable compounds for further enzymic detoxication (Parke and Ioannides, 1982). Such intermediates may exist as free radicals or interact with endogenous molecules or function as radical generators by interacting with molecular oxygen and other small molecules (Parke, 1981). Metabolism with cytochrome P450 may result in the formation of carbenes which form stable ligand complexes with cytochrome P450 e.g. methylenedioxyaryl compounds such as safrole. Hence, metabolism by microsomal mixed-function oxidases may result in detoxication, activation and formation of carbene complexes with cytochrome P450 following activation (Parke, 1981).

Enzyme Induction - Pretreatment of experimental animals with certain compounds was shown to induce liver microsomal enzyme activity (Brown et al, 1954). A variety of structurally unrelated compounds are now known to induce the activity of mixed-function oxidases. The most potent inducers are barbituate drugs, chlorinated hydrocarbons, insecticides and carcinogenic polycyclic hydrocarbons (Kupfer, 1970). Their only common property is their degree of lipophilicity (Parke, 1976). True enzyme induction results from de novo protein synthesis (Conney, 1967). Stimulation as distinct from induction may occur in vivo and in vitro, but only
for certain enzyme activities. Examples are biphenyl 2-hydroxylase (McPherson et al, 1974) and UDP-glucuronyltransferase (Wisnes, 1971). The consequences of induction are accelerated biotransformations and increased turnover of endogenous substances. Recently it has been demonstrated that both phenobarbitone and 3-methylcholanthrene increase due to de novo protein synthesis of cytochrome P-450 species. The total mRNA isolate from liver of a phenobarbitone-treated rat, when translated in vitro contained more protein than was immuno-precipitable with antibodies to phenobarbitone induced P-450 than liver from control animals (Colbert et al, 1979; Dubois & Waterman, 1979). However in most cases the stimulation of a particular enzyme activity by 3-methylcholanthrene or phenobarbitone has not rigorously been shown to be de novo protein synthesis.

Multiple Enzyme Forms - Pretreatment of animals with various inducers is known to produce differential effects on mixed-function oxidase enzyme activities, indicating the presence of multiple forms of cytochrome P450. The isozymes are characteristic of the particular tissue and animal species. After pretreatment with an inducer the forms are isolated, solubilised, purified and characterised by their immunological and physicochemical properties, their enzyme specificities, kinetics, and amino-acid sequences (Guengerich, 1979). The isozymes have different but overlapping substrate specificities (Guengerich, 1979), such that different forms of cytochrome P450 may metabolise a given substrate at comparable rates, but metabolise other substrates at vastly different rates; the enzyme variants of P450 have essentially the
same enzyme active site (Parke and Ioannides, 1982; Dus, 1982). Although 11 forms of cytochrome P450 in untreated rabbit liver microsomes were recently reported, only 6-8 forms have been adequately isolated and characterized from rat and rabbit liver, to be defined as distinct forms (Lu and West, 1978). However, it has been suggested that any individual mammal may have the capacity to produce thousands of unique cytochrome P450 haemoproteins (Nebert and Negishi, 1982).

Comparison of the inducing effects of phenobarbitone and 3-methylcholanthrene (Conney, 1967) shows phenobarbitone to stimulate a larger number of drug metabolizing pathways than 3-methylcholanthrene (Lu et al, 1976). Where substrates e.g. biphenyl, can be oxidised in a variety of positions the ratio of different hydroxylated products is altered by pretreatment with either inducer (Creaven and Parke, 1966). Phenobarbitone stimulates 4-hydroxylation of biphenyl, whereas 3-methylcholanthrene increases both 4- and 2-hydroxylation. Phenobarbitone does not cause an alteration in the wavelength of maximum absorption, whereas 3-methylcholanthrene causes the absorption maximum to decrease to 448nm (Alvares et al, 1967), for which reason the 3-methylcholanthrene form is known as cytochrome P448. This form is more distinct than other forms (Parke and Ioannides, 1982), as it exists naturally in the high-spin state, is induced by chemical carcinogens and is found in malignant cells, foetal and neonatal tissues. It is able to insert oxygen into bay regions and is therefore associated with production of reactive intermediates.
The physiological significance of multiple forms of cytochrome P450 lies in the potential for differing metabolisms. Differences in substrate specificity, regiospecificity and stereospecificity allow for activation or deactivation of a given chemical, which will determine the cytotoxic and carcinogenic action of a compound (Lu, 1979). Their clinical significance is important in polypharmacy and drug interactions; their significance in intermediary metabolism is in determining balances of the metabolic pathways; the differences in mixed-function oxidase activities between species, strains and individuals of different sex or age may be explained by multiple forms.

Physiological Factors Affecting Drug Metabolism

Species Differences - Differences in foreign compound metabolism occur between species due to genetic and environmental factors. Similarly different strains within a species, and individuals show genetically controlled differences (Evans et al., 1960).

Age - Foetal and newborn animals have a decreased ability to metabolise foreign compounds (Fouts and Adamson, 1959), and older rats show qualitative changes (Rikans and Notley, 1981) and generally slower rates of foreign compound metabolism, which is thought to be related to changes in the fatty acid composition of microsomal phospholipids (Rikans and Notley, 1981a).

Sex - Male rats generally have higher rates of foreign compound metabolism than female rats which is attributed to the anabolic action of androgens (Kato and Onoda, 1970) and to the presence of different amounts of one or more of the major forms of cytochrome P450 in the liver microsomes of male and female rats (Kamataki et
No sex difference is apparent for drug metabolizing enzymes, cytochrome P450 content and NADPH linked electron transport system of liver microsomes of rabbits, mice (although sex differences have been reported in certain strains), guinea pigs and hamsters (Kato, 1974). The magnitude of sex difference in the rat depends on the substrate used (Kato and Gillette, 1965). Hexobarbital hydroxylation, aminopyrine N-demethylation and pentobarbital oxidation are markedly sex dependent whereas aniline hydroxylation and zoxazolamine hydroxylation are less or are not sex dependent. N-Demethylation of N-methylaniline and p-nitroanisole are intermediate.

**Nutritional Status** - Foreign compound metabolism is sensitive to nutritional imbalances which are a major physiological factor in determining the body's metabolic reaction to a toxic compound. A brief review is now presented of the effect of the major nutrients on foreign compound metabolism.

**Starvation** - is frequently a prerequisite of chemotherapy or may ensue in pathological states. Kato and Gillette (1965) showed that starvation in male rats impairs only those enzymes that are largely sex-dependent, whilst in female rats these enzyme activities are increased by starvation. Subsequent work showed that starvation may interfere with the stimulatory effects of androgenic steroids in male rats whilst in female rats they may be due to slight increases in cytochrome P450 content and activity of the NADPH-linked electron transport system in liver microsomes (Kato and Onoda, 1970). Similar sex differences in response to starvation were reported by Gram et al (1970). Hepatic glucuronide conjugation of chloramphenicol was decreased in vivo by 18 - 24
hours fasting in male rats (Alvin and Dixit, 1974). Starvation markedly reduces the activity of mixed-function oxidases in the intestine (Wattenberg, 1971), with decreased activity of glucuronyltransferase (Marselos and Laitinen, 1975).

**Protein** - microsomal oxidations are generally decreased by inadequate protein intakes (Mgbodile and Campbell, 1972), with a concurrent decrease in hepatic microsomal protein and DNA concentrations. The liver cells tend to become larger and fewer in number (Campbell and Hayes, 1976). Fractionation of the microsomes from weanling male rats maintained on a 5% or 20% casein diet for 10 days, followed by reconstitution of components from each group to allow an independent examination of each component to be made, revealed that there was no significant effect of protein depletion on the phosphatidylcholine. The reductase and cytochrome P450 fractions obtained from the 5% casein group did not reconstitute equivalent control values indicating that the reaction between these two components could be the primary mechanism responsible for the effect of inadequate dietary protein (Nerurkar et al., 1978). The arrangement of components within the membrane was found to be more rigid in the protein depleted animals which would decrease translational mobility of the components (Campbell et al., 1979). Conflicting reports on the effect of dietary protein deficiency on conjugation reactions (Woodcock & Wood, 1971; Smith et al., 1973) indicate that the effect may be species related and dependent on the extent of protein depletion.

**Lipid** - contributes 30 - 40% by dry weight to the endoplasmic reticulum, most of which is phospholipid (Glaumann and Dallner, 1968). Deprivation of dietary lipid results in changes in
the content of microsomal phospholipid fatty acids and is associated with decreased metabolism of hexobarbital, aniline and ethylmorphine, and decreased content of cytochrome P450. A fat-free diet does not affect the activity of NADPH cytochrome C reductase so that the defect is at some stage beyond the initial oxidation of NADPH (Norred and Wade, 1972). Both the quantity and composition of dietary lipid is important in determining oxidative demethylation activities and cytochrome P450 levels (Rowe and Wills, 1976). Compared to a fat free diet, the rate of oxidative demethylation was higher with the addition of 10% corn oil. A similar finding (Century, 1969) suggests that an insufficient supply of polyunsaturated fatty acids is rate limiting for drug metabolism. However the effect was shown not to be an essential fatty acid deficiency (Marshall and McClean, 1971). Induction of cytochrome P450 in rat liver by phenobarbitone was found to be enhanced with increasing dietary concentration of highly unsaturated fatty acids (Marshall and McClean, 1971a). This was described as a 'permissive effect' since oils in the absence of phenobarbitone are only weak inducers. Fatty acid hydroperoxides or similar oxidation products were shown to play an essential role in induction of drug metabolizing enzymes in the liver (McClean and Marshall, 1971). The authors concluded that maximal induction of cytochrome P450 by phenobarbitone requires dietary peroxidised lipids. The content of lipid peroxide in rat hepatic endoplasmic reticulum and the rate of NADPH linked lipid peroxidation were found to be higher in rats fed 10% corn oil than 10% lard or a fat free diet (Rowe and Wills, 1976).

Carbohydrate - Sucrose feeding for 24-72 hours in place of a normal
chow diet, was found to decrease the activities of various mixed-function oxidases in male and female rats, together with a decrease in content of cytochromes P450 and b5, and NADPH cytochrome C reductase activity (Kato and Gillette, 1964). Similarly, Dickerson et al. (1971) found a 60% sucrose or glucose plus fructose diet when fed to rats, to decrease the activity of biphenyl-4-hydroxylase which correlated with lower levels of cytochrome P450. Xylitol feeding increases the hepatic amino-pyrine-N-demethylase activity, in mice, thought to be related to the concurrent increase in NADH (Smith, 1982).

Vegetarian Diets - A comparison of feeding a vegetarian and a meat diet in the long-term to male and female rats, on hepatic, small intestine and colonic mucosal mixed-function oxidases showed that meat diets increased hepatic benzo(a)pyrene hydroxylase and aminopyrine N-demethylase activities in male rats and the vegetarian diets increased the enzyme activities in female rats, abolishing the sex differences normally apparent in rats. The vegetarian diets were more effective in promoting large and small intestinal mixed-function oxidase activity in female rats than male rats. Protein and fat content of both diets were also varied but the source of the diet and sex of the animal were found to be more important determinants of mixed-function oxidase activities than the relative composition of fat and protein in the diets (Martin et al., 1980).

Drug-Nutrient Interactions - Diet and nutritional status are important in determining the body's reaction to foreign compounds and protection against harmful substances which may eventually or
immediately, and directly or indirectly be the cause of disease and illness. As drugs are classed as foreign compounds and are metabolized by this enzyme system, diet and nutritional status will equally affect the body's metabolism of a particular drug which will determine its bioavailability and the extent of its action. Anutrients present in the diet can induce the mixed-function oxidases particularly in the gut mucosa, which may alter the metabolism of some drugs and foreign compounds. The particular items of food eaten will affect the rate of absorption of drugs and other foreign compounds by altering the pH and motility of the gut, the ionic concentration, and the ratio of water and solid phases. Other food and drug interactions may occur: drugs may increase the toxicity of naturally occurring food substances by blocking specific metabolic pathways; chemical interactions between drugs and food components may lead to the production of toxic compounds; drugs may affect the gut flora and normal gut function; there may be synergistic effects between food components and drugs; drugs, especially when chronically administered, may lead to altered nutritional status, which in turn may lead to altered drug metabolism. For example, pellagra-like symptoms have been observed in patients treated with isoniazid (for tuberculosis) which disappear following combined treatment with niacin and pyridoxine (Dilorenzo, 1967). Isoniazid is a pyridoxine antagonist and causes isoniazid-pyridoxine-hydrazone to be excreted in the urine in amounts sufficient to deplete body stores of pyridoxal phosphate, the active form of the vitamin (Vilter, 1964). The availability of pyridoxine for synthesis of niacin from tryptophan is decreased, causing a secondary vitamin deficiency, that of niacin.
Low serum and red cell folate levels, and occasionally overt megaloblastic anaemia, occur in the majority of patients receiving long-term anticonvulsant therapy with diphenylhydantoin, phenobarbital and primidone (Reynolds, 1974). Phenothiazines and tricyclics also induce biochemical evidence of folic acid deficiency (Labadarios et al, 1978). It has been suggested that folic acid may be involved both as a coenzyme for drug metabolizing enzymes and in the synthesis of the enzymes themselves (Labadarios et al, 1978). Long term feeding to rats of anticonvulsant drugs in conjunction with a folate deficient diet was associated with impaired induction of cytochrome P450 (Labadarios, 1975). Hence chronic administration of any enzyme inducing drug would be increasingly likely to cause folate deficiency and consequently increased toxicity. The administration of folic acid to epileptics has been reported to aggravate their condition and disturb drug control of seizures, so that administration of folic acid supplements for therapeutic or prophylactic reasons remains controversial. This interesting drug-nutrient interaction is further investigated in Chapter Six.
AIMS

The aims of this study were:

1) To determine whether wheat bran is able to alter metabolic response, particularly of the mixed-function oxidase enzyme system of the liver and intestine, which may be related to its possible role in protecting the body from various diseases. Studies were designed to investigate more than one dietary level of bran and feeding periods were short-and long-term.

2) To determine the effect of bran on a physiological mechanism of protection against potential toxic insult i.e. the gastro-intestinal mucous barrier. Studies were designed to investigate more than one dietary level of bran and the effects of short- and long-term feeding.

3) To determine the effect of bran on the status of other nutrients: by measurement of the bone stores of minerals and circulating levels of 25-hydroxyvitamin D3.

4) To investigate the role of folic acid deficiency on drug-induced enzyme induction. Folic acid deficiency has been postulated as the cause of adverse reactions to anticonvulsant drugs after long-term therapy in epileptic patients. This interaction was further investigated.
Chapter Two

The Effect of Dietary Modification on the Hepatic Drug Metabolizing System in the Rat.
Introduction

The primary importance of diet in the aetiology of colon cancer is implicated by epidemiological evidence which shows an association in areas of high incidence with consumption of a high fat and a low fibre diet (Burkitt, 1971). The possible protective role of fibre against colon cancer is partly attributable to its physical action in the gut. The demonstration of a decreased incidence of chemically induced colon cancer by certain dietary fibres and isolated components in animal models has given a more positive indication of its protective action (Wilson et al., 1977; Wantabe et al., 1978). Further experiments with rats and mice have shown that dietary fibre protects against the toxic action of certain foreign chemicals e.g. sodium cyclamate, amaranth and 2,5-di-t-butylhydroquinone. When added to stock diets, which are high in cereal fibre, or semi-purified diets with an added fibrous component, there is a decreased incidence or absence of the toxic effects produced (slow weight gain, alopecia and diarrhoea) when the chemicals are added to a semi-purified diet (Kritchevsky, 1977).

Dietary fibre is known to alter the absorption of triglycerides and sterols from the intestine (Cummings, 1978). Fatty acid composition of ingested fats influences the composition of fatty acyl groups of membrane phospholipids, which are able to regulate the substrate binding properties of the microsomal drug metabolizing enzyme system, thus affecting the metabolism of
foreign compounds. Dietary fibres may contain unidentified components, probably anutrients, which may have the capacity to induce the microsomal drug metabolizing enzymes. Alternatively, adsorption by dietary fibre of xenobiotics or anutrients in the diet, hence slowing their absorption, may prevent the action of potential microsomal enzyme inducers or slow the body's exposure to certain foreign compounds.

A study of the effects of wheat bran on hepatic drug metabolism in the rat is now reported. The length of exposure required for high fibre diets to produce any beneficial effects is unknown. The optimal amount of dietary fibre that should be ingested daily is also unquantified. The only criteria by which a recommended daily intake for man has so far been assessed is related to the manifestation of its physiological properties (Spiller et al, 1977). It therefore seemed pertinent to examine the effect of two levels of wheat bran and also to study the effects of feeding diets in the short and long-term.

A diet containing a high level of saturated fat was included in some of the trials because of the association of high intakes of fat with cancer and atherosclerosis. Feeding a purified diet to rats results in lower activity of the mixed-function oxidase enzyme system in the liver (Brown et al, 1954), intestinal mucosa (Wattenberg, 1972a) and lung (Wattenberg, 1972), when compared to rats fed a stock diet. Hence, a stock diet (41B) was also included in some of the trials to compare any effects with those of the experimental, semipurified diets.
Experimental

Materials

The following chemicals were obtained from British Drug Houses Ltd (BDH): biphenyl, aniline hydrochloride, formaldehyde, p-aminophenol, bovine serum albumin and acetylacetone. Sigma was the supplier of: cytochrome C, 1-naphthol, NADP (nicotinamide dinucleotide phosphate), adenine/n-Heptane, diethyl ether and chloroform, all of Analar grade, were obtained from May and Baker, who very kindly donated the ethylmorphine hydrochloride. Ethoxyresorufin and resorufin were obtained from Pierce and Warrier. 2- and 4-Hydroxybiphenyl and Tween-80 (polyoxyethylene sorbitan mono-oleate) were supplied by Hopkins and Williams. 1-Naphthol-β-D-glucuronide was obtained from Koch and Light. NADH (nicotinamide adenine dinucleotide reduced) was purchased from Boehringer.

Copper reagent (protein determination) was prepared by the addition of 1% copper sulphate $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.5ml) and 2% trisodium citrate (0.5ml) to 1% sodium carbonate in 0.1M NaOH (50ml).

Animals

Male, weanling, Wistar Albino rats of the Surrey University breeding colony, were used for the short term feeding experiments (4, 7 & 12 wk). They were kept in polypropylene hanging cages made by North Kent Plastic Cages. The rats used in the long-term experiments (males and females) and the female rats maintained on the diets for 12 wk were supplied by the Flour Milling and Baking Research Association. Weanling Wistar Albino (COB) rats from Charles River, Manston, Kent were housed five per with wire mesh floors cage in plastic hanging cages at the Flour Milling and Baking
Research Association animal house until 3 wk prior to killing when they were transported to the animal house at Surrey University. Diet and water were available ad libitum to all rats. The temperature was kept at 37°C and the humidity at 50%. Lighting was on a 12 hr system.

Diets
The diets were formulated by the Flour Milling and Baking Research Association. The composition of the diets used is given in the Appendix to Chapter Two. The fibre-free diet was the experimental control. The concentrations of bran are equivalent to the amount of wheat bran contained in wholemeal bread which constitutes 17 and 34% of a diet by weight. Two levels are the minimum required to show a dose response. The stock diet, 41B was of vastly different composition to the experimental diets and therefore could not be considered as a control. It was included in the experimental project at the Flour Milling and Baking Research Association as a control of animal husbandry. The composition of the diets by analysis, for which acknowledgements are due to the Flour Milling and Baking Research Association, is also given in the Appendices. The diets were stored in sealed buckets at 4°C.

Methods
Preparation of liver subfractions - The rats were killed by cervical dislocation between 9-10.30 a.m. and the livers excised and washed in ice-cold 1.15% KCl to remove excess blood. They were blotted dry with filter paper, weighed, minced with scissors and homogenised with a Potter Elvehjem mechanically driven homogeniser,
in 0.1M Tris-HCl buffer pH 7.4 to give a 25%w/v homogenate. The vessel was surrounded by an ice bath while three up and down strokes were made. Homogenates were transferred to 50ml polycarbonate centrifuge tubes and centrifuged in an MSE High Speed 18 centrifuge at 10000g (av) for 20 min at 4°C to sediment cell debris, mitochondria and lysosomes. An aliquot of the supernatant was further centrifuged at 105000g (av) at 4°C for 60 min in an 8x50 ml fixed angle head rotor, Beckman L5-65 ultracentrifuge. This sedimented the microsomal pellet which, after removal of the soluble fraction (supernatant), was resuspended in 0.1M phosphate buffer pH 7.4, to give a 25%w/v suspension. When storage was required, microsomes were frozen at -40°C as a pellet covered by a small volume of buffer (Tris-HCl 0.1M pH 7.4), and resuspended the following day.

**Cytochrome P450 - (Omura and Sato, 1964)**

The microsomal suspension was diluted 1:3 with 0.1M phosphate buffer pH 7.4, the cytochrome P450 reduced with sodium dithionite and the solution divided equally between 2 cuvettes (3ml per cuvette). Carbon monoxide was gently bubbled through the test cuvette for 30 seconds and a difference spectrum was obtained between 400-500nm using an Pye Unicam SP1800 double beam spectrophotometer. Cytochrome P450 content was expressed as the difference in optical density between 450-490nm, using an extinction coefficient of 91mM⁻¹cm⁻¹.

**Cytochrome bs - (Ernste et al., 1962)**

The microsomal suspension, 25%w/v in 0.1M phosphate buffer pH 7.4 was further diluted 1:3 with the same buffer, and divided equally between two cuvettes (3ml per cuvette). To the test cuvette, 0.1mg
of NADH was added and a difference spectrum was recorded between 400-450nm using a Pye Unicam SP1800 double beam spectrophotometer. The difference in optical density between 410-426nm gave the concentration of cytochrome $b_5$ using an extinction coefficient of 185 mM$^{-1}$ cm$^{-1}$.

**NADPH Cytochrome C Reductase** - (Williams and Kamin, 1962)

Using a constant temperature cuvette, set at 37°C, in a Pye Unicam SP1800 double beam spectrophotometer, 1 mg of microsomal protein (105000g fraction) in 0.05M phosphate buffer pH 7.6 containing 1mM KCN, was incubated with 0.1 µmol cytochrome C for 2 min. Addition of 3 µmol NADPH initiated the reaction. The initial velocity at 550nm was taken as a measure of reductase activity using an extinction coefficient of 18.5 mM$^{-1}$ cm$^{-1}$.

**7-Ethoxyresorufin-O-Deethylase** - (Burke and Mayer, 1974)

Microsomal protein (1.0 mg; 105000g fraction) in 0.1M phosphate buffer pH 7.8 was incubated at 37°C with 1 µmol of ethoxyresorufin for 2 min in a constant temperature spectrofluorimeter cell. Addition of 0.5 µmol NADPH initiated the reaction. Fluorescence was measured at excitation wavelength 510nm emission wavelength 586nm and slit widths of 3.5nm using a Perkin-Elmer MPF-3 spectrofluorimeter. The reaction was followed for 2 min. Resorufin was used as the standard (0.1 nm).

**Biphenyl Hydroxylases** - (Creaven et al, 1965)

The incubation system consisted of 3 mg of microsomal protein (10000g fraction), 3.125 µmol biphenyl, 10 mg Tween-80, 10µmol MgCl$_2$, 1.5 µmol NADP, 40µmol Tris-HCl buffer pH 7.6, in a final volume of 2 ml.

After a preincubation period of 5 min at 37°C, the substrate was
added to initiate the reaction. Termination at 10 min was achieved by the addition of 2N HCl (0.5ml) and transfer of the tubes to an ice bath. Biphenyls were extracted into 10ml n-heptane for 10 min at room temperature followed by centrifugation using a Beckman J6 centrifuge at 2500rpm for 10 min. Aliquots of the heptane layer (2.0ml) were further extracted into 0.1M NaOH (10ml) and centrifuged as before. The heptane layer was removed by vacuum pump. Fluorescence of an aliquot of the aqueous layer (2.0ml) adjusted to pH5.5 by addition of 0.25M succinic acid (0.5ml) was recorded using a Perkin-Elmer MPF-3 spectrofluorimeter at excitation wavelength 275nm emission wavelength 338nm for the determination of 4-hydroxybiphenyl, and wavelengths of 295nm and 420nm respectively for determination of the 2-hydroxy isomer. Slit widths were set at 6nm. Blanks and standards (25µg 4-hydroxy- and 6µg 2-hydroxybiphenyl) were carried through the same procedure.

Aniline Hydroxylase - (Guarino et al, 1969)
The incubation system consisted of 3mg microsomal protein (10000g fraction) 20µmol aniline, 10µmol MgCl$_2$, 2µmol NADP, 20µmol glucose-6-phosphate and 150µmol Tris-HCl buffer pH 7.6, in a final volume of 2ml.

