UNIVERSITY OF SOUTHAMPTON

THE HYPOCHOLESTEROLAEMIC ACTION OF WHEAT BRAN AND A MOULD *(FUSARIUM)*

BY

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A thesis submitted to the University of Southampton
for the degree of Doctor of Philosophy, Department
of Physiology and Biochemistry, September, 1977

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND BIOCHEMISTRY

Doctor of Philosophy

THE HYPOCHOLESTEROLAEMIC ACTION OF WHEAT BRAN AND A MOULD* (FUSARIUM)

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Rats fed diets supplemented with cholesterol and sodium cholate and containing wheat bran (100 g/kg) or a mould (400 g/kg) had significantly lower concentrations of cholesterol in liver and plasma compared to control animals receiving a diet containing cellulose (100 g/kg) as a source of fibre. No differences in plasma or liver cholesterol concentrations were observed when rats were fed on low-cholesterol diets containing wheat bran, the mould or cellulose. The faecal excretion of bile acids in rats receiving bran and mould diets was greater than in rats fed diets containing cellulose: neutral sterol (cholesterol + coprostanol) excretion was also increased in the mould group. The half-life of cholesterol, measured as the rate of excretion of tritium from hypercholesterolaemic rats injected intraperitoneally with [H^3]-cholesterol, was similar in groups fed the mould (10.3 days) and bran (13.0 days) and slowest in rats fed a diet containing cellulose (20.7 days).

Wheat bran and chemical fibre preparations derived from bran bound bile salts *in vitro*. The mould, cellulose and bran lignin had low capacities to bind bile salts. No hypocholesterolaemic activity was observed in rats fed chemical fibre preparations of wheat bran. Bran and mould absorbed and retained more water than cellulose *in vitro*.

*See Appendix

The mould produced hypocholesterolaemia in hamsters fed on diets low in cholesterol after 45 days. The cholesterol-lowering effect of the mould was attributed to a diethyl ether soluble fraction.

The hypocholesterolaemic action of the mould was probably caused by a decrease in absorption of endogenous and dietary cholesterol and a decreased absorption of bile acids. The binding of bile acids to wheat bran may be one of the factors responsible for increasing bile acid excretion in rats fed on diets containing bran, thereby reducing body cholesterol.

Acknowledgements

I am deeply indebted to Professor T. G. Taylor and Doctor M. R. Turner for their invaluable advice and careful supervision throughout my studentship. I am also grateful for the provision of funds and facilities offered to me by Professor K. A. Munday at the Department of Physiology and Biochemistry.

The cooperation and assistance of the staff in the Animal House, University of Southampton, is gratefully acknowledged as is the stimulating environment created by my postgraduate colleagues.

Finally, I thank my wife, Viviane, and my parents for their encouragement and Mrs. J. Churchyard for the typing of this manuscript.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Dietary fibre is the portion of plant material which remains resistant to intestinal secretions. Fibre is not an inert substance which passes through the gut unchanged but it is a group of compounds displaying physical and chemical properties which alter gastro-intestinal function. Low intakes of dietary fibre have been associated with many "diseases of Western civilisation" including Ischaemic Heart Disease. Populations with raised values of serum cholesterol have a higher incidence of IHD whereas communities which have lower serum cholesterol concentrations often have a lower incidence.

Experimental evidence from animal models suggests that dietary fibre can act as a protective factor against high concentrations of cholesterol. The modes of action of fibre are yet unknown although changes in bile acid excretion and cholesterol absorption have been implicated as possible mechanisms.

The present chapter will review the subject cholesterol and bile acid homeostasis, the role of diet and cholesterol in the pathogenesis of atherosclerosis and finally, the role of dietary fibre in cholesterol homeostasis.

The literature reivew will be presented in the following form:

1.1 STEROL METABOLISM

A Cholesterol homeostasis

B Bile acid metabolism

1.2 ATHEROSCLEROSIS, CHOLESTEROL AND BILE ACIDS

A. Development of Atherosclerosis

B. Factors altering cholesterol homeostasis

C. Species variations

1.3 DIETARY FIBRE

A. Chemistry

B. Consumption of dietary fibre

C. Biological effects of dietary fibre

1.1 STEROL METABOLISM

A. REGULATION OF CHOLESTEROL CONCENTRATION

Fluctuations in the dietary intake of cholesterol can cause corresponding changes in the concentration of plasma cholesterol. Homeostatic mechanisms are available whereby 'normal' limits of plasma concentrations can be adhered to even when high dietary cholesterol intakes are present. Four mechanisms exist whereby the concentration of plasma cholesterol is regulated: a) limited absorption from the intestine; b) decreased endogenous synthesis; c) removal of cholesterol from the plasma for tissue storage and finally d) an increased hepatic conversion of cholesterol to bile acids.

The body has no facilities for disrupting the steroid nucleus (Chaikoff, 1952).

a) Absorption of dietary (and endogenous) cholesterol

(i) Source of cholesterol

Three sources of cholesterol are available for absorption from the small intestine, and the quantity of cholesterol available from each source is variable within individuals. The first source is the intake of dietary cholesterol, estimated by Ho and Taylor (1971), to be approximately 1 g per day in the adult male American. A second source is derived from