After a preincubation period of 5 min at 37°C, the reaction was initiated by addition of the substrate. Termination at 10 min was achieved by the addition of approximately 1g solid NaCl and transfer of the tubes to an ice bath. p-Aminophenol was extracted into peroxide-free ether (10ml) at room temperature, centrifuged using a Beckman J6 centrifuge at 2500rpm for 10 min, further extracted into alkaline phenol (4ml) for 40 min and centrifuged as before. The absorbance was measured at 620nm in a Cecil 272
spectrophotometer. Blanks and standards (0.05-0.25μmol p-aminophenol) were carried through the same procedure.

**Ethylmorphine-N-Demethylase** - (*Holtzman et al.*, 1968)

The incubation system consisted of 3mg microsomal protein (10000g fraction) 15μmol ethylmorphine hydrochloride, 10μmol glucose-6-phosphate, 0.2mg semicarbazide hydrochloride solution (pH7.0) and 150μmol Tris-HCl buffer pH7.6, in a final volume of 2.55ml.

After a 5 min preincubation period at 37°C, the reaction was initiated by addition of the substrate and terminated at 10 min by addition of 15mg zinc sulphate and the tubes transferred to an ice bath. Addition of 1.0ml of a saturated mixture of solutions of barium hydroxide and sodium tetraborate (2:1, v/v), precipitated all of the protein. After centrifugation, 2500rpm for 15 min (Beckman J6 centrifuge) an aliquot (2ml) of the supernatant was added to Nash reagent (2ml; Nash, 1953) and incubated at 37°C for 40 min. The absorbance was measured at 412nm using a Cecil 272 spectrophotometer. Blanks and standards (0.15-0.6μmol formaldehyde) were carried through the same procedure.

**1-Naphthol Glucuronyltransferase** - (*Bock*, 1974; *Shirkey*, 1977)

The incubation system consisted of 0.5mg microsomal protein (105000g fraction), 0.2μmol 1-naphthol, 2.5μmol MgCl₂, 0.5mg Brij-35, 5μmol UDPGA, and 0.05mmol Tris-HCl buffer pH7.6 in a final volume of 0.5ml.

After a 5 min pre-incubation period at 37°C, the reaction was initiated by the addition of 1-naphthol and was terminated at 4 min by addition of 0.05M glycine-trichloroacetic acid buffer pH2.2 (1.0ml), and the tubes were transferred to an ice bath. Unreacted
1-naphthol was extracted into chloroform (6ml) at room temperature and after centrifugation at 2500rpm for 10 min, an aliquot of the aqueous layer (1.0ml) was added to 1.0M glycine-NaOH buffer pH10.6 (1.0ml). Fluorescence was measured using a Perkin-Elmer MPF-3 Spectrofluorimeter at excitation wavelength 303nm, emission wavelength 334nm, with slit widths set at 6nm. Blanks and standards (5 - 20nmol) were carried through the same procedure.

**Protein Determination** - (Lowry et al, 1951)

Microsomal suspension 25%w/v was further diluted 1:20 with 0.5M NaOH to give a concentration of 600mg microsomal protein/ml. An aliquot (0.5ml) was added to an equal volume of 0.5M NaOH and freshly made copper reagent (5ml) was added. After vortex mixing the tubes were allowed to stand at room temperature for 10 min. Folin-Ciocalteau's phenol reagent was diluted 1:2 with distilled water and aliquots (0.5ml) added to the tubes and mixed within 1 second. The absorbance was recorded after 30 min using a Cecil 272 spectrophotometer. Bovine serum albumin (0-300μg) was used to construct a standard range of protein values.

**Statistical Analyses**

These were performed using Student's t test.

**Results**

No differences in food consumption or weight gain were observed between any of the groups of rats studied. All animals were healthy, and there were no obvious differences in behaviour or appearance between the groups.
The rats maintained on the 4.8% and 9.6% bran diets for 4 wk showed no changes in levels of hepatic cytochromes P450 and P448 or any of the mixed-function oxidases studied compared to the fibre-free controls (Table 2.1). Microsomal protein values were similar in all groups. A longer feeding period (7 wk) was found to produce no changes in these enzyme levels. The diet containing 8.5% beef suet was also found to produce no changes in the animals maintained on this diet compared to the fibre-free controls. There were no significant differences in microsomal protein content between the groups (Table 2.2). Diet 41B, the stock diet, was associated with higher levels of cytochrome P450, cytochrome P448, biphynyl-4-hydroxylase, and 7-ethoxyresorufin-0-deethylase in male and female rats maintained for 12 wk on the diet, compared to the fibre-free controls (Tables 2.3 & 2.4). 1-Naphthol glucuronyltransferase activity was increased in the male rats in the stock diet group compared to controls. The largest percentage increase above controls was seen for 7-ethoxyresorufin-0-deethylase in both male and female rats (Tables 2.3 & 2.4). This enzyme activity is attributed to cytochrome P448, an enzyme thought to be associated with the activation of carcinogenic molecules (Burke and Mayer, 1975). The male rats maintained on the 4.8% bran and 9.6% bran diets and the female rats maintained on the 9.6% bran diet had significantly higher 7-ethoxyresorufin-0-deethylase activities. Ethylmorphine-N-demethylase was significantly decreased in male rats maintained on the 9.6% bran diet. However, these changes did not follow any dose response effects.

In the male rats maintained for long-term (18 months) on the
stock diet, no increase in levels of cytochromes P450, \( b_5 \), or of mixed-function oxidase enzymes was apparent in comparison to controls, apart from an increased activity of 7-ethoxyresorufin-0-deethylase (Table 2.5). No changes were observed in the 9.6% bran and 8.5% beef suet groups compared to the fibre-free control group. Generally, all enzyme activities in the long-term study rats were lower than those observed in male rats at 4, 7 and 12 wk (Fig. 2.1). However 7-ethoxyresorufin-0-deethylase was not observed to decrease in activity in groups of rats maintained on the control and 9.6% bran diets (Fig. 2.1).
Table 2.1. Effect of Dietary Wheat Bran on the Hepatic Drug Metabolizing Enzyme System in the Rat Maintained on the Diets for 4 weeks.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>DIET GROUP</th>
<th>Fibre-free (Control)</th>
<th>4.8% Bran</th>
<th>9.6% Bran</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.86±0.08 (100%)</td>
<td>0.71±0.05 (83%)</td>
<td>0.82±0.04 (95%)</td>
</tr>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td></td>
<td>0.32±0.05 (100%)</td>
<td>0.27±0.02 (84%)</td>
<td>0.31±0.02 (97%)</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol/mg protein)</td>
<td></td>
<td>38.7±0.3 (100%)</td>
<td>40.3±5.0 (104%)</td>
<td>38.3±1.3 (99%)</td>
</tr>
<tr>
<td>NADPH Cytochrome C reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-Ethoxycoumarin-O-deethylation (nmol coumarin/mg protein/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (nmol 2-OH biphenyl/mg protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol p-aminophenol/mg protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylmorphine-N-demethylase (nmol formaldehyde/mg protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for four male animals. Figures in parentheses are percentage values of the control group.
Table 2.2. Effect of Dietary Wheat Bran and High Dietary Fat on the Hepatic Drug Metabolizing Enzyme System in the Rat, Maintained on the Diets for 7 weeks.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>DIET GROUP</th>
<th>Fibre Free (control)</th>
<th>4.8% Bran</th>
<th>9.6% Bran</th>
<th>0.5% Beef Suet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td></td>
<td>0.88±0.09 (100%)</td>
<td>1.02±0.04 (116%)</td>
<td>0.86±0.05 (98%)</td>
<td>0.80±0.08 (91%)</td>
</tr>
<tr>
<td>Cytochrome b5 (nmol/mg protein)</td>
<td></td>
<td>0.41±0.06 (100%)</td>
<td>0.49±0.03 (120%)</td>
<td>0.42±0.04 (102%)</td>
<td>0.39±0.03 (95%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-O-deethylase (nmol resorufin/mg protein/min)</td>
<td></td>
<td>0.042±0.004 (100%)</td>
<td>0.053±0.002 (126%)</td>
<td>0.039±0.004 (100%)</td>
<td>0.036±0.002 (92%)</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td></td>
<td>65±5 (100%)</td>
<td>61±1 (125%)</td>
<td>66±5 (102%)</td>
<td>52±6 (80%)</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (nmol 2-OH biphenyl/mg protein/hr)</td>
<td></td>
<td>7.64±0.73 (100%)</td>
<td>9.36±1.31 (123%)</td>
<td>7.58±0.61 (99%)</td>
<td>6.34±1.28 (83%)</td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol p-aminophenol/mg protein/hr)</td>
<td></td>
<td>61±6 (100%)</td>
<td>82±10 (134%)</td>
<td>70±7 (115%)</td>
<td>67±7 (110%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td></td>
<td>22.6±2.5 (100%)</td>
<td>20.4±1.5 (90%)</td>
<td>23.5±1.7 (104%)</td>
<td>28.5±2.7 (126%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for six male animals. Figures in parentheses are percentage values of the control group.
Table 2.3.  Effect of Dietary Wheat Bran and a Stock Diet (41B) on the Hepatic Drug Metabolizing Enzyme System in the Rat, Maintained on the Diets for 12 weeks.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Fibre-free (Control)</th>
<th>4.8% Bran</th>
<th>9.6% Bran</th>
<th>41B Stock Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td>0.57 ± 0.02 (100%)</td>
<td>0.58 ± 0.04 (102%)</td>
<td>0.55 ± 0.02 (96%)</td>
<td>0.70 ± 0.06 (123%)</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol/mg protein)</td>
<td>0.30 ± 0.03 (100%)</td>
<td>0.32 ± 0.06 (110%)</td>
<td>0.29 ± 0.02 (97%)</td>
<td>0.45 ± 0.02 (150%)***</td>
</tr>
<tr>
<td>NADPH Cytochrome C reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td>43.3 ± 5.0 (100%)</td>
<td>47.3 ± 6.3 (109%)</td>
<td>33.0 ± 3.0 (76%)</td>
<td>45.1 ± 3.2 (104%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-O-deethylase (nmol resorufin/mg protein/min)</td>
<td>0.025 ± 0.004 (100%)</td>
<td>0.047 ± 0.005 (188)***</td>
<td>0.039 ± 0.004 (156)***</td>
<td>0.14 ± 0.012 (560)***</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OHbiphenyl/mg protein/hr)</td>
<td>86 ± 5 (100%)</td>
<td>101 ± 6 (117%)</td>
<td>78 ± 10 (91%)</td>
<td>111 ± 6 (129) **</td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol p-aminophenol/mg protein/hr)</td>
<td>29 ± 2 (100%)</td>
<td>28 ± 2 (97%)</td>
<td>23 ± 3 (79%)</td>
<td>35 ± 4 (121%)</td>
</tr>
<tr>
<td>Ethylmorphine-N-demethylase (nmol formaldehyde/mg protein/hr)</td>
<td>0.57 ± 0.06 (100%)</td>
<td>0.49 ± 0.05 (86%)</td>
<td>0.42 ± 0.08 (74)***</td>
<td>0.61 ± 0.01 (101)</td>
</tr>
<tr>
<td>1-Naphthol glucuronyl transferase (nmol 1-naphthol glucuronide/mg protein/min)</td>
<td>78 ± 12 (100%)</td>
<td>102 ± 12 (131)</td>
<td>89 ± 10 (114)</td>
<td>106 ± 9 (136)</td>
</tr>
<tr>
<td>Micronenomal protein (mg/g liver)</td>
<td>24.1 ± 2.0 (100%)</td>
<td>23.0 ± 0.5 (95%)</td>
<td>30.9 ± 2.5 (114%)</td>
<td>27.8 ± 1.8 (115)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five male animals. Figures in parentheses are percentage value of control group. Value significantly different from control: *p<0.05; **p<0.02; ***p<0.01; ****p<0.001.
Table 2.4. Effect of Dietary Wheat Bran, High Dietary Fat and a Stock Diet on the Hepatic Drug Metabolizing Enzyme System in the Rat Maintained on the Diets for 12 weeks.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Fibre-free (control)</th>
<th>9.6% Bran</th>
<th>8.5% Beef suet</th>
<th>41B Stock Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.72 ± 0.06 (100%)</td>
<td>0.78 ± 0.09 (108%)</td>
<td>0.88 ± 0.14 (122%)</td>
<td>1.03 ± 0.05 (143%)**</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>0.52 ± 0.04 (100%)</td>
<td>0.63 ± 0.06 (121%)</td>
<td>0.62 ± 0.08 (119%)</td>
<td>0.70 ± 0.03 (135%)**</td>
</tr>
<tr>
<td>(nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH Cytochrome C</td>
<td>54.4 ± 8.8 (100%)</td>
<td>43.1 ± 4.3 (79%)</td>
<td>50.2 ± 4.9 (92%)</td>
<td>46.6 ± 2.6 (86%)</td>
</tr>
<tr>
<td>reductase (nmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cytochrome C reduced/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg protein/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-Ethoxyresorufin-O-</td>
<td>0.067 ± 0.017 (100%)</td>
<td>0.114 ± 0.017 (171%)</td>
<td>0.126 ± 0.024 (189%)*</td>
<td>0.150 ± 0.009 (225%)***</td>
</tr>
<tr>
<td>deethyelase (nmol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>resorufin/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl-4-hydroxy-</td>
<td>74 ± 6 (100%)</td>
<td>78 ± 11 (105%)</td>
<td>82 ± 32 (111%)</td>
<td>86 ± 6 (116%)</td>
</tr>
<tr>
<td>lase (nmol 4-OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biphenyl/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl-2-hydroxy-</td>
<td>1.7 ± 0.2 (100%)</td>
<td>1.8 ± 0.2 (106%)</td>
<td>1.8 ± 0.2 (106%)</td>
<td>1.9 ± 0.2 (111%)</td>
</tr>
<tr>
<td>lase (nmol 2-OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>biphenyl/mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline hydroxylase</td>
<td>48 ± 4 (100%)</td>
<td>45 ± 4 (94%)</td>
<td>43 ± 4 (90%)</td>
<td>40 ± 5 (83%)</td>
</tr>
<tr>
<td>(nmol p-aminophenol/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg protein/hr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Micronuclear protein</td>
<td>19.8 ± 1.2 (100%)</td>
<td>19.0 ± 1.8 (96%)</td>
<td>16.2 ± 1.3 (82%)</td>
<td>17.6 ± 1.7 (89%)</td>
</tr>
<tr>
<td>(mg/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five female animals.
Figures in parentheses are percentage values of the control group.
Value significantly different from control: *p<0.05; **p<0.005; ***p<0.001.
Table 2.5. Effect of Dietary Wheat Bran, High Dietary Fat and 41B Stock Diet on the Hepatic Drug Metabolizing Enzyme System of the Rat, Maintained on the Diets for 18 Months.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Fibre-free (Control)</th>
<th>9.6% Bran</th>
<th>8.5% Beef Suet</th>
<th>41B Stock Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P450 (nmol/mg protein)</td>
<td>0.35 ± 0.07 (100%)</td>
<td>0.29 ± 0.03 (83%)</td>
<td>0.30 ± 0.02 (86%)</td>
<td>0.30 ± 0.02 (86%)</td>
</tr>
<tr>
<td>Cytochrome b (nmol/mg protein)</td>
<td>0.20 ± 0.02 (100%)</td>
<td>0.21 ± 0.01 (105%)</td>
<td>0.19 ± 0.02 (95%)</td>
<td>0.23 ± 0.01 (115%)</td>
</tr>
<tr>
<td>NADPH Cytochrome C reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td>38.1 ± 4.5 (100%)</td>
<td>33.2 ± 2.6 (87%)</td>
<td>30.6 ± 3.0 (80%)</td>
<td>30.9 ± 3.5 (81%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-O-deethylase (nmol resorufin/mg protein/min)</td>
<td>0.029 ± 0.005 (100%)</td>
<td>0.045 ± 0.012 (155%)</td>
<td>0.045 ± 0.005 (155%)</td>
<td>0.080 ± 0.010 (276%)*</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>28 ± 3 (100%)</td>
<td>32 ± 4 (114%)</td>
<td>23 ± 3 (82%)</td>
<td>25 ± 3 (89%)</td>
</tr>
<tr>
<td>Aniline hydroxylase (nmol p-aminophenol/mg protein/hr)</td>
<td>11 ± 2 (100%)</td>
<td>13 ± 0.2 (118%)</td>
<td>13 ± 1 (118%)</td>
<td>14 ± 3 (127%)</td>
</tr>
<tr>
<td>Ethylmorphine-N-demethylase (nmol formaldehyde/mg protein/min)</td>
<td>0.12 ± 0.02 (100%)</td>
<td>0.16 ± 0.02 (133%)</td>
<td>0.12 ± 0.01 (100%)</td>
<td>0.18 ± 0.02 (139%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>35.7 ± 4.3 (100%)</td>
<td>34.4 ± 0.8 (96%)</td>
<td>37.8 ± 1.8 (106%)</td>
<td>40.4 ± 2.6 (113%)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for five male animals.
Figures in parentheses are percentage value of control group.
Value significantly different from control: *p<0.005.
Drug metabolism parameters in mature (18 months) compared to young rats (12 weeks) for 1: cytochrome P450, 2: cytochrome b5, 3: NADPH cytochrome C reductase, 4: ethoxyresorufin-O-deethylase, 5: biphenyl-4-hydroxylase, 6: aniline hydroxylase, 7: ethylmorphine-N-demethylase, 8: microsomal protein. Asterisks indicate values significantly different at 18 months compared to 12 weeks - * = p<0.05, ** = p<0.02, *** = p<0.01, **** = p<0.005 (Student's t-test). (See Table 2.3. § 2.5. for absolute values)
Discussion

The lack of effect of wheat bran on hepatic cytochrome P450 levels and mixed-function oxidases is in agreement with other workers (Smith-Barbaro et al, 1981a; Proia et al, 1981). An 11 month exposure to a diet containing 15% wheat bran produced no changes in hepatic cytochrome P450 or cytochrome b₅ levels when compared to control rats maintained on a diet containing 5% cellulose. Similar results were observed when 15% citrus pulp replaced wheat bran in the experimental diet. Hepatic 3-hydroxy-3-methylglutaryl (HMG) CoA reductase activity, the key rate-limiting enzyme in cholesterol synthesis, was unaffected by either of the two high fibres regimes compared to the control, indicating that these dietary fibres do not influence hepatic choledrogenesis (Smith-Barbaro et al, 1981a).

The addition of 10.4% cellulose to a purified diet was found to decrease hepatic cytochrome P450 levels and aniline hydroxylase and ethylmorphine-N-demethylase activities compared to the addition of 10.4% pectin or a cereal based diet, and fed to rats for 5 wk (Proia et al, 1981). Cytochrome b₅ and HMG CoA reductase levels were similar in rats maintained on the cellulose and pectin diets and lower than rats maintained on the cereal-based diets. This work can be criticised for not including a correct control diet, i.e. the purified diet without any fibrous component added. The authors concluded that cellulose and pectin have substantially different effects on the cytochrome P450 system in rat liver and that dietary fibre does not affect this system and the HMG coA
reductase system in a parallel manner. However these conclusions are not in agreement with those of Smith-Barbaro et al (1981a). The experiments differed by the length of exposure to the diets and the use of fibre components by Proia et al (1981) compared to the use of natural fibres by Smith-Barbaro et al (1981a).

In the reported experiment no dose response effects with dietary wheat bran content or consistent trends with the length of the feeding period were observed. It can be concluded from this work and other reports (Smith-Barbaro et al, 1981a) that wheat bran does not affect the hepatic microsomal mixed-function oxidase enzyme system.

Similarly no changes were observed in the 8.5% beef suet groups compared to the controls at any of the time points studied. Beef suet is composed of saturated and monounsaturated fat. This diet contained 2% corn oil, such that the beef suet component contributed approximately 18% and the corn oil approximately 4.2% of the calories. The control and bran diets contained 2% corn oil which contributed 5% of the calorific intake. The results here are in agreement with other evidence (Marshall & McLean, 1971a), indicating that the dietary level of saturated fat does not affect hepatic drug metabolizing enzyme activity when the dietary level of polyunsaturated fatty acids is adequate for maximal activity at both basal and induced levels.

The increased levels of cytochrome P450 and cytochrome b₅, and biphenyl-4-hydroxylase and 7-ethoxyresorufin-o-deethylase
activities in the male and female rats maintained on the stock diet for 12 wk compared to the control, semipurified diet group is a well-reported phenomenon. This effect is due to foreign residues, vegetable components, anutrients and natural xenobiotics (Wattenburg, 1971). A role of the hepatic drug metabolizing enzyme system is to eliminate naturally occurring xenobiotics, ingestion of which in large amounts would be expected to stimulate these readily-inducible enzymes (Parke, 1976). Feeding the stock diet over a longer period however results in an increased activity of 7-ethoxyresorufin-o-deethylase, an enzymic activity attributed to cytochrome P448 (Burke and Mayer, 1974). Kato and Takanaka (1968) reported that induction of the cytochrome P450 system by administration of phenobarbitone is less extensive in old than mature rats. More recent studies examining the extent to which aging impairs induction have produced conflicting evidence (Schmucker and Wang, 1980; McMartin et al, 1980; Gold and Widnell, 1974; Kao and Hudson, 1980). Inconsistent findings may be due to different strains, sex and age groups used for comparison, and the use of bedding and commercial diets which are known to alter levels of non-induced drug metabolizing enzymes.

More recent work (Rikans and Notley, 1981) has shown that qualitative changes in the microsomal enzyme system occur as the animals age, as well as modification of the induction process, supporting the evidence of Schmucker and Wang (1980) and McMartin et al (1980). Induction by methyltestosterone was found to be less in older animals (14-15 months) than in younger animals (3-5 months), whereas induction by phenobarbitone or B-naphthoflavone
was equivalent in both age groups. Benzphetamine-N-demethylase and aniline hydroxylase activities were decreased in older rats and nitroanisole-O-demethylation was enhanced (Rikans & Notley, 1981).

The same authors (Rikans and Notley, 1981a) found that aging from 5-14 months was accompanied by a decline in liver cytochromes P450 and b5, NADPH cytochrome C reductase and phospholipid content. The decline in microsomal monooxygenase activities was found to be substrate specific, indicating that the components which decline with age are not rate-limiting for the monooxygenation of all substrates. This may be explained by selective decreases in the activity of certain of the multiple forms of cytochrome P450. Evidence for a selective effect of aging on different forms of cytochrome P450 has been reported (McMartin et al, 1980).

In the experiments now reported, a significant decline of enzyme activities was observed for rats maintained on all diets studied at 18 months in comparison to rats fed for 4, 7 and 12 wk. The exception is a small increase in the activity of 7-ethoxyresorufin-O-deethylase in male control and 9.6% bran groups fed for 18 months compared to rats fed on these diets for 12 wk. A significant decline of activity of this enzyme was seen in the rats fed on the stock diet for 18 months in comparison to rats fed for 12 wk (males), indicating a less marked induction response. The measurements were made on different days which may account for some of the variation. Schmucker and Wang (1980) reported no age-dependent differences in non-induced microsomal protein concentration indicating no loss of endoplasmic reticulum. In the
reported experiment, higher values for microsomal protein were found in the fibre-free and stock diet groups of male rats maintained on the diets for 18 months.

The age-effects on 7-ethoxyresorufin-0-deethylase are interesting. The increased activity of this enzyme in rats maintained on the stock diet for 18 months, without an increased activity of any other enzymes may be due to a specific cytochrome P448 effect. This is supported by the maintenance of 7-ethoxyresorufin-0-deethylase activity in the rats fed on the experimental diets for 18 months where all other enzyme activities have decreased. It has been suggested that cytochrome P448 is the form responsible for carcinogenic activation (Burke & Mayer, 1975).

The role of the mixed-function oxidase system as a survival mechanism against foreign compounds has been postulated (Wattenberg, 1972), with the proviso that it is necessary to evaluate the overall balance of protective effects with potentially harmful ones. Exposure to naturally occurring xenobiotics, promoting increased xenobiotic metabolism, leads to a general increase in turnover and detoxication, and increased incidence of acute toxicity due to some activation of compounds by this system may also occur. A loss of capacity for induction of drug metabolizing enzymes with aging may be indicative of decreased adaptation of the survival system by a loss of protection against tumour formation which may be of significance in the aetiology of aging and disease. Elderly human subjects show decreased induction response to dichloralphenazone compared to young subjects (Salem et
A higher frequency of adverse drug reactions (Hurwitz, 1969) and a higher incidence of cancer is seen in the elderly.

In conclusion it appears that any protection afforded by wheat bran against colon cancer and other diseases (Burkitt, 1971) is not mediated through the hepatic mixed-function oxidase enzyme system. The association of aging with high incidence of cancers may be due to effects of aging on specific forms of cytochrome P450.
Chapter Three

The Effect of Dietary Wheat Bran on Intestinal Drug Metabolism and its Induction by Safrole in the Rat.
Introduction

The small intestine has wide scope for xenobiotic metabolism. Whilst its capacity for metabolic transformation (phase I metabolism) is lower than the liver it has a greater capacity for synthetic reactions (phase II metabolism), of which glucuronidation is the most active. The intestine assumes importance as an organ of foreign compound metabolism since it is the first tissue to come into contact with orally ingested xenobiotics and the gut is the major route of exposure of the body to these compounds. The conversion of a proportion of these xenobiotics by the intestine, to excretable compounds offers protection to the rest of the animal body.

The mixed-function oxidase enzymes of the gastro-intestinal tract are highly inducible. It has been suggested that most aryl hydrocarbon hydroxylase (AHH) activity catalysed by the mixed-function oxidase enzymes in the intestine, one of the two major portals of entry, results from continuous exposure to endogenous dietary inducers (Wattenberg, 1972a). Feeding rats a purified diet (casein, starch, corn oil, salt mix and vitamin supplement) compared to feeding purina rat chow results in substantially decreased benzo(a)pyrene hydroxylase activity in intestinal mucosa and lung (Billings and Wattenberg, 1972). Crude diets have been shown to enhance the microsomal metabolism of this substrate in the liver (Brown et al., 1954). The inducing activity of purina rat chow was traced to an inherent component, namely alfalfa grass (Wattenberg, 1972). Other vegetables were
subsequently shown to have inducing activity the most potent being
members of the Brassicaceae family - brussels sprouts, cabbage,
broccoli, turnips and cauliflower; spinach, dill and celery were
found to have similar effects. The potent compounds were
identified as indole-3-acetonitrile, indole-3-carbinol and
3,3'-diindolylmethane, metabolites of indolylmethylglucosinolate
(Loub et al., 1975). Flavones (Wattenberg et al., 1968) and isosafrole
(Lake and Parke, 1972) have been shown to induce mixed-function
oxidases in the intestine. Numerous other chemicals to which we
are exposed by dietary contamination and drugs are inducers of
intestinal mixed-function oxidase enzymes (Sell and Davison, 1973;
Wattenberg and Leong, 1965).

Within the gastro-intestinal tract, xenobiotics are exposed
to the same factors as dietary nutrients. Specific processes
operating in the lumen and wall of the gastro-intestinal tract
include secretion and cellular events; physicochemical conditions
differ from the mouth to the rectum due to pH, micellar formation
and ionic strength (Pekus, 1980). These processes and conditions
are in turn affected by the luminal contents. Dietary fibres are
known to alter physicochemical conditions by their bulking action,
adsorption of water and organic chemicals, their effect on transit
time, gut motility, and ion-exchange capacity.

In the previous chapter wheat bran was found not to induce
the mixed-function oxidases of the liver. Its effect on the more
readily inducible intestinal mixed-function oxidases has therefore
been investigated. A modified diet containing 28.8% bran, three
times the amount present in the 9.6% bran diet, was included in this experiment.

A proposed mechanism by which bran may protect against colorectal cancer is by the adsorption of carcinogens and precarcinogens in the intestinal tract (Burkitt, 1971). Safrole (4-allyl-1,2-methylenedioxybenzene) is a weak hepatocarcinogen (Homburger et al, 1961) and an inducer of hepatic and extrahepatic mixed-function oxidases (Parke and Rahman, 1970). It is the primary component of oil of sassafras and is a minor component of nutmeg, cinnamon, camphor and many other essential oils. The ability of wheat bran to adsorb safrole in vitro has been shown by Moore (1983), using wheat bran supplied by the Flour, Milling and Baking Research Association. There is approximately 60% adsorption at pH3. The effect of feeding safrole in the presence or absence of bran on the induction of intestinal mixed-function oxidases and a glucuronyltransferase enzyme was therefore investigated in order to determine whether the in vitro adsorption of safrole by bran occurred in vivo and was of physiological significance i.e. by decreasing the extent of interaction between the carcinogen and the mixed-function oxidase enzyme system.

Many technical difficulties are encountered when investigating extrahepatic drug metabolism, mainly because of the excessive connective tissue contaminants and differences in enzyme stability and co-factor requirements. Techniques employed in the study of intestinal drug metabolism include histochemical techniques, isolated loops, mucosal scrapings and isolated cells.
The latter two methods can be used for the preparation of sub-cellular structures. Difficulties arise because of contamination of the mucosal cells with cells of the brush border and terminal web, and with mucus. The rat especially produces copious quantities of mucus. Mucosal scraping preparations in the rat have been found to be unreliable for subfractionation (Shirkey, 1977). The use of isolated mucosal cells for sub-fractionation was found to be more consistent, giving reliable results (Shirkey, 1977). Hence this technique was used in this study, with only slight modifications.
Experimental

Materials

Safrole was purchased from Hopkins and Williams Ltd. Sucrose was manufactured by Tate and Lyle. EDTA was purchased from BDH Ltd. and glucose-6-phosphate dehydrogenase were purchased from Sigma. All other materials were obtained as described in Chapter Two.

Experimental Procedure

In the first experiment, rats (four per group) were maintained on the diets containing 4.8, 9.6 and 28.8% wheat bran for 4 wk. The latter level was achieved by addition of more of the same wheat bran to the 9.6% bran diet. The commencement of feeding was staggered such that two animals per group were killed on one day, and the final animal weights and feeding periods were the same for all animals. The results were pooled for analysis.

For the safrole study 0.25%(w/w) safrole was added to the fibre free and 9.6% bran diets. The unmodified fibre-free diet was the control. Four animals per group were used and each group was allowed a period of 5 days to adjust to the experimental diets before adding the safrole. The safrole-containing diets were introduced on day six. The animals were killed on day 13, thereby giving 8 days of feeding with safrole. All animals were killed on the same day.

Methods

Preparation of Intestinal Subfractions - (Modification of Shirkey, 1977)
Between 9-30-10-30 a.m. the rats were killed by cervical dislocation, the abdomen opened and the small intestine, excised from the duodenum to the caecum, was flushed with ice-cold 0.9% NaCl-20mM Tris pH7.4 buffer. The first 60cm of the proximal small intestine was cut into six 10cm lengths. Four rats were killed at one time and it was possible to deal with a total of 12 rats on one day. Further work was conducted at 4°C. The intestinal sections were everted onto six metal rods and vibrated vertically at 100Hz, 1.5mm (Vibromixer E1, A.G. Chemap, Zurich) for 1 min in 0.14M NaCl-5mM EDTA pH7.4 buffer solution to dislodge the intact epithelial cells uncontaminated with lamina propria. This solution was poured into six pre-weighed MSE 50ml centrifuge tubes and the cells were precipitated by centrifugation at 1000g for 2 min in an MSE Minor centrifuge. The supernatant was discarded and the cells weighed and resuspended in 9ml 75mM sucrose buffer pH7.4. The cells were homogenised with a Potter Elvehjem mechanically driven homogeniser with ten up and down strokes. Before the final stroke 9ml 0.52M sucrose buffer pH7.4 and 7ml 0.3M sucrose pH7.4 were added to render the solution 0.3M. The homogenate was centrifuged for 4 min at 1000g (max) in an MSE Minor centrifuge, and the supernatant retained. The precipitate was washed twice with 0.3M sucrose pH7.4 and the supernatants combined. Mitochondria and lysosomes were precipitated by centrifugation at 10000g(av) in an MSE High Speed 18 centrifuge. Microsomes were precipitated by centrifugation at 105000g(av) in a Beckman L5-65 centrifuge. The pellet was resuspended in 0.1M Tris buffer pH7.4 to give a concentration of 1g wet cell weight in 2.5ml Tris-HCl buffer.
Biphenyl-hydroxylases - (Creaven et al, 1965)

The incubation mixture consisted of 1.95\textmu mol NADP, 15\textmu mol glucose 6-phosphate, 1 unit glucose 6-phosphate dehydrogenase, 10\textmu mol MgCl\textsubscript{2}, 1mg microsomal protein (105000g fraction), 0.08mMol Tris-HCl buffer pH 7.6, 1.0 \textmu mol biphenyl and 0.08g Tween-80 in a final volume of 2ml.

The mixture was preincubated for 5 min in the absence of substrate which was added to start the reaction. Incubation was terminated at 7.5 min by addition of 2M HCl (0.5ml) and the tubes plunged into an ice-bath. The extraction procedure consisted of the addition of n-heptane (7ml) containing 1.15% isoamylalcohol and rotary extracting at room temperature for 10 min. After a 10 min centrifugation at 2500rpm in a Beckman J6 centrifuge, an aliquot of the heptane layer (2ml) was added to 0.1M NaOH (5ml) and similarly extracted and centrifuged. Fluorescence was measured in 2ml of the NaOH phase adjusted to pH5.5 by addition of 0.25M succinic acid (0.5ml) at excitation wavelength 275nm, emission wavelength 338nm for determination of 4-hydroxybiphenyl and excitation wavelength 295nm, emission wavelength 420nm for determination of the 2-hydroxy isomer using Perkin-Elmer MPF-3 spectrofluorimeter. Blanks and standards (25\textmu g 4-hydroxy- and 6\textmu g 2-hydroxybiphenyl) were carried through the same procedure. The optimal substrate concentration for biphenyl hydroxylases was found to be 1\textmu mol per incubation system, with an optimal incubation time of 7.5 min. Tween 80 was preferable to DMF (dimethyl formamide) for solubilising the biphenyl substrate as DMF was found to lead to precipitation, however small the volume added.

7-Ethoxyresorufin-O-Deethylase - (Burke and Mayer, 1974)
The procedure followed was identical to that described in Chapter Two except that 0.5mg microsomal protein (105000g fraction) was added.

1-Naphthol glucuronyltransferase, NADPH cytochrome C reductase (using 0.5mg microsomal protein, 105000g fraction), and protein determinations were carried out as described in Chapter Two. 1-Naphthol glucuronyltransferase activity was linear up to 7.5 min; the incubation time of 4 min was chosen in agreement with other workers (Shirkey, 1977; Dawson, 1980). NADPH cytochrome C reductase activity was linear within the range 0.27 to 0.68 mg microsomal protein.

7-Ethoxyresorufin-O-deethylase activity was linear over the range 0.27 to 0.54 mg microsomal protein.

Statistical analyses
These were performed using Student's t test.

Results

Growth of rats maintained on the diet containing 28.8% bran was similar to controls (Fig 3.1). These animals were healthy and identical to the other groups apart from the obvious increased quantity of faeces. Wheat bran at three dietary levels (4.8, 9.6 & 28.8%) was without effect on NADPH cytochrome C reductase, biphenyl-4-hydroxylase, 7-ethoxyresorufin-O-deethylase or 1-naphthol glucuronyltransferase activities compared to control values. Protein concentrations and the weight of cells obtained
were not significantly different between the groups (Table 3.1).

The growth of rats maintained on the diets containing safrole was less than the control group, with slightly lower weights in the fibre-free 0.25%(w/w) safrole group than the 9.6% bran 0.25%(w/w) safrole group, despite equivalent food intakes in these two groups (Fig 3.2 and Table 3.2). Safrole intake was therefore the same in both groups (Table 3.2). The weight of cells recovered and protein contents were slightly higher in the safrole groups than in controls but these changes were not significant (Table 3.3). Significant increases were found for NADPH cytochrome C reductase and 1-naphthol glucuronyltransferase activities in the safrole groups compared to controls, with a similar increase in activity for both groups (Table 3.3). Both the biphenyl 4-hydroxylase and 7-ethoxyresorufin-O-deethylase enzyme activities were increased in both safrole groups compared to controls, although the changes were not statistically significant. Biphenyl-2-hydroxylase and 7-ethoxyresorufin-O-deethylase activities were lower in the fibre-free 0.25%(w/w) safrole group than in the 9.6% bran 0.25%(w/w) safrole group, with the reverse being true for biphenyl 4-hydroxylase (Table 3.3).
Fig. 3.1. GROWTH CURVE OF RATS FED ON 28.8% BRAN CONTROL DIETS

Results are the means of four rats per group.
Table 3.1. Effect of Dietary Wheat Bran on Some Intestinal Drug Metabolizing Enzymes in the Rat
Maintained on the Diets for 4 weeks

<table>
<thead>
<tr>
<th>Enzyme Measured</th>
<th>Fibre-free (control)</th>
<th>4.8% Bran</th>
<th>9.6% Bran</th>
<th>28.8% Bran</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH Cytochrome C reductase (nmol Cytochrome C reduced/mg protein/min)</td>
<td>25 ± 6 (100%)</td>
<td>27 ± 2 (108%)</td>
<td>23 ± 3 (92%)</td>
<td>27 ± 1 (108%)</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>7.8 ± 1.5 (100%)</td>
<td>6.9 ± 1.9 (89%)</td>
<td>6.5 ± 2.7 (83%)</td>
<td>9.0 ± 3.5 (115%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-0-deethylase (pmol resorufin/mg protein/min)</td>
<td>6.9 ± 2.6 (100%)</td>
<td>5.4 ± 0.7 (78%)</td>
<td>6.9 ± 1.9 (100%)</td>
<td>6.4 ± 1.2 (93%)</td>
</tr>
<tr>
<td>1-Naphthol glucuronyl transferase (nmol 1-naphthol glucuronide/mg protein/min)</td>
<td>23.8 ± 2.5 (100%)</td>
<td>32.5 ± 2.8 (137%)</td>
<td>29.2 ± 6.2 (123%)</td>
<td>25.2 ± 3.9 (106%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g cells)</td>
<td>7.8 ± 0.6 (100%)</td>
<td>0.2 ± 0.5 (105%)</td>
<td>6.6 ± 0.9 (85%)</td>
<td>5.2 ± 1.1 (67%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM of four male animals.
Figures in parentheses are percentage value of control group.
Results are means of four rats per group. A period of 4 days on the respective diets without added safrole was allowed so that the animals were accustomed to the experimental diets; safrole feeding continued for a period of 8 days.
Table 3.2. Food and Safrole Intakes and Final Bodyweights (Rats)

<table>
<thead>
<tr>
<th>INTAKE</th>
<th>DIET GROUP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibre-Free</td>
<td>Fibre-Free +</td>
<td>9.6% Bran +</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.25% Safrole</td>
<td>0.25% Safrole</td>
</tr>
<tr>
<td>Food Intake</td>
<td>12.6 ± 1.4</td>
<td>11.1 ± 1.6</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>g/100g bodyweight/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safrole Intake</td>
<td>0.028 ± 0.004</td>
<td>0.028 ± 0.004</td>
<td></td>
</tr>
<tr>
<td>g/100g bodyweight/day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Safrole Intake in 8 days</td>
<td>0.198 ± 0.01</td>
<td>0.196 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>g/100g bodyweight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Bodyweight (g)</td>
<td>215 ± 2</td>
<td>198 ± 2</td>
<td>204 ± 3</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for four male animals. Safrole feeding period was 8 days.
<table>
<thead>
<tr>
<th>Enzyme Measured</th>
<th>DIET GROUP</th>
<th>Fibre-free Control</th>
<th>Fibre-free + 0.25% Safrole</th>
<th>9.6% Bran + 0.25% Safrole</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH Cytochrome C reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td>36.6±2.8 (100%)</td>
<td>50.3±3.9 (138)*</td>
<td>58.4±16.0 (160)*</td>
<td></td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>16.3±3.0 (100%)</td>
<td>43.8±14.5 (269)</td>
<td>33.7±9.4 (207)</td>
<td></td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (nmol 2-OH biphenyl/mg protein/hr)</td>
<td>6.6±2.4 (100%)</td>
<td>7.5±2.1 (114)</td>
<td>8.6±3.4 (131)</td>
<td></td>
</tr>
<tr>
<td>7-Ethoxyresorufin-O-deethylase (pmol resorufin/mg protein/min)</td>
<td>21.6±6.1 (100%)</td>
<td>32.6±8.2 (151)</td>
<td>48.9±15.5 (226)</td>
<td></td>
</tr>
<tr>
<td>1-Naphthol glucuronol transferase (nmol 1-naphtholglucuronide/mg protein/min)</td>
<td>33.8±5.5 (100%)</td>
<td>62.0±7.4 (183)**</td>
<td>68.9±9.2 (204)**</td>
<td></td>
</tr>
<tr>
<td>Microsomal protein (mg/g cells)</td>
<td>3.7±0.25 (100%)</td>
<td>4.4±0.6 (117)</td>
<td>4.3±0.72 (116)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for four male animals. Safrole was administered in the diet at 0.25% (w/v) for 8 days. Value significantly different from control: *p<0.05; **p<0.02.
Discussion

The modifications of the method of Shirkey (1977) for the preparation of intestinal epithelial microsomes are those of Dawson (1980), in which the highest reported value of rat intestinal cytochrome P450 was found. The modifications made were the omission of one washing at 1000g(max) and 1 washing at 10000g(av) which were thought to decrease the amount of non-microsomal protein sedimenting with the microsomes. A further modification was the use of six 10cm rods rather than three 20cm rods used by these workers (Shirkey, 1977; Dawson, 1980), for evertting the gut sections. This enabled the volume of solution into which the cells were shaken and collected to be decreased and therefore enabled the first centrifugation procedure to be completed more quickly.

Short term (4 wk) feeding of wheat bran up to 28.8% (w/w) of the diet did not alter the small intestinal phase I or phase II drug metabolizing enzyme activities. Smith-Barbaro et al (1981a) found that feeding rats a diet containing 15% wheat bran by weight for 11 months did not alter cytochrome P450 concentration but decreased cytochrome b\textsubscript{5} levels by 50% compared to a control diet containing 5% cellulose. Any protective effect of bran against colon cancer is not therefore mediated by a direct effect on liver or small intestinal mixed-function oxidase enzyme levels, producing the higher activities which are generally associated with lower tumour incidence (Wattenberg, 1972a).

The presence of a mixed-function oxidase system in the colon,
capable of activating carcinogens to their proximate reactive form has been reported (Strobel et al, 1980). Smith-Barbaro et al (1981a) found that feeding 15% wheat bran for 11 months decreased colonic cytochromes P450 and \( b_5 \) by 55%, compared to controls (5% cellulose). The exact role of this drug metabolizing system in colonic carcinogen detoxication is unknown, but it may be a major factor in determining the incidence of colorectal tumours.

These workers (Smith-Barbaro et al, 1981a) also found decreased activity of intestinal and colonic 3-hydroxy-3-methylglutaryl co A reductase activity on both a 15% wheat bran and 15% citrus pulp diet in comparison to the control diet containing 5% cellulose and fed for 11 months. The diet containing 15% citrus pulp was found to lower intestinal and colonic cytochromes P450 and \( b_5 \). This indicates that dietary fibres may depress intestinal cholesterogenesis, the organ which under high fibre feeding may become most sensitive to dietary manipulation (Smith-Barbaro et al, 1981a). Any protective effect of bran against colon cancer may be mediated through interaction with intestinal and colonic cholesterol metabolism which may influence the cytochrome P450 system, or it may be due to the physical actions of fibre i.e. altering the availability of potential carcinogenic molecules.

In rat liver, safrole and isosafrole have been shown to increase biphenyl 4- and 2-hydroxylases, glucuronyltransferase and cytochrome P450 levels (Parke and Rahman, 1971). Safrole induces benzo(a)pyrene hydroxylase activity in the rat small intestine and
has been shown to be a less potent inducer of extrahepatic drug metabolism than isosafrole (Lake, 1974). Safrole was found in the experiment now reported to increase rat intestinal NADPH cytochrome C reductase and 1-naphthol glucuronyltransferase activities and to increase biphenyl 4-hydroxylase and 7-ethoxyresorufin-O-deethylase activities, although these increases were not statistically significant. Biphenyl 2-hydroxylase activity was not increased. In the liver, both safrole and isosafrole enhance 4- and 2-biphenylhydroxylase activities but in extrahepatic tissues isosafrole only stimulates 4-hydroxylation. It appears that this is also true of safrole in the intestine.

The results indicate that 9.6%(w/w) wheat bran does not alter the capacity of intestinal drug metabolising enzyme induction by safrole, a natural dietary component, after an 8-day feeding period. Hence the interaction between safrole and cytochrome P450 is not reduced in the presence of bran, compared to a fibre-free diet.

The increased activity of 7-ethoxyresorufin-O-deethylase, though not significant, is interesting. Higher levels were found in the bran 94% wheat/0.25%(w/w) safrole-fed group than in the fibre-free 0.25%(w/w) safrole group. This enzyme utilises cytochrome P448, which is thought to be involved in microsomal activation of carcinogens (Burke and Mayer, 1975).

Other workers have shown that diets containing some fibrous components are protective against toxic substances and carcinogens
(Ershoff, 1972; Ershoff and Thurston, 1974; Wilson et al, 1977). They mostly conclude that the physical actions of fibre modify the toxicity of the substances. Binding studies in vitro have given support to this theory in some cases (Smith-Barbaro et al, 1981), but not in others (Takeda and Kiriyama, 1979). However, in some of the in vivo experiments, workers have compared semi-purified diets to stock rations, which is not experimentally valid since stock diets are of completely different composition apart from containing a high level of fibre. The physical actions of dietary fibre which are thought to protect against carcinogens and toxic substances depend on a variety of factors. Those pertinent are the chemical structure of the toxic substance and how it is metabolised and absorbed in the gastro-intestinal tract, and the influence of gastro-intestinal events on the compound which in turn are influenced by the type and amount of dietary fibre ingested. Interactions between the toxic substance and the particular type of dietary fibre are of major importance.

Thus wheat bran and citrus pulp have been reported to protect against dimethylhydrazine (DMH)-induced colon tumourigenesis (Wilson et al, 1977), probably by binding DMH (Smith-Barbaro et al, 1981). However, the protective effect against amaranth toxicity by gobo-fibre, obtained from the roots of the edible burdock, which does not adsorb amaranth (Takeda and Kiriyama, 1979), is probably due to slowing the passage of chyme through the small intestine, thereby counteracting the inhibition of digestive enzymes caused by the amaranth (Takeda et al, 1982). The protection afforded by dietary fibres is not uniform. The effects of toxic levels of
amino acids (5% glycine, 3% methionine and 5% tyrosine) fed to rats are not counteracted by the presence of gobo-fibre, probably because these compounds are easily absorbed (Takeda et al, 1979).

Safrole is lipophilic and fat soluble and is absorbed rapidly from the intestine (Benedetti et al, 1977). It is a weak hepatocarcinogen and a weak inducer of extrahepatic mixed-function oxidase enzyme activities. The results now reported indicate that adsorption of the compound by dietary wheat bran (9.6% (w/w)) does not affect the ability of safrole to induce the intestinal drug-metabolizing enzymes in vivo.
Chapter Four

The Effect of Dietary Modification on
Gastro-intestinal Glycoprotein Biosynthesis.
Introduction

Dietary fibre is associated with protection against certain gastro-intestinal diseases, including colorectal cancer and diverticulosis (Burkitt, 1971). The epithelial cells lining the gastro-intestinal tract have a very high rate of turnover and are continually being exfoliated and replaced (Croft et al, 1977). The tract is completely covered with a blanket layer of mucus which is also continually renewed (Teague, 1977). It is secreted by the cells lining the mucosa and forms a viscous, tenacious layer about 1 mm thick. It protects the delicate epithelium from mechanical and autolytic damage (Hollander, 1954), facilitates the passage of contents along the gastro-intestinal tract by lubrication, and may help to control the osmotic movement of fluids across the epithelial surface (Hafez, 1977). The ability to perform these functions resides in the chemical composition and structure which renders mucus resistant to attack and able to form water-insoluble and viscous gels (Edward, 1963) which act as a mesh, allowing retention of some substances whilst being permeable to others.

Mucus consists of water, glycoproteins, inorganic ions (sodium, potassium, calcium and chloride), proteins and DNA (Clamp, 1977). Its gel structure, which is ill-defined, depends primarily on the large glycoprotein molecules (Cohen and Gold, 1975), with molecular weights of several million (Clamp, 1977). Glycoproteins consist of conjugated proteins covalently linked to carbohydrate oligosaccharide chains. In gastro-intestinal mucous glycoprotein the polypeptide chains contain a high content of
serine, threonine, and proline, and a low content of aromatic and sulphur-containing amino acids. There are many hundreds of oligosaccharide chains per molecule containing less than 25 monosaccharides, usually about 8-10. The characteristic ones are L-fucose, D-galactose, N-acetylglucosamine, and N-acetylgalactosamine and acyl neuraminic acids. Uronic acids are absent and mannose and glucose are found only in small quantities. Most mucous glycoprotein of the gastro-intestinal tract contains over 50% carbohydrate (Clamp, 1977). The linkage of the carbohydrate to the polypeptide is an O-glycosidic linkage where N-acetyl glucosamine is joined from the carbon atom at position 1 to the hydroxy group of serine or threonine and the linkage atom is oxygen (Anderson et al., 1964).

The carbohydrate composition is of primary importance in determining the chemical and physical properties of mucus. The sugars are polar and tend to stabilise the glycoprotein in aqueous solutions; sialic acid residues impart a high negative charge to certain oligosaccharides; the sugars tend to be heavier than the amino acids thus affecting the density of the molecules.

Sialic acids are important constituents of glycoproteins. They are built of nine-carbon sugars and are predominantly N- and O-acyl derivatives of the \( \alpha \)-ketopolyhydroxyamino acid known as neuraminic acid, which can be viewed as a condensation product of mannosamine and pyruvic acid. The most widely distributed sialic acid is N-acetyl-neuraminic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosaminic acid).
Acyl neuraminic acids in glycoproteins are partly responsible for the high viscosity of mucilaginous secretions. They have low pK values and the carboxyl groups are fully dissociated at physiological pH values. The high density of the negatively charged carboxyl groups present on each mucin molecule impart to these molecules an extended rod—like charged polyelectrolyte structure, which is responsible for the very high viscosity of the aqueous solutions of the sialic-acid rich glycoproteins. If the sialic-acid groups are removed, the viscosity drops markedly. Hence any alteration in the terminal sialic acid moieties would produce mucus with considerably different properties. As sialic acids tend to occupy the terminal positions of oligosaccharide chains, they are also important in determining the half-life of glycoprotein molecules.

Cancers of the stomach and colon are associated with changes in mucus secretion (Clamp, 1977). Changes in the carbohydrate content of gastric mucus (Richmond et al., 1955) and blood group activity (Schrager and Oates, 1973) often occur with stomach cancer and there is often appearance of new (foetal) antigens in stomach and colon cancer (Clamp, 1977). Certain disease states may show changes in glycoprotein concentration and composition and changes in other constituents of mucus e.g. inorganic ions (Findlay and O'Connor, 1961).

The extent of intestinal glycosylation of glycoproteins is affected by age (Carlson et al., 1973), the region of the intestinal tract (Kim et al., 1975), administration of drugs (Forstner et
al, 1973; Lukie and Forstner, 1974), and by fasting and restraint stress (Dekanski et al., 1975).

The aim of the present work was to determine whether the experimental diets, fed for various periods, would alter the rates of mucus synthesis in the stomach, duodenum, and colon of the rat, by measuring the rate of incorporation of N-acetyl(\(^3\)H)glucosamine into mucosal homogenates from these sites. This sugar appears in three locations in the oligosaccharide chain: the N-acetyl glucosamine-asparagine linkage region, the core, and the terminal, non-reducing trisaccharides. Thus glucosamine incorporation occurs throughout the bio-synthetic process of glycoprotein molecules. In a previous study, N-acetylglucosamine was found to give the best incorporation rates of a series of labelled hexoses and amino sugars (Shillingford et al., 1974) into gastro-intestinal mucosal homogenates.

The effect of wheat bran at levels of 4.8, 9.6 and 28.8% (w/w) of the diet, 8.5% beef suet (w/w) and a stock diet (41B) compared to a fibre-free control diet on N-acetyl(\(^3\)H)glucosamine incorporation into mucosal homogenates was investigated in the rat fed for various periods of time on the diets. The incorporation of L-(\(^3\)H)serine into gastric and colonic mucosal homogenates was also measured to determine whether any changes occurred in synthesis of the polypeptide backbone of glycoprotein molecules with changes in dietary composition. Measurement of terminal sialic acids was undertaken to determine whether there were any changes in the type of mucus synthesised.
Experimental

Materials

N-Acetyl-D-[1\(^{3}\)H]glucosamine, (sp.act. 2.94Ci/mmol) and L-[3\(^{3}\)H]serine, (sp.act. 28Ci/mmol), were purchased from Amersham International PLC. Dimilume-30 and Soluene-350 were obtained from Packard Instrument Company. Periodic acid, butan-1-ol, 2-thiobarbituric acid and sodium arsenite were obtained from BDH Ltd. Phosphotungstic acid was purchased from Sigma, and all salts from BDH Ltd. Other materials were obtained as previously described.

The periodate reagent consisted of 25mM periodic acid in 0.06M H\(_2\)SO\(_4\) pH 1.2.

The sodium arsenite reagent consisted of 2%(w/v) sodium arsenite in 0.5M HCl.

The thiobarbituric acid reagent consisted of 0.1M thiobarbituric acid, brought to pH 9.0 with sodium hydroxide.

Animals

The rats used in the long-term studies (18 months) were Wistar Albinos (COB), from Charles River, Manston, Kent. They were housed at the Flour Milling and Baking Research Association animal unit until 2 wk before starting this experiment, when they were transferred to the University of Surrey animal unit. Female rats were used for the measurement of N-acetyl(\(^{3}\)H)glucosamine incorporation, and male rats for the measurement of the terminal sialic acids. All other rats used were male Wistar Albinos of the Surrey University breeding colony, and were weanling rats at the commencement of feeding.
Methods

Rate of Incorporation of N-acetyl(3H)glucosamine into glycoproteins
- (Lukie and Forstner, 1972; adapted by Okine et al., 1982)

Animals were killed by cervical dislocation, the abdomen opened and stomach, colon and the first 5 cm of the small intestine (duodenum) excised and placed in a beaker containing ice-cold 1.15% KCl to remove the food residues of these tissues. The mucosae were gently scraped with a microscope slide and suspended in modified Krebs-Ringer solution (15 ml) and homogenised with a Potter-Elvehjem mechanically driven homogeniser with ten up and down strokes. Aliquots of homogenates (5 ml) were incubated with 1 μCi of N-acetyl(3H)glucosamine or 2 μCi of L-(3H)serine for 2 hr at 37°C, and gassed every 20 min with 95% oxygen, 5% carbon dioxide. At 2 hr the reactions were terminated by addition of 20% (w/v) trichloroacetic acid containing 2% (w/v) phosphotungstic acid (5 ml). The tubes were left to stand overnight to ensure maximum precipitation. Centrifugation at 3000 rpm for 15 min deposited the precipitates which were washed twice with distilled water (5 ml) and twice with chloroform:methanol, 1:1 (v/v; 5 ml). The precipitate was transferred to scintillation vials, air dried and weighed. Distilled water (0.2 ml) and Soluene-350 (1.0 ml) were added and the protein allowed to digest. The scintillant (Dimilume) was added (10 ml) and the radioactivity counted in an LKB-Wallac Ultrabeta 2002 scintillation counter. All estimations were performed in duplicate. Non-specific binding of the glucosamine was corrected using incubation systems in which the trichloroacetic acid-phosphotungstic acid reagent was added.
immediately after the addition of the radio-labelled material and before incubation was commenced. Affinity chromatography on sephadex has shown the precipitated mucus fraction to contain the label only in mucus glycoprotein (Johnston, 1977).

The quench curve was determined by addition of chloroform (0.0 - 1.2 ml) to Dimilume-30 (10 ml), Soluene-350 (1.0 ml) water (0.2 ml) and $^3$H-hexadecane standard ($1.64 \times 10^5$ dpm), to enable conversion of cpm to dpm, by the external standard efficiency ratio.

**Measurement of N-acetylneuraminic acid (NANA)**

**Sample preparation:**
Mucosal scrapings of stomach, duodenum and colon, obtained as described above, were homogenised in 5 mM EDTA (15 ml) and glycoproteins precipitated by the addition of 10% trichloroacetic acid containing 1% (w/v) phosphotungstic acid (5 ml). The precipitate was washed twice with distilled water (5 ml) and twice with chloroform:methanol, 1:1 (v/v; 5 ml), air dried and weighed. The precipitate was homogenised in distilled water (10 mg precipitate/ml) and if storage was required this preparation was stored at -40°C.

**Hydrolysis procedure:**
Equal volumes of sample and 0.05 M H$_2$SO$_4$ were sealed under nitrogen in a glass ampoule and heated at 80°C in an oven for 1 hr. The sample was filtered.

**Estimation of N-Acetylneuraminic acid:** (Aminoff, 1961).
Aliquots of the sample (0.5 ml) were treated with periodate reagent (0.25 ml) for 30 min. The excess periodate was reduced with sodium
arsenite reagent (0.2ml), and the tubes left to stand until the yellow colour of the liberated iodine had disappeared. Thiobarbituric acid reagent (2ml) was added and the samples covered and heated in a boiling water bath for 7.5min. The solutions were cooled and extracted with butan-1-ol (5ml) containing 5%(v/v) 12M HCl. Separation of the two phases was facilitated by a short centrifugation at 3000rpm for 10 min, using a Beckman J6 centrifuge. The absorbance was read at 549nm using a Cecil 272 spectrophotometer. Blanks and standards (5-40µg N-acetyleneuraminic acid) were carried through the same procedure.

There is a second absorbance maximum at 532nm due to deoxyribose (2-DR) for which a correction was made since it interfered with absorption at 549nm. The correction factor was obtained by determination of the molecular extinction coefficients of NANA and 2-DR and substituting them into the following equation:

$$\mu\text{mol NANA} = \left[ \frac{E_3}{E_2E_3 - E_1E_4} \times \text{OD}_{549nm} - \frac{E_4}{E_2E_3 - E_1E_4} \times \text{OD}_{532nm} \right] \times 4.3$$

where

\[E_1 = \text{mol. ext. coeff. of NANA at 532 nm} \times 10^{-3}\]
\[E_2 = \text{mol. ext. coeff. of NANA at 549 nm} \times 10^{-3}\]
\[E_3 = \text{mol. ext. coeff. of 2DR at 532 nm} \times 10^{-3}\]
\[E_4 = \text{mol. ext. coeff. of 2DR at 549 nm} \times 10^{-3}\]
Statistical analyses
These were performed using Student's t test.

Results

The quench curve for tritium is shown in Fig 4.1. The rate of incorporation of radiolabelled N-acetylglucosamine and L-serine was found to be linear for incubation periods of up to 3h (Figs. 4.2 & 4.3).

After 4 wk exposure to the experimental diets the rats maintained on the 4.8% and 9.6% bran, and 8.5% beef suet diets were found to have significantly higher incorporation of N-acetyl(3H)glucosamine in stomach mucosal glycoproteins in comparison to the fibre-free control group (Table 4.1). No changes were observed at 8 wk (Table 4.1). Duodenal mucus production was not affected by any of the diets after 4 or 8 wk of feeding (Table 4.2). Colonic mucus production was significantly increased in rats maintained on the 8.5% beef suet diet for 4 wk, but no effect was observed after 8 wk of feeding. The 4.8% and 9.6% bran diets did not significantly alter colonic mucus production after 4 or 8 wk of exposure to the diets (Table 4.3).

The long-term study in female rats showed a significant increase of gastric mucosal incorporation of N-(3H)acetylglucosamine in those rats maintained on the 9.6% bran diet (Table 4.1). The 8.5% beef suet group had the same
increase compared to the fibre-free control group but, because of the large animal variation, the result was not significant for this group (Table 4.1). Similarly the increase observed in rats maintained on the stock diet was found not to be significant. A significant increase in sugar incorporation in animals maintained on the stock diet was observed for duodenal mucus production (Table 4.2). In the colon the stock diet was again found to significantly increase the production of mucus (Table 4.3). The rats maintained on the 9.6% bran and 8.5% beef suet diets had increased incorporations compared to the fibre-free controls, but these were not statistically significant.

The growth of the rats maintained on the 28.8% bran diet followed the same curve as the control group (fibre-free) over this time period (8 wk, Fig. 4.4). This diet was found not to affect the incorporation of N-acetyl$^{(3)}$H$\text{glucosamine}$ at any of the intestinal sites studied after an 8 wk feeding period (Table 4.4).

Incorporation of L-serine, which indicates the rate of polypeptide synthesis was not altered by dietary wheat bran at either level, or the stock diet, after 12 wk of feeding, in the stomach mucosa compared to the fibre-free control group. In the colon the 4.8% bran diet similarly did not alter the incorporation of L-$^{(3)}$H$\text{serine}$ into the mucosal glycoproteins in comparison to the fibre-free control group (Table 4.5).
The content of terminal sialic acid in the acid-precipitated glycoproteins was not affected by dietary wheat bran, dietary beef suet or the stock diet after 4 wk or 18 months of exposure to the diets, in gastric or duodenal mucosal glycoproteins (Table 4.6 & 4.7). In colonic glycoproteins, 4.8% bran was found to significantly increase the sialic acid content and 9.6% bran to increase it to the same extent, though not reaching levels of significance due to animal variation (Table 4.8).
Fig. 4.1. QUENCH CURVE FOR [3H]-HEXADECANE STANDARD

Δ (³H)Hexadecane Standard.
Fig. 4.2. N-ACETYL GLUCOSAMINE INCORPORATION IN MUCOSAL GLYCOPROTEIN

Incorporations are given for gastric, colonic and duodenal mucosal glycoproteins in the rat.
Fig. 4.3. L-SERINE INCORPORATION INTO MUCOSAL GLYCOPROTEIN

TIME OF INCUBATION (hr)

- Gastric mucosal glycoprotein

- Colonic mucosal glycoprotein
Table 4.1. Effect of Wheat Bran, Beef Suet and a Stock diet on the Incorporation of N-acetyl-glucosamine in the Rat Gastric Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Length of feeding period</th>
<th>Rate of (^{3}\text{H})N-acetylglucosamine incorporation (pmol/mg glycoprotein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks (males)</td>
<td>8 weeks (males)</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td>0.13±0.11</td>
<td>0.27±0.14</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>2.13±0.39***</td>
<td>0.27±0.15</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>2.67±0.64**</td>
<td>0.25±0.10</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>1.27±0.43*</td>
<td>0.17±0.09 (4)</td>
</tr>
<tr>
<td>41B Stock diet</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 rats per group unless otherwise indicated (numbers in parentheses).
Value significantly different from control: *p<0.05, **p<0.01, ***p<0.005.
Table 4.2. Effect of Wheat Bran, Beef Suet and Stock Diet on the incorporation of N-acetylglucosamine in the Rat Duodenal Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Rate of $[^3]$H-N-acetylglucosamine incorporation (pmol/mg glycoprotein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of feeding period</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td></td>
</tr>
<tr>
<td>4.8% bran</td>
<td></td>
</tr>
<tr>
<td>9.6% bran</td>
<td></td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td></td>
</tr>
<tr>
<td>41B Stock diet</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 rats per group unless otherwise indicated (numbers in parentheses).
Value significantly different from control: *p<0.02.
### Table 4.3. Effect of Wheat Bran, Beef Suet and a Stock Diet on the Incorporation of N-acetylglucosamine into Rat Colonic Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Length of feeding period</th>
<th>Rate of (³H)N-acetylglucosamine incorporation (pmol/mg glycoprotein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 weeks (males)</td>
<td>8 weeks (males)</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td>0.65±0.14</td>
<td>1.00±0.38</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>0.65±0.22</td>
<td>0.66±0.17</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>0.55±0.20</td>
<td>0.64±0.13</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>2.04±0.58 **</td>
<td>1.47±0.19(4)</td>
</tr>
<tr>
<td>41B Stock diet</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 rats per group unless otherwise indicated (numbers in parentheses).

Values significantly different from control:
*p<0.05,  **p<0.02.
Fig. 4.4. GROWTH CURVE OF RATS FED ON 28.8% BRAN + CONTROL DIETS

Results are means of five rats per group.
Table 4.4. Effect of High Levels of Dietary Wheat Bran on the Incorporation of N-acetylglucosamine into Rat Gastric Duodenal and Colonic Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Rate of (^{3}\text{H})N-acetylglucosamine incorporation (pmol/mg glycoprotein/hr)</th>
<th>Tissue</th>
<th>Stomach</th>
<th>Duodenum</th>
<th>Colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre-free</td>
<td></td>
<td></td>
<td>2.68±0.50</td>
<td>1.38±0.86</td>
<td>1.14±0.12</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>2.09±0.60</td>
<td>1.33±0.29</td>
<td>1.24±0.26</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 animals per group. Male rats were maintained on the diets for an 8-week period.
Table 4.5. Effect of Wheat Bran and a Stock Diet on the Incorporation of L-Serine into Rat Gastric and Colonic Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Tissue</th>
<th>Rate of Incorporation of $^{3}$H-L-Serine (mol x 10$^{-15}$/mg glycoprotein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stomach</td>
<td>Colon</td>
</tr>
<tr>
<td>Fibre-free</td>
<td>9.1±2.0</td>
<td>2.7±0.6</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.8% bran</td>
<td>8.6±1.9</td>
<td>3.3±0.9</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>12.8±4.0</td>
<td>-</td>
</tr>
<tr>
<td>41B Stock</td>
<td>8.2±0.6</td>
<td>-</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are means ± SEM for 5 rats per group. Male rats were maintained on the diets for 12 weeks.
Table 4.6. Effect of Wheat Bran, Beef Suet and a Stock Diet on the Sialic Acid Content of Rat Gastric Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Length of feeding period</th>
<th>Sialic Acid Content (nmol/mg glycoprotein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Weeks (males)</td>
<td>18 months (males)</td>
</tr>
<tr>
<td>Fibre-free</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.39±0.09</td>
<td>0.48±0.07(4)</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>0.55±0.12</td>
<td>-</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>0.34±0.08</td>
<td>0.49±0.08(3)</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>0.35±0.07</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>41B Stock</td>
<td>-</td>
<td>0.27±0.03(4)</td>
</tr>
<tr>
<td>Diet</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 rats per group unless otherwise indicated (numbers in parentheses).
Table 4.7. Effect of Wheat Bran, Beef Suet and a Stock Diet on the Sialic Acid Content of Rat Duodenal Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Sialic Acid Content (nmol/mg glycoprotein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of feeding period</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td></td>
</tr>
<tr>
<td>4.8% bran</td>
<td></td>
</tr>
<tr>
<td>9.6% bran</td>
<td></td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td></td>
</tr>
<tr>
<td>41B Stock Diet</td>
<td></td>
</tr>
</tbody>
</table>

Results are the mean ± SEM for 5 rats per group unless otherwise indicated (numbers in parentheses).
Table 4.8. Effect of Wheat Bran, Beef Suet and a Stock Diet on the Sialic Acid Content of Rat Colonic Mucosal Glycoproteins.

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Sialic Acid Content (nmol/mg glycoprotein) (Males)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre-free Control</td>
<td>0.77±0.02(4)</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>1.83±0.03 *</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>1.84±0.05</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>0.80±0.02</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 animals per group unless otherwise indicated (numbers in parentheses). Value significantly different from control: *p<0.05.
The rats were maintained on the diets for 4 weeks.
Discussion

The assay of incorporation of N-acetyl(3H)glucosamine into intestinal mucosal glycoproteins shows wide animal variation. Animals are not fasted before killing as this considerably depresses the rate of incorporation (Dekanski et al, 1975). At the time of killing the animals have variable contents of food residues in the stomach, duodenum and colon. It is not known what extent of variation in mucus production this produces. Mucosal contamination of the scraping is another variable parameter which may lead to discrepancies between samples.

The gastro-intestinal tract is a difficult organ to study because of such factors as extremes of pH, variable enzyme content, and microsomal contribution of intestinal matter (Hafez, 1977). Luminal fluids may contain secretions from various other organs which enter into the tract. The rate of incorporation into duodenal mucosal glycoproteins shows a low activity after 8 wk of feeding, which suggests that autolysis may have occurred due to the presence of digestive enzymes in the preparation. Microrganisms present in the colonic preparations may utilise the N-acetyl(3H)glucosamine, another source of variation between animals.

Wheat bran was found to significantly increase incorporation of N-acetyl(3H)glucosamine into gastric mucosal glycoproteins only, after 4 wk and 18 months of feeding. The bran-containing diets are more bulky than the control diet and would be expected to require
more secretion to achieve the same level of liquidisation, which occurs before the contents leave the stomach, as the fibre-free diet. Also, the bran may adsorb the secretions which would cause a greater requirement for mucus production. Similarly, the high fat diet on entering the stomach would produce more viscous contents than the control diet requiring an increased volume of secretion from the stomach to achieve the same level of liquidisation. This may explain the significant increase seen after 4 wk, and the increase after 18 months of feeding. A significant increase in N-acetyl(3H)glucosamine incorporation was seen in the colon mucosal homogenate after 4 wk and 18 months of feeding with the 8.5% beef suet diet.

The increased incorporation of N-acetyl(3H)glucosamine in the rats maintained on the 8.5% beef suet diet is in contrast to results observed by Biol et al. (1981). These authors found a high fat diet (10% linoleic or oleic acid), fed to rats injected ip with 14Cglucosamine, to inhibit incorporation of 14Cglucosamine into microsomal pellets of gastro-intestinal tissue. No effect on incorporation of ip injected (14C)fucose or (14C)galactose, after 1 wk of feeding was observed. This feeding period is considerably shorter than the periods chosen in the experiment now reported. Study of several of the glycosyltransferases indicated that high fat diets (10-30% fat) inhibited fucosyl-transferase activity in vitro after a 4 wk feeding period. The types of fat used to supplement the diet for the in vivo study were unsaturated, whereas beef suet, used in the experiment now reported, contains saturated fat. Biol et al. (1981) found the corresponding saturated fat
(stearic) to produce the same decrease in fucosyltransferase activity in vitro.

The stock diet (41B) was found to increase the incorporation of N-acetyl$^3$H)glucosamine at all sites studied, after 18 months of feeding in the female rats. These increases reached levels of significance in the colon and duodenum. The stock diet has a high dietary fibre content (17.6% NDF, Appendix I). As the bran diets only produced significant increases in the stomach it seemed pertinent to determine whether a higher dietary level of bran (28.8%,w/w) would produce similar results to those of the stock diet, thus elucidating the possible role of the higher dietary fibre content of the stock diet in increasing the rate of incorporation of the radiolabelled sugar into the mucosal homogenates. However the 28.8% bran diet, after 8wk of feeding, was observed not to produce an increase in the rate of incorporation of N-acetyl$^3$H)glucosamine into mucosal homogenates at any of the intestinal sites studied, indicating that bran, even at this high level, does not increase the rate of glycosylation. The stock diet is of variable composition and the increased production of mucus caused by this diet may be due to an undefined component.

The incorporation of L-serine into mucosal glycoproteins is a measure of polypeptide synthesis. This was found to be unaffected by two levels of wheat bran and the stock diet in the stomach, and by the 4.8% bran diet in the colon, after a 12wk feeding period.
Measurement of the terminal sialic acids in the rats maintained on the 4.8 and 9.6% bran diets and 8.5% beef suet diet for 4 wk (males), and the 9.6% bran, 8.5% beef suet and the stock diets for 18 months (females) showed no significant changes in comparison to the control fibre-free group in the stomach and duodenum glycoproteins, indicating that the character of the mucus is not altered at these sites by the diets. A significant increase was observed in the colonic mucosal glycoproteins in the rats maintained on the 4.8% and 9.6% bran diets for 4 wk. This suggests that the mucus would be more viscous. A more viscous mucus may give better protection to the underlying mucosa by forming a thicker barrier.

Changes in mucus composition in disease states are well-reported. These changes may be quantitative affecting the volume of mucus secreted, quantitative changes in the concentration of constituents, increased loss of the mucus producing cells or abnormalities of the synthetic process (Clamp, 1977). Histochemical studies have shown changes in intestinal mucus cells in ulcerative colitis and Crohn's disease (Lev and Spicer, 1965), where hyperplastic goblet cells contain more sulpho-mucin than normal intestinal goblet cells. Greco et al (1967) found staining patterns of goblet cells at various stages of ulcerative colitis to differ. At early stages the goblet cells contained only neutral mucins, in advanced cases they contained weakly acidic mucins, which was indicative of a slower cell turnover. Filipe (1969) compared the distribution of mucins to ulcerative colitis and Crohn's disease to normal controls. The controls showed a
predominance of sulphated mucosubstances and absence of neutral mucins in the lower two-thirds of the crypts while the goblet cells of the upper third of the crypts contained a mixture of sulphated and non-sulphated acid mucopolysaccharides with a variable but small amount of neutral mucins. In severe cases of ulcerative colitis a decrease of sulphated and acid non-sulphated mucins was found. In patients with Crohn's disease the reduction of mucosubstances was found in only a few cases. An increased production of mucus was reported by Goodman et al (1975) in patients with Crohn's disease. These authors reported a 50% higher level of glucosamine synthetase in intestinal biopsies of patients with the disease compared to normal controls. Glucosamine synthetase catalyses the synthesis of glucosamine, the rate limiting reaction in gastro-intestinal glycoprotein biosynthesis (Jerzy Glass & Slomiany, 1977). Lev et al (1969) reported less cytoplasmic mucus in gastric cancer cells than in the normal gastric surface epithelium or goblet cells of the intestinal mucosa.

A reduction of the carbohydrate fraction of human colonic neoplasm compared to normal adjacent mucosa was reported by Kim et al (1974) which appeared to be directly related to the reduction of certain specific glycosyltransferases. Decreased activity of sialyltransferase for example reduces the cohesive properties of mucus. Amino acids in the protein core of glycoprotein of both malignant and normal specimens appear to be of similar composition whereas the glycoproteins from malignant colons have a wider monosaccharide variation than normal colons (Farke, 1978).
Many diseases of the gastro-intestinal tract are thought to be related to diet. Any quantitative or qualitative effect of dietary alteration on mucus biosynthesis would be of significance when considering causation of these diseases. Biol et al (1981) found high carbohydrate diets produced no effect on the in vivo incorporation of $^{14}\text{C}$-glucosamine, $^{14}\text{C}$-fucose or $^{14}\text{C}$-galactosamine into the microsomal pellet of intestinal mucosal cells. High protein diets were shown to increase the activity of several glycosyltransferase enzymes whereas high carbohydrate and high fat diets inhibited fucosyl-transferase. In the present study, bran appeared to increase mucus production in the stomach and to alter the sialic acid content in colonic mucosal glycoproteins. Beef suet (8.5%) appeared to increase mucus production in the stomach and the colon in the short-term, with no effect on the sialic acid content. The stock diet was associated with increased production of mucus at the three sites studied. The effects are only indications and no firm conclusions can be drawn from the results now presented.
Chapter Five

The Effect of Wheat Bran on Vitamin D Status and Bone Mineral stores in the Rat.
Introduction

Little research of the effect of dietary fibre on the absorption of vitamins and vitamin status has been conducted. Cummings (1978) has stated that since there is a general association of malnutrition and vitamin deficiency with staple diets, which are generally high in fibre content, and fibre has the capacity to adsorb organic components, this is an important area of research.

Vitamin D is a fat soluble vitamin and its absorption from the diet is similar to that of cholesterol, lipid soluble drugs and fatty acids (Hollander, 1981). In the gastro-intestinal tract these compounds interact with bile acids which solubilise and convey them to the absorptive membrane where they become partitioned into the monomeric phase and penetrate the lipid cell membrane. Both lymph and portal blood transport vitamin D away from the intestine (Sitrin et al., 1982). The primary area of absorption is unclear — Norman and Deluca (1963) proposed that it was the distal small intestine, whereas Schachter et al. (1964) concluded the proximal small intestine to be the major site of absorption. Both endogenous and exogenous vitamin D undergo enterohepatic circulation (Hollander, 1981).

The hypolipidemic effects of certain dietary fibres is thought to be due to binding of cholesterol and bile salts in the intestine thereby increasing faecal excretion of bile acids and salts, lowering blood cholesterol levels and draining hepatic
cholesterol pools. In vitro work has substantiated this theory. Vitamin D is likely to be subject to the same interactions with dietary fibre. Its dietary concentration is considerably lower than the other sterols so that any loss could be of significance to the organism.

Vitamin D is the only vitamin known to act as a hormone. It induces the formation of specific messenger RNA's which code for proteins mediating its effects (Eisenstein and Passavay, 1964). It is also endogenously synthesised in the skin from 7-dehydrocholesterol (Deluca, 1978), by a photochemical reaction utilising ultraviolet light forming precholecalciferol which undergoes thermal isomerisation to cholecalciferol (Havinga, 1973). It circulates in the plasma bound to an α-globulin as 25-hydroxyvitamin D₃. The hydroxylation reaction occurs in the liver (Horsting and Deluca, 1969). The kidney further hydroxylates this metabolite to 1,25-dihydroxyvitamin D₃, the active form of the vitamin (Deluca & Schnoes, 1976). Measurement of plasma levels of 25-hydroxyvitamin D₃ is thus an indicator of bodily reserves of pro-hormone, since conversion to the active form is closely controlled to meet physiological requirements. The function of vitamin D in calcium and phosphorus homeostasis is to elevate serum concentrations of both ions by acting on the intestine to increase absorption, on bone to mobilise stores of these minerals and on kidney to improve renal reabsorption.

The effects of dietary fibre on mineral balance are complex and the relevance of adverse interactions on the health of
populations is difficult to elucidate. Some fibres increase requirements for certain minerals depending on which type of fibre and which mineral is under consideration (Sandstead et al., 1977; Ismail-Beigi et al., 1977; Kelsay et al., 1979). Mineral status is dependent upon other physiological parameters - vitamin D status, and protein and vitamin C intakes (Cummings, 1981). The body is able to adapt to low intakes of some minerals (Malm, 1958).

Calcium availability from foods of plant origin is inferior to its availability from milk (Ambrecht and Wasserman, 1976; James et al., 1978; Ranhotra et al., 1980). Phytic acid (Myo-inositol-1,2,3,4,5,6-hexakis-dihydrogen phosphate) is one of the factors shown to be responsible (Mellanby, 1925; McCance and Widdowson, 1942). Phytic acid has been found to interfere with absorption of phosphorus (Taylor, 1965), zinc (Reinhold, 1975), and iron (Davies and Nightingale, 1975). The presence of phytase (myo-inositol hexaphosphate phosphohydrolase) in human gut, which hydrolyses phytate esters and liberates bound metals, can prevent the potential loss of some minerals. Losses of phytate also occur during breadmaking since phytase is present in all sections of the wheat grain (Peers, 1953). In vitro studies have shown that fibre itself is able to bind calcium and zinc (Reinhold et al., 1975; James et al., 1978). The uronic acid content has been found to correlate with mineral binding (James et al., 1978). The association of wholemeal bread with disturbances in mineral metabolism is important since wholemeal bread is a staple food in the diets of many populations. In many Western countries, white bread is fortified with iron and calcium, whereas wholemeal bread
The experimental work now reported was designed to investigate the effect of wheat bran either as a separate item in the rat diet or included as wholemeal bread, on the circulating levels of 25-hydroxyvitamin D₃ and the bone mineral stores. This experimental work was conducted at the Flour Milling and Baking Research Association.
Experimental Materials

26(27)\(^{3}\)H-hydroxycholecalciferol, specific activity 22.3\text{ci/mmole} was obtained from Amersham International PLC. It was stored in ethanol at -20°C at a concentration of 2\text{\mu ci/ml ethanol} and diluted for use to a concentration of 80\text{pg/10\mu l}. 25-hydroxyvitamin D was a gift from Dr. D.E.M. Lawson of the Dunn Nutritional Laboratory at Cambridge, but can be obtained commercially from Philips-Duphar, Research Dept, Weesp, Nr. Amsterdam, The Netherlands. It was diluted for use to a concentration of 64\text{ng/ml ethanol} and stored at -20°C. Charcoal (Norit G.S.X.) was obtained from Hopkin and Williams. Dextran, Sodium barbital and Triton X-100 were obtained from Sigma, toluene and acetic acid from May and Baker and PPO and DMPOPOP from Packard Instrument Company. Ethanol 99.8\%(v/v) min. was purchased from James Burrough Ltd. Chloroform, nitric acid (69-72\%) and lanthanum chloride were purchased from BDH Ltd.

The Dextran coated charcoal suspension consisted of 9.0g charcoal and 0.9g Dextran-20 in 300\text{ml barbital acetate buffer pH}8.6.

The scintillant consisted of Triton - 600\text{ml}, toluene - 1400 \text{ml}, PPO - 10g and DMPOPOP - 0.2g.

Vitamin D-deficient serum was originally taken from rats maintained for 4 \text{wk} on a vitamin D deficient diet. Examination of the bones of these animals showed rickets to be present. This serum was used as a source of vitamin D binding protein. It was subsequently found that animals maintained for only one week on the diet had equivalent binding capacity. The serum was stored at -20°C and diluted 1:10000 for use in barbital-acetate buffer pH8.6, and maintained at 4°C.
Animals

Male and female, weanling, Wistar Albino (COB) rats from Charles River, Manston, Kent, were maintained five per cage, in wire bottom hanging cages. Food and water were available ad libitum.

Experimental Procedure

Vitamin D3 Determination

Samples of tail vein blood were taken from male and female rats maintained on the following diets for 12 wk - fibre-free (control), 34% white bread, 34% wholemeal bread, 4.8% bran and 9.6% bran. The percentage composition and analysis of the diets is given in Appendix 1. The vitamin D content of the diets was approximately 500I.U. vitamin D/kg diet, which represents an adequate intake for normal vitamin D metabolism. Extracts of plasma were prepared by mixing 0.5ml of plasma with 2ml of ethanol and leaving at 4°C for 30min. After this time the samples were centrifuged at 1500rpm, for 10min, in a Mistral 6L centrifuge, and the extract was ready for use (Belsey et al, 1974).

Bone Mineral Measurements

Rats maintained on the following diets for 5 months - fibre-free (control), 17% white bread, 34% white bread, 17% wholemeal bread, 34% wholemeal bread, 4.8% bran, 9.6% bran, 8.5% beef suet and 41B stock diet, were killed by cervical dislocation and the hind leg removed and stored at -20°C until required. The diets contained adequate levels of all minerals.
Methods

Measurement of serum 25-hydroxycholecalciferol (Modification of Preece et al (1974)).

The incubation mixture consisted of 80pg 26(27)H-25-hydroxycholecalciferol, plasma extract (45μl), barbital acetate buffer (500μl) pH8.6 containing 1:10000 dilution of the vitamin D deficient serum. The incubation was carried out at 4°C overnight (16 hr). After this time 10μl of the charcoal suspension was added and the tubes left to stand for 15min at 4°C. The tubes were then centrifuged at 2650rpm, 4°C, for 15 min using a Mistral 6L centrifuge. Aliquots of the supernatant (500μl) were put into scintillation vials with scintillant (10ml) and water (100μl). Counts were made for 5min. All samples were measured in quadruplicate. Non-specific binding counts were determined by omission of the vitamin D deficient serum from the incubation mixture. This value was subtracted from all samples. Recoveries of plasma extraction were measured by adding 10μl of tracer to each sample. All recoveries were greater than 93%. The standard curve was prepared from a serial dilution in 80% ethanol of 25-hydroxycholecalciferol to give a total of eight standards with concentrations ranging from 0.25ng/ml to 32 ng/ml.
Measurement of Bone Minerals
(Turnland et al., 1979; Kang et al., 1979)
The legs were thawed, the soft tissues removed using scissors and a scalpel and the bone weighed. A hole was drilled in each end of the bone using a fine drill and the bone reweighed, so that each bone could be labelled with a numbered aluminium tag. Chloroform extraction was carried out for 24 hr using the Soxhlet extraction technique, after which the bones were dried in an oven at 100°C for 24 hr or until constant weight was obtained. They were then ashed in a muffle furnace at 500°C for 24 hr in acid-washed evaporating dishes until they were white and brittle. The ashed bones were reweighed. Nitric acid 25% (2 ml) was added to the evaporating dish containing the bone and the dish and contents were gently warmed until the bone had dissolved. The solution was filtered into a 100 ml plastic volumetric flask containing 5% lanthanum chloride (4 ml). Each dish was washed with 10% nitric acid (25 ml) which was boiled and added to the volumetric flask. The volume was made up to 100 ml with deionised water, rinsing and boiling the dish each time to ensure complete removal of the contents. The solution was then ready for atomic absorption analysis with appropriate dilution, using a Perkin Elmer 560 atomic absorption spectrophotometer (air-acetylene flame).

Statistical Analyses
These were performed using Student's t test.
Results

Measurement of Plasma Vitamin D$_{3}$ - The quench curve for tritium, for which acknowledgements are due to Dr. B. Thewlis, of the Flour Milling and Baking Research Association, is shown in Fig. 5.1. The machine efficiency was found to be 52.6% and the counting efficiency varied from 17.4-28.3%. Non-specific binding counts were on average 4.87% ± 1.98% of the total counts. Extraction efficiencies of the plasma samples were on average 98.3% ± 5.2%. A typical standard curve is shown in Fig. 5.2.

Large variation was found between animals within the same dietary groups. Bran, fed either as a supplement or in the form of wholemeal bread, was found not to significantly affect plasma 25-hydroxycholecalciferol concentrations in male or female rats in comparison to controls (fibre-free diet). Neither level of bran supplementation (4.8 or 9.6%) was found to produce a significant effect. Similarly there were no significant differences between the male or female rats maintained on the 34% white bread diet when compared to the controls (Table 5.1). Female rats were observed to have lower plasma 25-hydroxycholecalciferol levels than the male rats in the same dietary groups, although these differences were not significant.

Bone Mineral Measurements - The mineral concentrations are expressed in mg/gm dried bone, and mg/gm ashed bone which refer to the bone weight after Soxhlet extraction and the weight after ashing respectively. The weights of the femur bones from the various diet groups were not significantly different to the control group (fibre-free), when expressed as dried or ashed bone weight,
except for the female rats maintained on the 17% wholemeal bread diet; the dried bone weights were significantly lower than the control (Table 5.2). Plasma concentrations of calcium, magnesium and phosphorus were measured and no significant differences were found between any of the diet groups in comparison to the fibre-free diet group (Moore, 1983).

No significant differences were observed between the diet groups for the bone calcium concentrations when the results were related to the dried bone weight in either the male or the female groups (Table 5.3). When the results were related to ashed bone weight the female rats maintained on the 17% white bread diet, 9.6% bran diet, 8.5% beef suet and 41B stock diet had significantly lower calcium concentrations than the fibre-free controls. The male rats showed no significant differences to the control group for calcium concentrations in relation to the ashed bone weight.

Magnesium concentration was not altered significantly in any of the diet groups in comparison to the fibre-free control group in either the female or male rats whether related to the dried bone weight or to the ashed bone weight (Table 5.4).

Phosphorus concentration was found to be significantly higher in the female rats maintained on the 17% wholemeal bread diet when compared to the fibre-free control group and related to dried bone weight but not when related to the ashed bone weight. No other significant differences were found between the other diet groups and controls for either the male or female rats when related to dried or ashed bone weights (Table 5.5).
PER CENT COUNTING EFFICIENCY

AUTOMATIC EXTERNAL STANDARD ×1000

QUENCH CURVE FOR TRITIUM STANDARD

Fig. 5.1. $^{3}$H- Hexadecane Standard
Fig. 5.2. **25-HYDROXYCHOLECALCIFEROL STANDARD CURVE**

25-HCC = 25-hydroxycholecalciferol
### Table 5.1. Measurement of Plasma 25-Hydroxycholecalciferol Concentration in Male and Female rats.

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Male Rats</th>
<th>Female Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre-free Control</td>
<td>65 ± 13</td>
<td>46 ± 12</td>
</tr>
<tr>
<td>34% White bread</td>
<td>57 ± 16</td>
<td>49 ± 13</td>
</tr>
<tr>
<td>34% Wholemeal bread</td>
<td>63 ± 20</td>
<td>49 ± 17</td>
</tr>
<tr>
<td>4.8% Bran</td>
<td>55 ± 13</td>
<td>63 ± 24</td>
</tr>
<tr>
<td>9.6% Bran</td>
<td>60 ± 26</td>
<td>41 ± 18</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 5 animals. The rats were maintained on the experimental diets for 3 months.
Table 5.2. Dried and Ashed Weights of Femur Bones in the Rat Maintained on the Experimental Diets for 3 Months.

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MALE RATS</td>
</tr>
<tr>
<td></td>
<td>Dried Bone</td>
</tr>
<tr>
<td>Fibre-free control</td>
<td>0.65±0.02</td>
</tr>
<tr>
<td>17% white bread</td>
<td>0.62±0.03</td>
</tr>
<tr>
<td>34% white bread</td>
<td>0.62±0.03</td>
</tr>
<tr>
<td>17% wholemeal bread</td>
<td>0.62±0.04</td>
</tr>
<tr>
<td>34% wholemeal bread</td>
<td>0.55±0.08</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>0.63±0.03</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>0.63±0.03</td>
</tr>
<tr>
<td>41B Stock Diet</td>
<td>0.67±0.03</td>
</tr>
</tbody>
</table>

Results are means ± SEM for 3-5 animals.

Value significantly different from control: *p < 0.02
### Table 5.3. Calcium concentration in Dried and Ashed Femur Bones of Rats Maintained on the Experimental Diets for 3 Months

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>mg Calcium/g bone</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MALE RATS</td>
<td>FEMALE RATS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dried Bone</td>
<td>Ashed Bone</td>
<td>Dried Bone</td>
</tr>
<tr>
<td>Fibre-free (control)</td>
<td>262±15</td>
<td>377±15</td>
<td>295±29</td>
</tr>
<tr>
<td>17% white bread</td>
<td>233±6</td>
<td>354±7</td>
<td>299±49</td>
</tr>
<tr>
<td>34% white bread</td>
<td>252±4</td>
<td>396±7</td>
<td>250±13</td>
</tr>
<tr>
<td>17% wholemeal bread</td>
<td>245±18</td>
<td>365±18</td>
<td>292±9</td>
</tr>
<tr>
<td>34% wholemeal bread</td>
<td>247±14</td>
<td>409±16</td>
<td>262±4</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>249±3</td>
<td>381±3</td>
<td>286±21</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>237±19</td>
<td>355±34</td>
<td>250±7</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>251±9</td>
<td>386±14</td>
<td>247±14</td>
</tr>
<tr>
<td>41B Stock diet</td>
<td>263±4</td>
<td>402±5</td>
<td>245±10</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 4-5 rats.

Value significantly different from controls: * p < 0.05; ** p < 0.02.
Table 5.4. Magnesium Concentration in Dried and Ashed Femur Bones of Rats Maintained on the Experimental Diets for 3 Months.

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>MALE RATS</th>
<th></th>
<th>FEMALE RATS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dried Bone</td>
<td>Ashed Bone</td>
<td>Dried Bone</td>
<td>Ashed Bone</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td>4.7±0.2</td>
<td>6.9±0.4</td>
<td>4.2±0.4</td>
<td>7.0±0.8</td>
</tr>
<tr>
<td>17% white bread</td>
<td>4.2±0.3</td>
<td>6.4±0.5</td>
<td>4.1±0.4</td>
<td>6.3±0.7</td>
</tr>
<tr>
<td>34% white bread</td>
<td>4.6±0.3</td>
<td>7.2±0.5</td>
<td>5.0±0.7</td>
<td>7.8±1.0</td>
</tr>
<tr>
<td>17% wholemeal bread</td>
<td>4.2±0.4</td>
<td>6.5±0.6</td>
<td>4.1±0.4</td>
<td>6.3±0.6</td>
</tr>
<tr>
<td>34% wholemeal bread</td>
<td>3.8±0.9</td>
<td>5.9±1.2</td>
<td>3.5±0.4</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>4.6±0.3</td>
<td>7.1±0.4</td>
<td>5.4±0.5</td>
<td>7.0±0.5</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>4.6±0.2</td>
<td>6.9±0.3</td>
<td>4.3±0.2</td>
<td>6.7±0.7</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>4.4±0.3</td>
<td>6.7±0.3</td>
<td>4.4±0.4</td>
<td>6.6±0.7</td>
</tr>
<tr>
<td>41B Stock diet</td>
<td>3.6±0.6</td>
<td>5.5±1.0</td>
<td>4.3±0.3</td>
<td>6.5±0.5</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 4-5 animals.
Table 5.5. Phosphorus Concentration in Dried and Ashed Femur Bones of Rats Maintained on the Experimental Diets for 3 Months.

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>mg Phosphorus/g bone</th>
<th>MALE RATS</th>
<th>FEMALE RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dried Bone</td>
<td>Ashed Bone</td>
</tr>
<tr>
<td>Fibre-free Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>125±4</td>
<td>185±7</td>
<td>109±4</td>
</tr>
<tr>
<td>17% white bread</td>
<td>115±12</td>
<td>189±18</td>
<td>116±8</td>
</tr>
<tr>
<td>34% white bread</td>
<td>123±3</td>
<td>194±6</td>
<td>137±19</td>
</tr>
<tr>
<td>17% wholemeal bread</td>
<td>112±8</td>
<td>197±30</td>
<td>125±5*</td>
</tr>
<tr>
<td>34% wholemeal bread</td>
<td>109±15</td>
<td>191±39</td>
<td>109±11</td>
</tr>
<tr>
<td>4.8% bran</td>
<td>129±3</td>
<td>194±4</td>
<td>117±7</td>
</tr>
<tr>
<td>9.6% bran</td>
<td>125±4</td>
<td>194±6</td>
<td>116±9</td>
</tr>
<tr>
<td>8.5% beef suet</td>
<td>126±5</td>
<td>191±7</td>
<td>116±10</td>
</tr>
<tr>
<td>41B Stock Diet</td>
<td>100±10</td>
<td>153±16</td>
<td>125±15</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for 4-5 animals.

Value significantly different from control: *p < 0.05.
Discussion

The modifications of the method of Preece et al. (1974) were the omission of the sample extraction and chromatographic step which were replaced by the ethanolic extraction procedure of Belsey et al. (1974). The 1:20000(v/v) dilution of the vitamin D-deficient serum was replaced with a 1:10000(v/v) dilution, which was found to give more consistent results. The omission of 1% bovine serum albumin was found to be without effect. The volume of sample added was 45μl rather than 50μl and the assay was performed in quadruplicate rather than triplicate.

The method of Belsey et al. (1974) is a rapid assay (incubation time 2hr, 4°C.) for 25-hydroxycholecalciferol in which it was shown that the vitamin D-transport protein from vitamin D-deficient serum preferentially binds $^3$H-25-hydroxycholecalciferol for which the binding half-time was found to be very low (min), contrasting with the binding half-time of $^3$H-cholecalciferol which was found to be very high (days). Cholesterol and 25-hydroxycholesterol produced no displacement of $^3$H-25-hydroxycholecalciferol. Vitamin D analogues which do cross-react are at low serum concentration (Belsey et al., 1974a). The overnight incubation used in the assay now reported would incur less than 15% maximum binding attributable to cholecalciferol.

The presence of ethanol in the incubation system solubilises the steroids avoiding the need for a lipoprotein carrier which
requires several days to reach equilibrium (Belsey et al., 1971). Morris and Peacock (1976) used a level of 10% ethanol extract in the incubation system above which plasma proteins are precipitated, and below which the $^3$H-25-hydroxycholecalciferol is insufficiently solubilised. Haddad and Chyu (1971) used a level of 7% ethanol. In the experiment now reported the level of 8.29% ethanol was used, a lower level than Preece et al. (1974) who used a level of 10.7%.

The results indicate that wheat bran at dietary levels of 4.8 and 9.6% do not alter the circulating levels of 25-hydroxycholecalciferol, in male or female rats, compared to a fibre-free control diet, and that 34% white bread and 34% wholemeal bread are similarly without effect. It can be concluded that wheat bran at these levels has no gross effect on absorption and subsequent hepatic hydroxylation of dietary cholecalciferol in rats receiving an adequate supply of the vitamin.

Work in vitro has shown that lignin, pectin and some types of bran bind bile acids and bile salts (Eastwood & Hamilton, 1968; Kritchevsky, 1978; Vahouny, 1980) whereas cellulose has little ability to sequester bile acids (Story and Kritchevsky, 1976a; Vahouny et al., 1978). Eastwood and Hamilton (1968) proposed that the nature of the bonding between bile acids or bile salts and lignin is hydrophobic constituting partitioning of the bile acids and salts between the fibre and the aqueous phases. Lignin has also been shown to bind other hydrophobic compounds e.g. phenol (Stamm and Millett, 1941). Vitamin D is a hydrophobic molecule since it has only one oxygen function. Eastwood and Hamilton
(1968) proposed that the more hydrophobic the molecules the more easily they are bound.

Investigations in vivo have shown that bran is ineffective as a hypolipidaemic and hypocholesterolaemic agent (Kay and Truswell, 1977a) so that its ability to bind bile acids and cholesterol in vitro bears little relationship to its activity in vivo (Calvert and Yeates, 1981). Jenkins et al (1975) and Cummings et al (1976) have reported increased faecal excretion of steroids with increased concentration of dietary wheat fibre. Walters et al (1975) conversely reported no effect. The increase in faecal fat excretion which accompanies increased consumption of wholewheat products is thought not to be significant in terms of the overall digestibility and absorption of fat (Cummings, 1978). Lowsowsky (1978) argues that since the level of faecal fat after the consumption of quite high levels of bran is not much greater than controls, and bran has a high lipid content - 6.29% (Cummings et al, 1976), much of the additional faecal fat may be unabsorbed material from the bran.

In the light of the action of bran in vivo and the results observed in the reported experiment it would appear that wheat bran does not adversely affect serum 25-hydroxycholecalciferol concentration.

Other dietary fibres have been reported to be hypolipidaemic in vivo and to have the capacity to bind bile acids and salts in vitro. Such fibres may be more likely to produce adverse effects
on dietary vitamin D absorption. Pectin has been shown to be hypocholesterolaemic in rats (Wells and Ershoff, 1961; Story et al, 1977), and to increase the excretion of bile acids but not sterols in rats fed 1% cholesterol (Leveille & Sauberlich, 1966). Guar gum (Chang and Johnson, 1976) and carageenan, another vegetable gum (Ershoff and Wells, 1962; Tsai et al, 1976) have been shown to decrease cholesterol levels in experimental animals. In man, pectin (Keys et al, 1961; Jenkins et al, 1975b) has been reported to be hypocholesterolaemic whereas Fahrenbach & Riccardi (1965) found no effect. Guar gum has been reported to lower cholesterol levels in man (Fahrenbach & Riccardi, 1965; Jenkins et al, 1975b).

Dietary fibres associated with hypolipidaemia and hypocholesterolaemia may exert adverse effects on vitamin D absorption. However, man's facility for endogenous synthesis of cholecalciferol provides the majority of his requirements. Therefore any adverse effect on the availability of dietary vitamin D would be of little consequence except where certain groups of the population have poor vitamin D status. The recognised groups at risk are the indigenous elderly at home (Lawson et al, 1979) and hospitalised (Corless et al, 1975), children and adult women of the immigrant Asian population (Holmes et al, 1973), and pregnant Asian women (Bashmir et al, 1981). Lawson et al (1979) found that from December to February serum vitamin D levels of some elderly people fell to levels associated with osteomalacia, although no clinical symptoms were apparent. Ford et al (1972) were the first to report lower vitamin D levels in Asian immigrants in the United Kingdom in comparison to the indigenous population, which were considered to
be due to maintenance of their traditional eating habits and dress (Preece et al., 1973). Phytate was suggested as a possible cause (Dunnigan et al., 1975).

A dietary supply of vitamin D is important where there is a low rate of formation in the skin which occurs in house-bound and hospitalised elderly people (Lawson, 1981) and Asian women wearing their traditional costumes in this country. Dietary intakes of vitamin D in the United Kingdom are insufficient to maintain plasma 25-hydroxycholecalciferol at saturated levels in the elderly (Lawson, 1981). In this situation any adverse effects of dietary fibre on vitamin D absorption could exacerbate vitamin D deficiency and possibly precipitate osteomalacia. In the elderly, dietary fibre may be advised for prophylactic reasons. In the event of vitamin D deficiency, increased exposure to sunlight will make good any dietary insufficiency. Exposure to artificial ultraviolet irradiation has proved to be effective for geriatric patients in hospital (Corless et al., 1978).

The two ways of expressing the bone mineral concentrations relate to the dried calcified organic matrix of the bone with the lipid content removed (dried bone), and to the total mineral content with all organic components removed (ashed bone). The organic matrix consists largely of collagen (90%), glycoproteins, phosphoproteins, proteoglycans and peptides. It forms a framework for the deposition of hydroxyapatite, the basic structure of bone mineral. Other components of bone mineral include amorphous calcium phosphate (Eanes and Posner, 1970), calcium carbonate,
magnesium, sodium, fluorine; traces of iron, copper, lead, manganese, tin, aluminium, strontium and boron have been detected (Eastoe, 1961).

Magnesium concentration in the femur bone was found to be unaffected by dietary bran supplementation, or the addition of wholemeal bread to the diet. Similarly the white bread, beef suet and stock diets were without effect on the magnesium concentration. About half to two-thirds of the body's magnesium is present in the bone of which only 20% is exchangeable (Foster, 1968). It resides in the hydration shell rather than the hydroxyapatite crystals (Neuman and Neuman, 1958).

Phosphorus levels were significantly higher in the female rats maintained on the 17% wholemeal bread diet when expressed /gm dried bone. This effect was probably related to the lighter weight of the dried bone observed in this group. Wholemeal bread contains approximately four times the amount of phosphorus found in white bread. The effect was not seen in the female rats maintained on the 34% wholemeal bread diet or in the male rats maintained on the wholemeal bread diets. About 88% of the body's phosphorus is concentrated in the skeleton, and its homeostatic regulation is controlled by the actions of vitamin D, parathyroid hormone and calcitonin.

The significant decreases in calcium concentration (ashed bone weight) observed in female rats maintained on 34% white bread, 9.6% bran, 8.5% beef suet and the 41B stock diets do not show any
changes that are consistent with dietary changes. The 8.5% beef suet diet and 34% white bread diet contain less fibre than the other diets so that these changes are not related to fibre concentration. Also white bread is fortified with calcium and would not be expected to produce a decrease in bone calcium concentration. These changes are representative of a very small change in total body calcium since 99% is stored in the skeleton.

It can be concluded that wholemeal bread at levels of 17 and 34% of the diet and wheat bran at levels of 4.8 and 9.6% of the diet do not produce any deleterious effects on mineral status in male and female rats maintained on a well-balanced diet, as determined by measurement of concentrations of these minerals in the femur bone.

The condition of hypogonadal dwarfism caused by zinc deficiency, and anaemia caused by iron deficiency in Iran has been attributed to the consumption of large amounts of unleavened bread (bazari bread), made from high extraction flour (Haghshenas et al, 1972). A comparison of the ingestion of bazari and white breads has demonstrated the nutritional inferiority of bazari because of the poor availability of minerals, shown by increased faecal losses, even though bazari bread contains greater quantities of zinc, calcium, magnesium and phosphorus than white bread (Reinhold et al, 1976). Hence the diet of the villagers contained adequate zinc, magnesium, iron and calcium but because of poor availability deficiency states occurred. Mineral binding is thought to be due to phytic acid and uronic acids present in some sources of dietary
fibre. Purified phytate ingestion in humans is associated with increased faecal zinc. Plasma zinc and serum iron were lowered initially but returned to normal levels with continued ingestion of phytate; calcium and serum phosphate levels were decreased and remained at low levels (Reinhold et al, 1975). The available carboxyl groups of the acidic polysaccharides, which represent the cation exchange properties of dietary fibre, also bind the dietary metal ions. The relative importance of binding by fibre and phytate is as yet undetermined. Addition of fibre to the diet has also been shown to enhance losses of magnesium, calcium, phosphorus, sodium and potassium (Southgate et al, 1976; Reinhold et al, 1976). Sandstead et al, (1977) found no relation between dietary fibre and faecal zinc or copper in a metabolic balance experiment. The significance of the reported adverse effects of dietary fibre on mineral status will be governed by the intake of fibre and minerals, and the prevailing mineral status and the body's ability to adapt to any decrease in mineral supply. The present work shows that dietary wheat bran is without effect on bone mineral concentrations in male and female rats consuming adequate intakes of minerals.
Chapter Six

The Effect of Folate Deficiency on Microsomal Enzyme Induction in the Rat.
Introduction

Long term therapy with anticonvulsant drugs is known to alter the metabolism of endogenous substrates and to increase drug metabolism probably by induction of liver enzymes, especially cytochrome P450. Effects include altered vitamin D metabolism with decreased hepatic hydroxylation to 25-hydroxycholecalciferol (Silver et al, 1972), which may result in hypocalcaemia (Richens and Rowe, 1970a) and precipitate rickets or osteomalacia (Dent et al, 1970; Stamp, 1974); folic acid deficiency (Mannheimer et al, 1952; Richens, 1972) which may be associated with megaloblastic change and macrocytic anaemia (Reynolds, 1972); increased excretion of 6β-hydroxycortisol (Werk et al, 1964; Brooks et al, 1972); induction of sex hormone metabolism (Kuntzman, 1969); raised serum γ-glutamyl transpeptidase and liver alkaline phosphatase (Richens and Rowe, 1970); increased urinary excretion of D-gluconic acid and decreased levels of serum bilirubin (Thompson et al, 1969). Effects on drug metabolism are increased turnover, causing decreased effects of anticoagulants (Macdonald and Robinson, 1968), antiasthmatics (Brooks et al, 1972), antidepressants, contraceptive pills (Mumford, 1974) and doxycycline (Penttila et al, 1974).

An important consequence of long-term anticonvulsant therapy is sudden increased drug sensitivity or toxicity in some patients apparently stabilised on anticonvulsants (Davis and Labadarios, 1976). The metabolic interaction thought to be related to this phenomenon is the depression of folic acid levels (Labadarios, 1975; Fig. 1). The mechanisms by which anticonvulsants
Fig. 6.1. Long-term Effects of Drug Administration on Folate Metabolism and Toxic Side Effects of Drugs.

Long-term Administration

Induction of Drug-Metabolizing Enzymes

Increased Requirement for Folate

Increased Rate of Drug Metabolism

Folate Deficiency

Decreased Enzyme Induction

Decreased Rate of Drug Metabolism

Impaired Haematopoiesis

Embryotoxic Effects

Drug Toxicity

Increased Susceptibility to Toxic Chemicals

(After Parke and Ioannides, 1981)
cause folic acid deficiency are uncertain. The postulated mechanisms are inhibition of intestinal conjugase by anticonvulsant drugs (Hoffbrand and Nechelles, 1968), interference of enterocyte uptake of monoglutamate (Dahlke and Mertens-Rosler, 1967) and a higher dietary requirement due to increased utilisation by enzyme induction (Maxwell et al., 1972). Some drugs may be acting as folic acid antagonists (Hawkins and Meynell, 1954; Reynolds, 1968). Folic acid supplementation (15-30mg daily) may cause a reversion of seizure control (Chanarin et al., 1960), which would support this theory. However, it may be explained by decreased serum phenytoin concentration, which is caused by repletion of folate (Baylis et al., 1971; Richens, 1975). Borderline intakes of folic acid in hospitalised patients have been reported (Ibbotson et al., 1967; Labadarios et al., 1978). Hence folic acid deficiency which manifests after long-term anticonvulsant therapy could be multifactorial, the pertinent causes being inadequate intake, reduced absorption and increased utilisation.

Animal experimentation has shown that rats maintained on a folate deficient diet containing 500 mg/kg phenobarbitone do not exhibit the induced cytochrome P450 levels observed in controls (Labadarios, 1975). The control diet contained the same level of phenobarbitone and was either folate-supplemented or semi-supplemented, the latter being the minimum amount required to cure symptoms of folate deficiency, (Asenjo, 1948). Labadarios proposed that the synthesis of nucleic acids and essential enzyme proteins may be decreased in rats maintained on the folate-deficient diet, since folic acid is thought to be a
co-factor in the biosynthesis of purine and pyrimidine nucleotides.

To further investigate this important drug-nutrient interaction the study was undertaken of the effect on hepatic mixed-function oxidase enzyme activities of administration to folate deficient rats of phenobarbitone, an anticonvulsant drug and powerful microsomal enzyme inducer, and 3-methylcholanthrene, a polycyclic, aromatic hydrocarbon. These two compounds stimulate the metabolism of drugs, carcinogens and other substrates of liver microsomes by different mechanisms. 3-Methylcholanthrene stimulates the metabolism of fewer substrates and induces a different form of cytochrome P450 to phenobarbitone, namely cytochrome P448, which is thought to be involved in the activation of carcinogens (Burke and Mayer, 1975). Cytochrome P448 differs from cytochrome P450 in its molecular weight, terminal amino acid composition, antigenicity (with no immunological cross-reactivity) and substrate specificity (Ryan et al., 1979). The microsomal cytochromes P450 and P448, NADPH cytochrome C reductase and mixed-function oxidase enzyme activities were measured to determine the effects of any interaction between folate deficiency and enzyme induction. Levels of microsomal and total haem were measured to determine whether decreased cytochrome P450 synthesis (Labadarios, 1975) was due to altered haem metabolism, rather than to effects on the apoprotein moiety of cytochrome P450. Serum and red cell folate, serum vitamin B₁₂ levels, haematocrit, haemoglobin and red cell counts were measured in order to determine the presence and extent of folate deficiency. Hepatic enzymes involved in phase II metabolism were also measured, an area not previously investigated.
Experimental

Materials
Folate and vitamin $B_2$ determinations were made using a dual radioassay kit purchased from Amersham International PLC. Cyanomethaemoglobin, heparin, Drabkin's reagent, pyridine, p-nitrophenol, potassium hydroxide and phenbarbitone-sodium were obtained from BDH Ltd. 3-Methylcholanthrene and folic acid were supplied by Sigma. Benzphetamine was a gift from the Upjohn Company, Kalamazoo, Michagan. All other materials were obtained as previously reported in Chapter Two.

Diets
Cellulose powder, corn starch, corn oil, vitamin- and fat-free casein and folic acid-free vitamin/mineral premix were supplied by Special Diet Services Ltd. Hydrogenated vegetable oil was purchased from J.Sainsbury's PLC.

Animals
Wistar albino, weanling, male rats, from the University of Surrey breeding colony, were housed in wire-bottomed cages. Food and water were available ad libitum.

Diet composition
The diet was composed as follows: (Thompson et al 1972; Labadarios, 1975)

Corn starch.................................................46%
Vitamin- and fat-free casein.....................20%
Hydrogenated vegetable oil ..........17%
Cellulose powder ..................10%
Corn oil ............................2%
Folic acid-free vitamin/mineral premix ...5%

The folate supplemented diet was prepared by adding 1.0mg folic acid in the monoglutamate form /kg diet. Acknowledgements are due to Special Diet Services Ltd for the folic acid analyses of the diets. The deficient diet was found to contain less than 0.2mg/kg and the supplemented diet to contain 1.2mg/kg by microbiological assay using Lactobacillus casei. The diets were stored at 4°C in sealed buckets.

The rats were maintained on the diets for periods of 13 - 18 wk. During the feeding period samples of tail vein blood were taken at intervals to determine serum and red cell folate levels and serum vitamin B₁₂ levels. Animals were weighed and dietary intakes assessed weekly.

On the morning of the termination of the experiment, between 9-10.30 a.m., prior to cervical dislocation, each animal was anaesthetised with ether and blood was collected by cardiac puncture. An aliquot was taken to provide serum for folate and vitamin B₁₂ determinations. The remainder was heparinised and used for red blood cell counts, red cell folate, haematocrit and haemoglobin determinations. Preparation of liver subfractions was as previously described in Chapter Two.
Dosing Regimes

Phenobarbitone - the low dose was administered in the drinking water as a 1% (w/v) solution for 4 days. At the end of the experiment the animals had been maintained on the diets for 13 wk. The high dose was administered by ip injection at 80mg/kg for 14 days. At the end of the experiment the animals had been maintained on the diet for 18 wk.

3-Methylcholanthrene - was dissolved in corn oil and administered by ip injection at 25mg/kg for 7 days. Control animals received an equivalent volume of corn oil. At the end of the experiment the animals had been maintained on the diets for 15 wk.
Methods

Cytochrome P450, cytochrome b₅, NADPH cytochrome C reductase, 7-ethoxyresorufin-o-deethylase, biphenyl 4- and 2-hydroxylases, 1-naphthol glucuronyltransferase and microsomal protein determinations were made as previously described in Chapter Two.

Folate and Vitamin B₁₂ Measurement - The principle of the assay was as follows:

Prior to assay, folate and vitamin B₁₂ were released from their serum binding proteins by incubation at room temperature, pH 12.8-13.0, with a denaturation reagent containing potassium cyanide. The samples were incubated at pH 9.5 with folate binding protein and hog intrinsic factor. Serum folate competes with ¹²⁵I-labelled folate for sites on folate binding protein, and serum vitamin B₁₂ competes with ⁵⁷Co-labelled vitamin B₁₂ for sites on intrinsic factor. The protein-bound fraction was separated by adsorption of the free fraction onto coated charcoal, and precipitated by centrifugation. Counts were made of the free fraction on a multigamma counter (LKB). The serum folate, vitamin B₁₂, and red cell folate concentrations were interpolated from a standard curve.

Haematocrit - was determined by centrifugation (10 min, MSE haematocrit centrifuge) of fresh whole blood drawn into heparinised haematocrit tubes and sealed. Determinations were made in duplicate.

Haemoglobin - was determined by the cyanomethaemoglobin method. (Drabkin & Austin, 1932). Whole blood (20µl) was diluted with Drabkin's reagent (4ml) and vortex mixed. The absorbance was
determined at 540nm against a blank of Drabkin's reagent using an SP500 spectrophotometer. The standard, cyanomethaemoglobin, contained 57.2mg/100ml which is equivalent to 11.5mgHb/ml blood. Determinations were made in duplicate.

Red Blood Cell Counts - were determined using a Coulter Counter. Whole blood was diluted 1:50000 with Isoton. Duplicate counts were made, and correction was made for coincidence.

Microrosomal Haem (Schenkman et al, 1975)

Washed microsomes were prepared and the pellet resuspended in 0.1M phosphate buffer pH7.4 at a concentration of 12.5%. Aliquots (3ml) were added to pyridine (2ml) and 1M NaOH (1ml) and divided between two cuvettes. Formation of the pyridine haemochromogen was commenced by the addition of sodium dithionite and recorded as a difference spectrum between 550nm and 660nm using an SP18 spectrophotometer. Haem content was calculated by measuring the difference in absorbance between 557nm and 575nm using an extinction coefficient of 32.4 mM⁻¹ cm⁻¹. The haem content of 1% liver homogenate was similarly determined (protein range 0.1-1.0 mg).

Benzphetamine-N-demethylase

The method used was identical to that for determination of ethylmorphine-N-demethylase except that 0.4 µmol of benzphetamine were used in the incubation system in place of ethylmorphine hydrochloride.

p-Nitrophenol glucuronyltransferase

The method measures the rate of disappearance of the substrate. The incubation mixture consisted of 0.1 µmol of p-nitrophenol, 0.15-0.25 mg microsomal protein (10000g fraction), 0.6mg Brij, 3
µmol of UDPGA and 0.125 mmol Tris-HCl buffer pH 7.4, in a final volume of 0.6 ml.

After a 5 min pre-incubation at 37°C, UDPGA was added to initiate the reaction. Termination at 4 min was achieved by the addition of an aliquot of 0.1M trichloroacetic acid (2.4ml) and plunging the tubes into an ice bath. A 20 min centrifugation at 4000 rpm using a Beckman J6 centrifuge, precipitated the protein. After addition of 10M KOH (0.06ml) the absorbance at 400nm was determined using a Cecil 272 Spectrophotometer, against a blank in which the substrate was omitted. The standard contained the original concentration of p-nitrophenol, achieved by the omission of UDPGA during the incubation.

Statistical Analyses

These were performed using Student's t test.

Results

The rats fed on the folate deficient diet were consistently of lower weight than rats fed on the folate supplemented diet, although the results were not statistically significant (Fig 6.2). There was no difference in food intake between the two groups.

Red cell folate was observed to decline more rapidly than serum concentration (Fig 6.3). After about 12 wk of feeding, serum folate levels were within the same range as those found to be associated with lower induced levels of cytochrome P450 (Labadarios, 1975). Haematocrit, haemoglobin, red blood cell
counts, mean corpuscular haemoglobin concentration, and mean corpuscular volumes in the folate deficient rats were equivalent to the controls (Tables 6.1, 6.4, & 6.7), and examination of blood smears after 12 and 18 wk similarly indicated that megaloblastic anaemia was not present. After 13 wk of feeding, the levels of cytochromes P450 and $b_5$, and NADPH cytochrome C reductase activity were not altered in the folate deficient group compared to the controls (Table 6.2). Microsomal haem was higher in the folate deficient group, but this effect was not significant (Table 6.2). The activity of biphenyl-4-hydroxylase was found to be significantly lower in the folate deficient group, but no changes were observed for the other mixed-function oxidase enzymes, or for the phase II conjugation enzymes studied (Table 6.3). After 15 wk of feeding there were similarly no changes in cytochrome P450 or $b_5$, NADPH cytochrome C reductase activity or microsomal haem content (Table 6.8). Biphenyl-4-hydroxylase was decreased in the folate deficient group, but the effect was not significant (Table 6.9). There were no changes in the activities of the other mixed-function oxidase enzymes or the glucuronyltransferases.

The administration of phenobarbitone at 1 gm /litre in the drinking water for 4 days to rats fed on the folate deficient and folate supplemented diets was found to induce cytochrome P450 to an equivalent extent in both groups (Table 6.2). Microsomal haem was similarly increased in both treated groups (Table 6.2). Biphenyl-4-hydroxylase showed a higher level of activity in the folate supplemented phenobarbitone group than the folate deficient phenobarbitone treated group (Table 6.3). No differences were
observed for the other mixed-function oxidase enzyme activities or glucuronyltransferases (Table 6.3).

The longer pre-treatment with phenobarbitone, 80 mg/kg ip for 14 days, to rats maintained on the folate deficient and folate supplemented diets produced equivalent levels of induction in both groups for cytochromes P450 and b5, NADPH cytochrome C reductase activity, microsomal haem (Table 6.5), microsomal oxidase enzyme activities and glucuronide-conjugation enzyme activities (Table 6.6). At the commencement of dosing the control group contained three animals, one of which died 3 days prior to the end of the experiment.

Treatment with 3-methylcholanthrene (80mg/kg, for 7 days) was found to produce equivalent levels of cytochromes P450 and b5, NADPH cytochrome C reductase activity and microsomal haem content in the folate deficient and folate supplemented groups (Table 6.8). Biphenyl-2-hydroxylase and 7-ethoxyresorufin-0-deethylase activities, which are greatly enhanced by 3-methylcholanthrene treatment in comparison to phenobarbitone treatment, showed no statistically significant differences between the folate supplemented and folate deficient groups (Table 6.9). The other mixed-function oxidase enzymes and the glucuronyltransferase enzymes similarly showed no statistically significant differences in activity (Table 6.9).
Fig. 6.2. GROWTH CURVE OF RATS MAINTAINED ON EXPERIMENTAL DIETS
Results are means of five rats per group from each diet group measured at each time point. The value of the folate deficient group is expressed as a percentage of the folate supplemented group for both serum and red cell folate levels.
Table 6.1. Effect of Folate Deficiency and Phenobarbitone Treatment on Serum and Red Cell Folate Levels, and other Blood Parameters in the Rat.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate Supplemented Control</td>
</tr>
<tr>
<td>Serum folate (ng/ml)</td>
<td>24.5±2.3 (100%)</td>
</tr>
<tr>
<td>Red Cell folate (ng/ml)</td>
<td>1666±438 (100%)</td>
</tr>
<tr>
<td>Serum B12 (pg/ml)</td>
<td>1036±65 (100%)</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>42± 1 (100%)</td>
</tr>
<tr>
<td>Haemoglobin (g/100ml blood)</td>
<td>14.5±0.3 (100%)</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>34.5±1.3 (100%)</td>
</tr>
<tr>
<td>RBC x 10^6 (cells/ mm³)</td>
<td>7.48±0.8 (100%)</td>
</tr>
<tr>
<td>MCV (µm)</td>
<td>57.0±6.0 (100%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 13 weeks. Phenobarbitone was administered at 0.1% (w/v) in the drinking water for 4 days prior to killing the animals. Figures in parentheses are percentage values of the appropriate control group.
MCHC : Mean Corpuscular Haemoglobin Concentration;
RBC : Red Blood Cells Count;
MCV : Mean Corpuscular Volume.
Value significantly different from control: * p< 0.05;
** p< 0.02;
*** p< 0.005;
**** p< 0.001;
Table 6.2. Effect of Folate Deficiency on Some Components of the Hepatic Microsomal Drug Metabolizing Enzyme System and their Induction by Phenobarbitone in the Rat.

<table>
<thead>
<tr>
<th>Component Measured</th>
<th>TREATMENT GROUP</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate Supplemented</td>
<td>Folate Deficient</td>
<td>Folate Supplemented</td>
<td>Folate Deficient</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td>0.1% phenobarbitone</td>
<td>0.1% phenobarbitone</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P₄₅₀ (ng/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.78±0.05 (100%)</td>
<td>0.73±0.03 (94%)</td>
<td>1.82±0.11 (100%)</td>
<td>1.69±0.1 (93%)</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b₅ (ng/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.49±0.04 (100%)</td>
<td>0.43±0.01 (88%)</td>
<td>0.55±0.03 (100%)</td>
<td>0.49±0.03 (89%)</td>
<td></td>
</tr>
<tr>
<td>NADPH Cytochrome C Reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td>47.5±4.0 (100%)</td>
<td>48.7±7.7 (103%)</td>
<td>43.1±4.0 (100%)</td>
<td>55.7±11.5 (130%)</td>
<td></td>
</tr>
<tr>
<td>Microsomal haem (nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.62±0.04 (100%)</td>
<td>0.92±0.1 (148%)</td>
<td>1.31±0.05 (100%)</td>
<td>1.37±0.25 (105%)</td>
<td></td>
</tr>
<tr>
<td>Total haem (nmol/mg liver protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.64±0.04 (100%)</td>
<td>0.62±0.11 (97%)</td>
<td>0.48±0.05 (100%)</td>
<td>0.72±0.09 (150%)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 13 weeks. Phenobarbitone was administered at 0.1% (w/v) in the drinking water for 4 days prior to killing the animals. Figures in parentheses are percentage values of the appropriate control group.
Table 6.3. Effect of Folate Deficiency on Some Hepatic Drug Metabolizing Enzymes and Their Induction by Phenobarbitone in the Rat

<table>
<thead>
<tr>
<th>Enzyme Measured</th>
<th>TREATMENT GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate Supplemented Control</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>17.3±0.6 (100%)</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (nmol 2-OH biphenyl/mg protein/hr)</td>
<td>1.4±0.6 (100%)</td>
</tr>
<tr>
<td>Benzphetamine-N-demethylase (μmol formaldehyde/mg protein/hr)</td>
<td>0.32±0.04 (100%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-0-deethylase (nmol resorufin/mg protein/hr)</td>
<td>0.050±0.006 (100%)</td>
</tr>
<tr>
<td>1-Naphthol glucuronyl transferase (nmol 1-naphtholglucuronide/mg protein/min)</td>
<td>39.8±2.0 (100%)</td>
</tr>
<tr>
<td>P-Nitrophenol glucuronyl transferase (nmol p-NP used/mg protein/min)</td>
<td>8.8±0.7 (100%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>31.7±1.5 (100%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 13 weeks. Phenobarbitone was administered at 0.1% (w/v) in the drinking water for 4 days prior to killing the animals. Figures in parentheses are percentage values of the appropriate control group. Value significantly different from control: *p<0.01.
Table 6.4. Effect of a Longer Phenobarbitone Treatment on Serum and Red Cell Folate Levels, and other Blood Parameters in the Rat Maintained on a Folate Deficient Diet.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Treatment Group</th>
<th>Folate Supplemented 80mg phenobarbitone/kg</th>
<th>Folate Deficient 80mg phenobarbitone/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (ng/ml)</td>
<td>10.4 ± 0.6 (100%)</td>
<td>4.6 ± 0.8 (44%)*</td>
<td></td>
</tr>
<tr>
<td>Red Cell Folate (ng/ml)</td>
<td>793 ± 241 (100%)</td>
<td>288 ± 11 (36%)**</td>
<td></td>
</tr>
<tr>
<td>Serum B₁₂ (pg/ml)</td>
<td>931 ± 181 (100%)</td>
<td>832 ± 68 (90%)</td>
<td></td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43 ± 7 (100%)</td>
<td>42 ± 2 (98%)</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/100ml blood)</td>
<td>12.9 ± 1.7 (100%)</td>
<td>13.2 ± 1.2 (102%)</td>
<td></td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>30.0 ± 0.5 (100%)</td>
<td>31.1 ± 2.7 (104%)</td>
<td></td>
</tr>
<tr>
<td>RBCC x 10⁶ (Cells/mm³)</td>
<td>9.34 ± 2.3 (100%)</td>
<td>9.52 ± 1.0 (102%)</td>
<td></td>
</tr>
<tr>
<td>MCV (µm)</td>
<td>46.5 ± 3.8 (100%)</td>
<td>44.6 ± 3.2 (96%)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for two male animals (control) and five male animals (deficient) maintained on the diets for 18 weeks. Figures in parentheses are percentage value of control group. Phenobarbitone was administered at 80mg/kg bodyweight by daily I.P. injection for 14 days.

MCHC : Mean Corpuscular Haemoglobin Concentration.
RBCC : Red Blood Cell Count.
MCV : Mean Corpuscular Volume.
Value significantly different from control: * p < 0.005
** p < 0.001
### Table 6.5. Effect of a Longer Phenobarbitone Treatment on some Components of the Hepatic Microsomal Drug Metabolizing Enzyme System in the Rat Maintained on a Folate Deficient Diet.

<table>
<thead>
<tr>
<th>Component Measured</th>
<th>Treatment Group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Folate Supplemented</td>
<td>Folate Deficient</td>
</tr>
<tr>
<td></td>
<td>80mg phenobarbitone/kg</td>
<td>80mg phenobarbitone/kg</td>
</tr>
<tr>
<td>Cytochrome P450 (ng/mg protein)</td>
<td>2.80 ± 0.37 (100%)</td>
<td>2.16 ± 0.17 (77%)</td>
</tr>
<tr>
<td>Cytochrome b5 (ng/mg protein)</td>
<td>0.65 ± 0.05 (100%)</td>
<td>0.58 ± 0.02 (89%)</td>
</tr>
<tr>
<td>NADPH Cytochrome C reductase. (nmol Cytochrome C reduced/mg protein/min)</td>
<td>60 ± 1 (100%)</td>
<td>64 ± 0.3 (106%)</td>
</tr>
<tr>
<td>Microsomal haem (µmol/mg protein)</td>
<td>1.80 ± 0.06 (100%)</td>
<td>2.00 ± 0.14 (111%)</td>
</tr>
<tr>
<td>Total haem (µmol/mg liver protein)</td>
<td>1.41 ± 0.14 (100%)</td>
<td>1.45 ± 0.28 (107%)</td>
</tr>
</tbody>
</table>

Results are Mean ± SEM for 2 male animals (control) and 5 male animals (deficient), maintained on the diets for 18 weeks. Phenobarbitone was administered at 80mg/kg bodyweight by daily I.P. injection for 14 days and the animals killed 24 hours after the last administration. Figures in parentheses are percentage values of control.
Table 6.6. Effect of a Longer Phenobarbitone Treatment on Some Hepatic Drug Metabolizing Enzymes and their Induction in the Rat Maintained on a Folate Deficient Diet.

<table>
<thead>
<tr>
<th>Enzyme Measured</th>
<th>Folate Supplemented 80mg phenobarbitone/kg</th>
<th>Folate Deficient 80mg phenobarbitone/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>2'-Hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>62.4±5.0 (100%)</td>
<td>55.8±2.6 (90%)</td>
</tr>
<tr>
<td>Benzphetamine-N-demethylase (µmol formaldehyde/mg protein/hr)</td>
<td>0.35±0.02 (100%)</td>
<td>0.33±0.03 (94%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-o-deethylase (nmol resorufin/mg protein/hr)</td>
<td>0.035±0.01 (100%)</td>
<td>0.030±0.02 (96%)</td>
</tr>
<tr>
<td>1-Naphthol glucuronyl transferase (nmol 1-naphtholglucuronide/mg protein/min)</td>
<td>48.7±2.8 (100%)</td>
<td>44.6±3.7 (92%)</td>
</tr>
<tr>
<td>P-Nitrophenolglucuronyl transferase (nmol PNP used/mg protein/min)</td>
<td>33.5±5 (100%)</td>
<td>33±9 (100%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>39.4±2.2 (100%)</td>
<td>42.6±0.9 (108%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for two male animals (control) and five male animals (deficient), maintained for 18 weeks on the diets. Figures in parentheses are percentage values of control groups.

*Phenobarbitone was administered at 80 mg/kg bodyweight by daily IP injection for 14 days and the animals killed 24 hr after the last administration.
Table 6.7.  Effect of Folate Deficiency and 3-Methylcholanthrene (3-MC) Treatment on Serum and Red Cell Folate Levels, and other Blood Parameters in the Rat.

<table>
<thead>
<tr>
<th>Parameter Measured</th>
<th>Folate Supplemented (Control)</th>
<th>Folate Deficient</th>
<th>Folate Supplemented 25mg/kg 3-MC</th>
<th>Folate Deficient 25mg/kg 3-MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum folate (ng/ml)</td>
<td>18.2±1.1 (100%)</td>
<td>3.6±0.4 (20%***</td>
<td>25.6±3.2 (100%)</td>
<td>6.1±1.0 (24%***</td>
</tr>
<tr>
<td>Red Cell folate (ng/ml)</td>
<td>904±155 (100%)</td>
<td>301±2 (33%**</td>
<td>1066±42 (100%)</td>
<td>367±33 (34%***</td>
</tr>
<tr>
<td>Serum B12 (pg/ml)</td>
<td>1008±106 (100%)</td>
<td>692±56 (69%†</td>
<td>1038±41 (100%)</td>
<td>972±118 (94%)</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>44±7 (100%)</td>
<td>43±7 (98%)</td>
<td>44±6 (100%)</td>
<td>43±3 (98%)</td>
</tr>
<tr>
<td>Haemoglobin (g/100ml blood)</td>
<td>14.2±0.5 (100%)</td>
<td>13.3±1.4 (94%)</td>
<td>14.4±0.4 (100%)</td>
<td>13.9±1.0 (96%)</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>32.5±2.5 (100%)</td>
<td>31.3±1.4 (96%)</td>
<td>33.0±0.8 (100%)</td>
<td>32.0±1.9 (97%)</td>
</tr>
<tr>
<td>RBC x 10^6 (cells/mm³)</td>
<td>10.12±1.0 (100%)</td>
<td>8.80±1.8 (87%)</td>
<td>10.98±1.52 (100%)</td>
<td>8.98±1.1 (82%)</td>
</tr>
<tr>
<td>MCV (µm)</td>
<td>43.3±4.1 (100%)</td>
<td>49.8±9.8 (115%)</td>
<td>40.5±6.3 (100%)</td>
<td>47.7±4.0 (118%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 15 weeks. 3-Methylcholanthrene in corn oil was administered at 25 mg/kg bodyweight by daily IP injection and the animals were killed 24 hours after the last administration. Figures in parentheses are percentage values of appropriate control group. MCHC: Mean corpuscular haemoglobin concentration; RBC: Red blood cell count; MCV: Mean corpuscular volume. Value significantly different from control: *p<0.05; **p<0.005; ***p<0.001.
Table 6.8. Effect of Folate Deficiency on some Components of the Hepatic Microsomal Drug Metabolizing Enzyme System and their Induction by 3-Methylcholanthrene (3-MC) in the Rat.

<table>
<thead>
<tr>
<th>Component Measured</th>
<th>TREATMENT GROUP</th>
<th>Folate Supplemented (Control)</th>
<th>Folate Deficient</th>
<th>Folate Supplemented 25mg 3-MC/kg</th>
<th>Folate Deficient 25mg 3-MC/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P₄₅₀  (ng/mg protein)</td>
<td>0.82±0.11 (100%)</td>
<td>0.67±0.03 (82%)</td>
<td>1.27±0.09 (100%)</td>
<td>1.10±0.08 (87%)</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b₅  (ng/mg protein)</td>
<td>0.45±0.05 (100%)</td>
<td>0.39±0.01 (87%)</td>
<td>0.70±0.02 (100%)</td>
<td>0.60±0.02 (86%)</td>
<td></td>
</tr>
<tr>
<td>NADPH Cytochrome C Reductase (nmol cytochrome C reduced/mg protein/min)</td>
<td>48.3±1.0 (100%)</td>
<td>38.0±0.3 (79%)</td>
<td>45.5±1.2 (100%)</td>
<td>47.3±4.0 (104%)</td>
<td></td>
</tr>
<tr>
<td>Microsomal haem (µmol/mg protein)</td>
<td>1.19±0.1 (100%)</td>
<td>1.06±0.21 (89%)</td>
<td>1.76±0.10 (100%)</td>
<td>2.09±0.77 (119%)</td>
<td></td>
</tr>
<tr>
<td>Total haem (µmol/mg liver protein)</td>
<td>0.60±0.05 (100%)</td>
<td>0.52±0.02 (87%)</td>
<td>0.56±0.03 (100%)</td>
<td>0.59±0.04 (105%)</td>
<td></td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 15 weeks. 3-Methylcholanthrene in corn oil was administered at 25mg 3-MC/kg bodyweight by daily IP injection for 7 days and the animals were killed 24 hours after the last administration.

Figures in parentheses are percentage values of the appropriate control group.
Table 6.9. Effect of Folate Deficiency on Some Hepatic Drug Metabolizing Enzymes and their Induction by 3-Methylcholanthrene (3-MC) in the Rat.

<table>
<thead>
<tr>
<th>Enzyme Measured</th>
<th>TREATMENT GROUP</th>
<th>Folate Deficient</th>
<th>Folate Supplemented</th>
<th>Folate Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Control)</td>
<td></td>
<td>25mg/kg 3-MC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (nmol 4-OH biphenyl/mg protein/hr)</td>
<td>50.4±7.7 (100%)</td>
<td>34.4±3.1 (68%)</td>
<td>67.9±3.3 (100%)</td>
<td>67.6±4.9 (99%)</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (nmol 2-OH biphenyl/mg protein/hr)</td>
<td>0.12±0.03 (100%)</td>
<td>0.14±0.02 (117%)</td>
<td>3.30±0.26 (100%)</td>
<td>3.24±0.26 (98%)</td>
</tr>
<tr>
<td>Benzphetamine-N-demethylase (umol formaldehyde/mg protein/hr)</td>
<td>0.11±0.03 (100%)</td>
<td>0.12±0.01 (110%)</td>
<td>0.10±0.02 (100%)</td>
<td>0.08±0.02 (80%)</td>
</tr>
<tr>
<td>7-Ethoxyresorufin-o-deethylase (nmol resorufin/mg protein/hr)</td>
<td>0.057±0.010 (100%)</td>
<td>0.055±0.011 (97%)</td>
<td>7.48±1.141 (100%)</td>
<td>5.89±0.821 (79%)</td>
</tr>
<tr>
<td>1-Naphthol glucuronyl transferase (nmol 1-naphthol glucuronide/mg protein/min)</td>
<td>38.5±7.4 (100%)</td>
<td>28.2±2.6 (73%)</td>
<td>188.3±12.1 (100%)</td>
<td>171.1±13.7 (91%)</td>
</tr>
<tr>
<td>p-Nitrophenol glucuronyl transferase (nmol PNP used/mg protein/min)</td>
<td>28.8±3.9 (100%)</td>
<td>30.4±1.7 (106%)</td>
<td>37.3±5.9 (100%)</td>
<td>26.2±1.4 (70%)</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>31.0±2.9 (100%)</td>
<td>33.5±3.1 (108%)</td>
<td>32.1±2.0 (100%)</td>
<td>34.3±2.5 (107%)</td>
</tr>
</tbody>
</table>

Results are mean ± SEM for five male animals maintained on the diets for 15 weeks. 3-Methylcholanthrene in corn oil was administered at 25mg/kg bodyweight by daily IP injection for 7 days, and the animals were killed 24 hours after the last administration.

Figures in parentheses are percentage values of the appropriate control group.
Discussion

The measurement of circulating serum and red cell folate levels were used to judge a state of folate depletion. In the experiment now reported the serum and red cell folate levels in the folate deficient group had declined to 50% of the folate supplemented group by 12 wk of feeding, and thereafter declined more rapidly to approximately 30% (14 - 15 wk) and then stayed at approximately this level. Red cell folate, which is considerably higher than serum folate, is less affected by short-term dietary changes and is an accurate indication of body folate reserves. Decreased blood folate activity indicates a severe depletion of tissue stores (Grossowicz et al, 1964). Experimental rats fed a folate deficient diet for 20 days were found to have decreased liver and kidney folate levels after 10 days, but blood levels were not seen to decrease until tissue levels had fallen. Chanarin et al (1969) have also shown that hepatic levels decline more rapidly than serum folate levels in rats maintained on a folate deficient diet containing sulphanilamide for 12 wk. Antibiotic may be added to experimental diets to speed up and heighten the attainment of folate deficiency in the rat since the de novo synthesis of folic acid by gut microflora is thought to be a reason for the difficulty encountered in inducing folate deficiency in experimental rats (Teply et al, 1947). Coprophagy is also thought to contribute to this problem. It has been reported (Kodicek and Carpenter, 1950) that 1% sulphathiazole is haemolytic. Serum from patients receiving antibiotics has been shown to inhibit the growth of Lactobacillus Casei, the bacteria used for microbiological
assessment of folic acid (Waters and Mollin, 1961). In the experiment now reported the rats were maintained in wire-bottomed cages. Since drugs were administered to induce enzyme levels, antibiotics were not added to the diets, in order to avoid drug interactions.

The major circulating form of folic acid in serum is 5-methyltetrahydrofolic acid. Hence results of radioassay methods and microbiological assay using Lactobacillus Casei are directly comparable. Red cell values are considerably higher when measured by radioassay as this measures total folate, including the peptide-bound fraction which accounts for 86-90% of the total red cell folate, whereas Lactobacillus Casei does not respond to polyglutamates or peptide bound derivatives.

The results of this study indicate that a long-term dietary deficiency of folic acid in the rat, resulting in low serum and red cell folate levels, does not alter the capacity for induction of hepatic microsomal cytochromes, or phase I or phase II drug metabolizing enzymes when compared to a control group of rats with normal folate levels.

Labadarios (1975) hypothesised that the lower level of induced cytochrome P450 in rats maintained on a folate deficient diet containing 500 mg/kg phenobarbitone for 12 wk (approximately 40mg/kg/day for 12 wk), was related to folate deficiency. The results now reported with comparable serum folate levels to those achieved by Labadarios (1975) indicate that induction of
cytochromes P450 and \textsuperscript{5} microsomal haem, biphenyl 4-hydroxylase and 7-ethoxyresorufin-0-deethylase activities is possible. The differences may be due to the length of time for which the experimental rats were exposed to the phenobarbitone - 12 wk where decreased induction was reported and periods of 4 days and 2 wk where induction was equivalent to induced control values. This indicates that there is a further complication resulting from long-term administration of phenobarbitone that is causing the decreased levels of cytochrome P450 observed by Labadarios (1975) in rats receiving phenobarbitone supplemented, folate deficient diets.

It is possible that, in the experiment now reported, the serum folate levels have not reached the critical level at which enzyme induction is decreased. However, it was observed that individual animals with serum folate levels as low as 1.8 ng/ml serum had induced levels of cytochromes P450 and \textsuperscript{5} microsomal haem, and induced activities of biphenyl hydroxylases, 7-ethoxyresorufin-0-deethylase and glucuronyl transferases that were similar to control values. The serum folate value at which Labadarios (1975) found decreased levels of induction was 4.3 ng/ml serum ± 0.2. The average serum folate value of the phenobarbitone treated group (80 mg/kg, 14 days) and maintained on the folate deficient diet was 4.6 ng/ml serum. Long-term exposure to anticonvulsant drugs would be associated with long-term metabolic disturbances, causing strain on certain metabolic pools, especially haem. This may eventually drain the metabolic pools whereas with short-term exposure they are adequate for enzyme
induction to occur.

Folic acid is involved in the biosynthesis of cytochromes (Parke, 1978a). It is a cofactor in both apocytochrome P450 biosynthesis and haem synthesis (Clement et al., 1981). In the first stage of haem synthesis, succinyl CoA and glycine condense to form δ-aminolaevulinic acid. Conversion of serine to glycine represents the most efficient supply of glycine in the body, and this requires tetrahydrofolate as coenzyme. A decreased ability of folate deficient rats to incorporate labelled formate into serine in vivo (Elwyn and Sprinson, 1950) and a lower capacity of tissue preparations to convert serine to glycine (Vilenkina, 1952) have been reported. δ-Amino laevulinic acid synthetase has a high Km value for glycine and experimental depletion prevents activity of the enzyme. In a state of glycine depletion, this will be the rate limiting step for haem synthesis (Tephly et al., 1973). Glycine is an abundant component of many foods. The amount of amino acid coming from the diet, and amino acid turnover and synthesis in the tissues represent many times the quantities present free in the body at one time, such that pools of free amino acids are small in relation to the amount of protein in the tissues (Munro and Portugal, 1972).

Cytochrome P450 synthesis in rat liver accounts for 70% of haem synthesised under normal metabolism. Long-term exposure to inducing drugs will put a considerable strain on the pathways involved in its synthesis. Long-term exposure to anticonvulsant drugs together with a limiting supply of folic acid, may result in
an inadequate supply of haem, after all the available haem has been used up, because of reduced glycine pools. The situation will arise where haem synthesis cannot keep pace with requirements. Clement et al (1981) reported a significant depression of intestinal haem content in guinea pigs maintained on a folate deficient diet, and the activity of intestinal 7-ethoxyresorufin-O-deethylase was also significantly decreased. However the significant depression of NADPH cytochrome C reductase activity was suggestive of gut morphological changes.

An activator for uroporphyrinogen I synthetase, which catalyses the conversion of porphyrobilinogen to uroporphyrinogen III, has been identified (Piper and Van Lier, 1977), which may be a pteroylglutamate derivative. This may regulate the activity of uroporphyrinogen I synthetase, which is thought to become the rate limiting enzyme if the activity of \( \delta \)-aminolaevulinic acid is increased (Bonkowsky et al, 1979). This is another possible mechanism by which folic acid deficiency may decrease haem synthesis.

Apoprotein synthesis is generally thought to be the primary and rate limiting event in the control of cytochrome P450 formation (Correia & Mayer, 1975). Under normal conditions, the availability of haem is not rate limiting for cytochrome P450 synthesis, or when phenobarbitone or 3-methylcholanthrene are administered (Sardana et al, 1976). However, if haem synthesis is experimentally inhibited during formation and assembly of cytochrome P450, the total apocytochrome present on the microsomal membrane is decreased (Bhat
et al., 1977). In the event of decreased haem synthesis in the folate deficient animals, this will become rate limiting for cytochrome P450 formation.

In the experiment now reported, folate depletion was observed not to alter hepatic haem levels in rats maintained on a folate deficient diet compared to rats maintained on a folate supplemented diet, or to decrease the induced levels of hepatic haem in animals treated with phenobarbitone or 3-methylcholanthrene. This indicates that the effects of short-term drug exposure did not affect haem supply indicating that the substrates and coenzymes of haem synthesis were adequate for increased synthesis to take place. It appears therefore that a longer time of exposure to cytochrome P450 inducing agents is necessary for the lesions in the synthetic pathway to become apparent. Under conditions of longer exposure there is a greater total demand for haem synthesis and a continual depletion of substrates which will be in reduced supply because of folate deficiency. The decreased supply of haem may then become rate limiting for the de novo synthesis of cytochrome P450.
Chapter Seven

General Discussion
The Dietary Fibre Hypothesis

It is considered by Southgate (1982) that the original dietary fibre hypothesis, which states that a fibre-rich diet is protective against the diseases common in affluent Western societies, e.g. colon cancer, diverticular disease, diabetes and obesity, and that a low fibre diet may be causative in the aetiology of such diseases, (Southgate, 1982; Trowell, 1975; Burkitt & Trowell, 1975), relates to types of diet. It argues that the difference between protective and non-protective diets is determined by the amounts of plant cell wall material they provide and that the protective agent is, or is derived from, the plant cell walls in the diet. As fibre-containing foods are generally of low energy density and high bulk, increasing the intake of dietary fibre will make it more unlikely that excess energy will be consumed, and the percentage contribution to the diet from fat, protein and refined carbohydrates is likely to be reduced. Hence some of the benefits of a high fibre diet may be attributable to the indirect effects of reduced consumption of other dietary components. The diets consumed by communities with a low incidence of the diseases against which dietary fibre is thought to be protective have other characteristics - they are low in fat, refined sucrose and salt, and are high in starch (Perissé et al., 1969),

Studies to test this hypothesis have concentrated on the role of dietary fibre. The two main approaches are epidemiological studies of the relationship between intake of dietary fibre and
incidence of disease, or studies aimed at elucidating the mechanism of action of dietary fibre. The latter approach may involve feeding a particular fibre or a purified component and measuring a biological response or investigating the relationships between the biological structures of fibres and their functions.

Problems of Dietary Fibre Studies - Investigation of the mechanisms of action of dietary fibre is a slow process requiring long-term and carefully controlled experiments.

For the purposes of dietary fibre research it is important to study each source of dietary fibre individually. The importance of such an approach lies in the necessity to explain the effects in terms of individual dietary fibres. Ideally experiments should aim to provide nutritionally adequate diets and result in purification of the active component of a dietary fibre, detailed physical and chemical characterisation of the component and quantitative measurement of the effects produced (Lang & Briggs, 1976). Investigations of individual components of such fibre sources, though providing potentially useful information, are fraught with difficulties since the purification procedures cause changes in the physical properties of the components so that they behave differently than when in their natural state in the plant cell wall. Similarly such procedures as drying, grinding, freeze-drying and cooking influence the behaviour of fibres in the gastro-intestinal tract. As dietary fibre is associated with other nutrients, any change in dietary fibre content from one source will encompass changes in other nutrients so that it will be difficult
to identify the causal factor of any observed effects. Hence purification studies have inherent problems and fibre in its natural state contains other nutritional components, so that it is difficult to identify the causal agent of any observed effect. Ideally, experiments would be designed to determine the effect of each individual fibre component in a natural fibre source, but this approach is non-physiological. However it may give some indications of effects of the various dietary fibre components.

The Rat as an Animal Model for Dietary Fibre Studies - There are species differences in the extent of digestion of a dietary fibre source and these variations may cause difficulties when extrapolating from rat to man. In the rat, fibre is generally poorly digested (Cummings, 1981a), whereas in man most fibres are digested to a greater extent. However, because of the high cellulose and lignin content of wheat bran it is poorly digested in man. Digestion of wheat bran in the rat includes epidermal disruption, interior cross tube and aleurone cell distortion, and hydrolysis of protein substances between internal tissues. Little other digestion occurs apart from bacterial degradation of endosperm remnants (George et al., 1979). Most studies indicate that cellulose is not digested in the rat to any appreciable extent, which justifies the description of cellulose as a non-nutritive fibre and non-nutritive bulk (Lang & Briggs, 1976).

Mixed-Function Oxidases and Toxicity - The mixed-function oxidase enzyme system is responsible for the metabolism of foreign compounds (Parke, 1968). Such reactions generally lead to the
deactivation of drugs and detoxication of environmental chemicals by producing metabolites with greater polarity and lower lipid solubility than the parent compound. However, microsomal oxygenation in some cases leads to the formation of reactive intermediates (Gelboin, 1969; Oesch et al., 1972) which have similar or greater lipid solubility than the parent compound, are electrophilic and may attack the nucleophilic centres of DNA and other biological macromolecules (Miller & Miller, 1969). This forms the basis of mutagenicity and carcinogenicity of such metabolites. Microsomal enzyme activities mediated by cytochrome P448 are believed to be involved in the formation of such reactive intermediates (Burke & Mayer, 1975; Parke & Ioannides, 1982).

It is now generally accepted that metabolites ultimately responsible for toxicity are often reactive intermediates. They may be only minor metabolites, but are more important toxicologically than the major metabolites (Hawkins, 1981). Hence, induction of cytochrome P450 and increased rates of mixed-function oxidations which result from exposure to certain drugs, environmental chemicals and dietary anutrients, may be beneficial if the foreign compound is rendered inactive by such metabolism, but harmful if toxic reactive metabolites are produced.

Apart from the mixed-function oxidase enzyme system which metabolises xenobiotics, a number of other enzymes in the cell e.g. glucuronyltransferases, epoxide hydrolase, glutathione-s-transferase, which further detoxify foreign compounds, exert a significant influence on the fate of foreign
compounds in an organism. These enzymes convert mutagenic, electrophilic intermediates into non-toxic substances that do not react with biological macromolecules. If phase II metabolism is greater than the rate of conversion of a carcinogen to its reactive intermediates by phase I metabolism then the toxic effects will be prevented.

Diet and Carcinogenesis - The evidence that a substantial proportion of human cancers is related to diet (Miller et al., 1980) has come from epidemiological studies. These include studies of migrants, correlational studies of various dietary factors with the incidence of cancers and case-control studies in various populations. Carcinogenesis is a multistage process, the successive stages of which may involve qualitatively different events that can be enhanced or inhibited by different types of environmental factors (Weinstein et al., 1980). The probability that these events will occur is influenced by factors in the host which are independent of the carcinogenic stimuli i.e. age, sex, genetic constitution, present and past environment, nutritional status, endocrine activity, immunological function and function of carcinogen-metabolizing enzymes, DNA repair enzymes and cell growth control processes. Modification of these factors may eventually be important in reducing the risk of cancer. The natural history of neoplastic development can be divided into initiation, which encompasses interaction of a genotoxic agent with DNA to yield abnormal genetic material and promotion, where epigenetic agents increase the development of lesions initiated by genotoxic carcinogens; progression to invasion of adjacent tissues and
metastasis in distant tissues may subsequently occur. Actual and potential regulation of these processes is at the stage of promotion. Promoting agents exhibit both threshold and maximal activity and their action is reversible (Pitot, 1982). Hence the identification of promoting agents in the environment is important in understanding the nature and prevention of development of human cancer. That nutrition plays a role in tumour promotion was reported by Rusch et al (1945), and Boutwell et al (1949). Tryptophan has been shown to be an effective promoting agent in chemically-induced bladder carcinogenesis in the rat (Cohen et al, 1979), but its administration actually inhibits hepatocarcinogenesis initiated by azo dyes and N-nitrosodiethylamine (Evarts & Brown, 1977). Fatty acids, predominantly unsaturated, have been indicated as promoting agents in the genesis of experimental neoplasia of the liver, mammary gland and forestomach (Diamond et al, 1980; Carroll & Hopkins, 1979). Bile acids have been implicated as promoting agents in experimental colonic cancer in the rat; a variety of halogenated aromatic compounds and several synthetic chemicals including saccharin, butylated hydroxytoluene and cyclamate, have exhibited promoting activity in bladder and liver carcinogenesis (Diamond et al, 1980). Identification of the many promoting agents to which man is exposed from the environment is of great importance, since they undoubtedly play a significant role in the development of human cancers. It is probable that all humans possess some initiated cells, from birth or earlier, due to ambient environmental factors, such as background radiation, diet and therapeutic intervention. Promotion may therefore be one of the
major factors in the genesis of clinical cancers and nutrition may be a major influence on its extent.

Diet and Colon Cancer - Examination of the environmental factors that possibly contribute to the occurrence of colonic cancer indicates that diet plays a major role (Armstrong & Doll, 1975; Graham & Mettlin, 1979). The active species is most likely to be generated in the colon or colonic tissue from substrates present in a high risk diet (Thompson & Hill, 1982). Examination of luminal contents has not yet revealed any potentially active substrate; the substrates investigated include nitrosamines (Venitt, 1981), tryptophan and tyrosine (Hill, 1980; Bone, 1976), neutral steroids (Wynder & Reddy, 1978) and bile acids (Hill, 1977a). The presence of faecal mutagens has been demonstrated by various investigators. Bruce et al (1977) have shown the faeces of humans consuming a high fat diet to contain compounds causing direct mutagenesis of TA98 and TA100 in the Ames test, and have demonstrated that increased dietary fibre, α-tocopherol or vitamin C consumption reduced faecal mutagens. Kuhnlein et al (1981) demonstrated that vegetarians have lower levels of faecal mutagens than non-vegetarians. Certain compounds have been reported to be non-carcinogenic or non-mutagenic alone, but to enhance the tumorigenic or mutagenic properties of carcinogens (Reddy et al, 1980).

The protective effect of dietary fibre against colonic cancers may be due to adsorption, dilution or altered metabolism of co-carcinogens, promoters or as yet unidentified carcinogens (Cummings et al, 1979a). Different types of non-nutritive fibres
possess specific binding properties and different bulking and
diluting effects. However, by modifying the luminal environment
dietary fibres may protectively influence the action of genotoxic
and epigenetic carcinogens, thus feasibly reducing the risk of
colon cancer.

Mucus and Gastro-intestinal Disease - The mucous barrier can be
considered as a protective layer to the underlying delicate mucosa
against insult from foreign toxic compounds. If the normal
physiological functions of the mucus are altered, its protective
effect on the epithelium may be changed either to the benefit or
detriment of the organism. Mucus is believed to contribute to the
thickness of the unstirred layer in the intestine (Nimmerfall &
Rosenthaler, 1980), which may in greater quantities reduce the
absorption rate especially of lipid soluble compounds
(Thompson, 1978a). The methodology used in the present work was
possibly inadequately sensitive to detect any changes caused by
bran in mucus production, and more specific determinations are
required to elucidate further the effect of bran on changes in
mucus production and composition. If bran were to increase mucus
production, this would be beneficial by increasing the protection
of the underlying epithelium against toxic chemicals and
carcinogenic metabolites. This effect would be especially
beneficial in the colon where a carcinogen activating cytochrome
P450 system has been detected (Strobel et al., 1980). It is possible
that a carcinogen-detoxifying system is also present in the colonic
wall, and since bacterial metabolism may produce carcinogenic
metabolites, a mucus layer offering maximal protection to the colon
wall would be beneficial.

Nutritional status and Drug-Nutrient Interactions - The response of an organism to a toxic substance will vary widely depending on many factors e.g. the species, the toxic substance, the dose, the route of administration, the presence of synergists or antagonists, and many other factors. Nutritional well-being is required for normal functioning of the metabolic detoxifying system of foreign compounds. The choice of a well-balanced diet containing a variety of foods should supply adequate nutrients and also limit the intake of undesirable substances. An adequate diet will provide full stores of all materials required for growth and repair (Bender, 1980). If the intake falls below the needs of an individual, the body stores will be depleted. At first there will be no effect on health and only biochemical examination would detect this. Eventually normal functioning of organs and tissues will be impaired, again only shown biochemically, and with no visible signs of ill-health. This situation is termed sub-clinical malnutrition. Clinical signs will appear only when the deficiency is sufficiently severe to cause damage to body tissues. Related to the ill-defined area of sub-clinical malnutrition is covert malnutrition (Bender, 1975), where a given level of a nutrient appears adequate but is revealed not to be when the individual is stressed. Covert malnutrition can thus be defined theoretically but its presence will not be determined in an individual prior to an event. Exposure to foreign compounds may cause stress to an individual. Response to continued exposure may involve adaptation of metabolic responses, a situation where stores of nutrients and
metabolic intermediates may determine the limitations of such adaptation. An adequate diet is thus important when long-term exposure to drugs occurs. Since organisms are continually exposed to many toxic and foreign compounds, an adequate diet may offset adverse reactions.

In addition to interactions between foods and foreign compounds which may alter the potential toxic effect of a foreign compound, nutritional status is important in determining the activity of the biochemical defence systems. Adverse effects of dietary fibre on the balance of some minerals has been reported. The prevailing mineral status will affect the outcome of any such adverse effect of dietary fibre.

However, the nutrient requirements of individuals are not known. The recommended daily intakes of nutrients are based on the measured average requirements of small numbers of individual subjects with a large safety factor added on. Similarly, the nutrient intakes of individuals are not known (Bender, 1980). The recommended daily intake of dietary fibre is more difficult to determine than other nutrients, especially as it is composed of a variety of different substances, the importance of the relative amounts of which is unknown.

The Importance of Nutrition in Toxicological Studies - The major importance of nutritional status as one of the many factors influencing the biological effects of foreign compounds is well-recognised even though the mechanisms by which dietary factors
influence the toxicology of a compound are not well-understood. Conventional experimental studies designed to evaluate the toxicity of a compound, such as drugs, food additives, and environmental chemicals, are generally conducted with animals maintained on a nutritionally balanced diet. Although data generated from these studies provides valuable information on the toxicity of the test compounds under such conditions, their relevance for man is limited because of the wide variation in nutritional status and dietary intakes of the human population.

Conclusions of the present study - The role of diet in causation of disease is now the subject of intensive scientific investigation. Much scientific research has been stimulated by the hypothesis of Burkitt (1971) that a lack of dietary fibre in the diet, especially bran, is central to the mode of causation of many of the diseases of affluence including colo-rectal cancer, atherosclerosis, diverticular disease, diabetes, obesity and ulcerative colitis. Despite vastly increasing our understanding of the composition, chemical, physical and physiological effects of dietary fibre, no conclusive biochemical evidence has been elucidated which substantiates this theory. The long-term health effects of increasing dietary fibre content are not fully established (Talbot, 1980).

It can be concluded from the present study that wheat bran does not induce components of the hepatic or intestinal mixed-functional oxidase enzyme system in the rat. This indicates that the postulated protective effect of bran against the diseases
of affluence is not mediated by altering the direction of metabolism or substrates of the mixed-function oxidase enzyme system which generally detoxifies foreign compounds but is also responsible for the production of toxic and carcinogenic metabolites. Similarly a high fat diet did not induce components of the hepatic mixed-function oxidase system. A lack of effect of wheat bran on bone mineral stores of calcium, magnesium and phosphorus was shown in the rat receiving adequate dietary supplies of these minerals. It can be concluded that the reported adverse effects of dietary fibre on mineral status were not substantiated in this experiment under conditions of adequate nutrition. Studies of the effect of wheat bran on gastro-intestinal mucus production were generally inconclusive. Conclusions from the further investigation of the drug-nutrient interaction between folic acid deficiency and anticonvulsant treatment resulting in eventual drug toxicity, suggest that reduced cytochrome P450 induction is not related to short-term folic acid deficiency but may be determined by prolonged deficiency of to some further complication resulting from long-term ingestion of these drugs.
REFERENCES
Endocrinol. Metab. 38, 1046-1051.


Corless, D., Beer, M., Boucher, B.J., Gupta, S.P. & Cohen, R.D.


Cummings, J.H., Hill, M.J., Jivraj, T., Houston, H., Branch, W.J.


Guarino, A.M., Gram, T.E., Gigon, P.L., Greene, F.E. & Gillette,

Hill, M.J. (1977) in Topics in Gastroenterology, 5. Ed Truelove,


91-103.


Parke, D.V. (1978a) in World Review of Nutrition &
Preece, M.A., O'riordan, J.L.H., Lawson, D.E.M. & Kodicek, E.
Proia, A.D., McNamara, D.J., Edwards, K.D.G. & Anderson, K.E.
Trowell, H.C., Southgate, D.A.T., Wolever, T.M.S., Leeds, A.R.,
Tsai, A.C., Ellis, J., Kelley, J.J., Lin, R.S.C. & Robson, J.R.K.
1175-1188.


Vahouny, G.V. (1980) in Nutrition and Food Science. Ed Santos,
W., Lopes, N., Barbosa, J.J., Chaves, D. & Valente, J.C., Plenum
Vahouny, G.V., Roy, T., Gallo, L.L., Story, J.A., Kritchevsky, D.,
Nutr. 31, S208-S211.
825-829.
829-835.
123-130.
Van Soest, P.J. & Robertson, J.B. (1976) in Marabou Symposium :
Food and Fibre. Naringsforskning Suppl. 14, 12-22.
Venitt, S. (1981) in Progression in Mutation Research. Ed Kappas,


653-666.

APPENDICES
Appendix I (1) to chapters 2 and 5

Percentage Composition of the Experimental Diets by Weight

<table>
<thead>
<tr>
<th>Component</th>
<th>Diet</th>
<th>Percentage by Weight of Each Component</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet</td>
<td>Fibre 4.8% Bran 9.6% Bran 8.5% Beef</td>
<td>17% White Bread</td>
<td>34% White Bread</td>
<td>17% Wholemeal Bread</td>
<td>34% Wholemeal Bread</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Control)</td>
<td>0.0% 0.0% 0.0% 0.0% 0.0% 0.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bread:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wholemeal</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td></td>
<td>71.2</td>
<td>67.0</td>
<td>62.8</td>
<td>62.7</td>
<td>57.0</td>
<td>42.0</td>
<td>57.0</td>
</tr>
<tr>
<td>Gluten</td>
<td></td>
<td>8.9</td>
<td>8.3</td>
<td>7.7</td>
<td>8.9</td>
<td>6.4</td>
<td>3.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Casein</td>
<td></td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Egg</td>
<td></td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Corn Oil</td>
<td></td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral mix</td>
<td></td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>L-Threonine</td>
<td></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Lysine-hydrochloride</td>
<td></td>
<td>0.3</td>
<td>0.29</td>
<td>0.29</td>
<td>0.29</td>
<td>0.29</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>Wheat Bran</td>
<td></td>
<td>-</td>
<td>4.8</td>
<td>9.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Beef Suet</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Composition of Diet 41B

- 49.67% digestible carbohydrate
- 12.73% digestible crude protein
- 2.42% digestible crude oil
- 4.78% crude fibre
Appendix I (ii): to chapters 2 and 5

Composition of the Experimental Diets by Analysis

<table>
<thead>
<tr>
<th>Diet</th>
<th>Moisture %</th>
<th>Ash %</th>
<th>Nitrogen %</th>
<th>Fat %</th>
<th>NDF %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibre Free</td>
<td>9.6±0.9</td>
<td>3.9±0.3</td>
<td>2.7±0.2</td>
<td>4.6±0.3</td>
<td>0.07±0.04</td>
</tr>
<tr>
<td>17% White Bread</td>
<td>10.0±0.7</td>
<td>4.2±0.3</td>
<td>2.7±0.1</td>
<td>4.6±0.4</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>34% White Bread</td>
<td>10.2±1.0</td>
<td>4.3±0.3</td>
<td>2.7±0.1</td>
<td>5.0±0.5</td>
<td>0.8±0.3</td>
</tr>
<tr>
<td>17% Wholemeal Bread</td>
<td>9.9±0.8</td>
<td>4.2±0.3</td>
<td>2.7±0.2</td>
<td>4.9±0.5</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>34% Wholemeal Bread</td>
<td>10.3±1.2</td>
<td>4.5±0.2</td>
<td>2.8±0.1</td>
<td>5.0±0.4</td>
<td>3.7±0.4</td>
</tr>
<tr>
<td>4.8% Bran</td>
<td>9.8±0.8</td>
<td>4.1±0.2</td>
<td>2.7±0.1</td>
<td>4.6±0.4</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>9.6% Bran</td>
<td>9.9±0.8</td>
<td>4.4±0.3</td>
<td>2.7±0.1</td>
<td>4.7±0.4</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>8.5% Beef Suet</td>
<td>8.8±0.7</td>
<td>3.8±0.2</td>
<td>2.6±0.1</td>
<td>11.4±1.6</td>
<td>0.15±0.14</td>
</tr>
<tr>
<td>41B Stock Diet</td>
<td>12.6±0.7</td>
<td>6.0±1.0</td>
<td>2.4±0.2</td>
<td>4.0±0.5</td>
<td>17.6±0.25</td>
</tr>
</tbody>
</table>

NDF - Neutral Detergent Fibre
Values are mean ± SD of 21 batches of diet.
Acknowledgements are due to the Flour Milling and Baking Research Association for Supplying this data.
Appendix II: to chapter 2

Bodyweight and Liver Weight Data

<table>
<thead>
<tr>
<th>DIET GROUP</th>
<th>4.8%</th>
<th>9.6%</th>
<th>8.5%</th>
<th>41B Stock diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding Period</td>
<td>Fibre-Free (Control)</td>
<td>Bran</td>
<td>Bran</td>
<td>Beefsuet</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>4 weeks - Mean ± SEM for four Male rats</th>
<th>7 weeks - Mean ± SEM for six Male rats</th>
<th>12 weeks - Mean ± SEM for five Male rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>69±4</td>
<td>70±3.3</td>
<td>70±4.6</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>240±4</td>
<td>231±5</td>
<td>236±4</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>171±3</td>
<td>161±4</td>
<td>166±5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.0±0.3</td>
<td>8.5±0.2</td>
<td>9.7±0.6</td>
</tr>
<tr>
<td>Relative liver weight (g/100g bodyweight)</td>
<td>3.75±0.1</td>
<td>3.68±0.1</td>
<td>4.11±0.3</td>
</tr>
</tbody>
</table>
Appendix II: to chapter 2 Contd ...

**Bodyweight and Liver Weight Data**

<table>
<thead>
<tr>
<th>DIET GROUP</th>
<th>Fibre-5% Free (Control)</th>
<th>5% Bran</th>
<th>5% Suet</th>
<th>41B Stock Diet</th>
</tr>
</thead>
</table>

### 12 weeks - Mean ± SEM for five female rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Fibre-5% Free</th>
<th>5% Bran</th>
<th>5% Suet</th>
<th>41B Stock Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight</td>
<td>44±0.4</td>
<td>44±0.4</td>
<td>44±0.4</td>
<td>44±0.4</td>
</tr>
<tr>
<td>Final body weight</td>
<td>248±8</td>
<td>252±5</td>
<td>262±15</td>
<td>276±8</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>204</td>
<td>208</td>
<td>218</td>
<td>232</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>7.6±0.6</td>
<td>7.7±0.5</td>
<td>7.6±0.5</td>
<td>8.8±0.6</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>3.1±</td>
<td>3.1±</td>
<td>2.9±</td>
<td>3.2±</td>
</tr>
</tbody>
</table>

### 18 months - Mean ± SEM for five male rats

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Fibre-5% Free</th>
<th>5% Bran</th>
<th>5% Suet</th>
<th>41B Stock Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight</td>
<td>49±0.4</td>
<td>49±0.4</td>
<td>49±0.4</td>
<td>49±0.4</td>
</tr>
<tr>
<td>Final body weight</td>
<td>652±36</td>
<td>682±43</td>
<td>767±47</td>
<td>659±34</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>603</td>
<td>633</td>
<td>718</td>
<td>610</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>20.5±0.5</td>
<td>20.5±1.5</td>
<td>21.0±1.8</td>
<td>21.3±0.6</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>3.17±0.13</td>
<td>3.05±0.28</td>
<td>2.74±0.18</td>
<td>3.15±0.17</td>
</tr>
</tbody>
</table>

* Mean and SEM for batches of rats on delivery to FMBRA (based on total deliveries of 750 of each sex in the batches used to supply the above experiments).
Appendix III: to chapter 6

Bodyweight and Liverweight Data

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Induction Treatment</th>
<th>Folate Supplemented</th>
<th>Folate Deficient</th>
<th>Folate Supplemented with Induction Treatment</th>
<th>Folate Deficient with Induction Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbitone 0.1% (w/v) in drinking water for 4 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Bodyweight (g)</td>
<td>429±7</td>
<td>424±25</td>
<td>473±13</td>
<td>406±13</td>
<td></td>
</tr>
<tr>
<td>Liverweight (g)</td>
<td>14.4±0.5</td>
<td>14.3±0.9*</td>
<td>22.0±1.1</td>
<td>18.2±0.9</td>
<td></td>
</tr>
<tr>
<td>Relative Liverweight (g/100g bodyweight)</td>
<td>3.35±0.1</td>
<td>3.36±0.4</td>
<td>4.63±0.1</td>
<td>4.48±0.2</td>
<td></td>
</tr>
<tr>
<td>Phenobarbitone 80 mg/kg for 14 days (I.P.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Bodyweight (g)</td>
<td>521±32 (2)</td>
<td></td>
<td>473±18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liverweight (g)</td>
<td>20.9±1.4 (2)</td>
<td></td>
<td>18.8±1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative Liverweight (g/100g bodyweight)</td>
<td>4.05±0.05 (2)</td>
<td></td>
<td>4.02±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Methylcholanthrene 25 mg/kg bodyweight for 7 days (I.P.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Bodyweight (g)</td>
<td>474±8</td>
<td>471±10</td>
<td>497±23</td>
<td>447±22</td>
<td></td>
</tr>
<tr>
<td>Liverweight (g)</td>
<td>12.9±0.3</td>
<td>13.2±0.4</td>
<td>18.8±1.2</td>
<td>16.7±0.7</td>
<td></td>
</tr>
<tr>
<td>Relative Liverweight (g/100g bodyweight)</td>
<td>2.74±0.06</td>
<td>2.81±0.10</td>
<td>3.77±0.09</td>
<td>3.74±0.10</td>
<td></td>
</tr>
</tbody>
</table>

Results are means and SEM of five male animals (unless otherwise indicated)

Value significantly different from Control: * p < 0.02
<table>
<thead>
<tr>
<th>Component</th>
<th>%w/w</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>11.0</td>
</tr>
<tr>
<td>Starch</td>
<td>14.1</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>27.3</td>
</tr>
<tr>
<td>hexoses</td>
<td>5.5</td>
</tr>
<tr>
<td>pentoses</td>
<td>20.9</td>
</tr>
<tr>
<td>uronic acids</td>
<td>0.85</td>
</tr>
<tr>
<td>Cellulose</td>
<td>9.45</td>
</tr>
<tr>
<td>hexoses</td>
<td>8.23</td>
</tr>
<tr>
<td>pentoses</td>
<td>0.41</td>
</tr>
<tr>
<td>uronic acids</td>
<td>0.82</td>
</tr>
<tr>
<td>Lignin</td>
<td>4.8</td>
</tr>
<tr>
<td>Total DF</td>
<td>41.6</td>
</tr>
</tbody>
</table>

Acknowledgements are due to FMBRA for supplying this data.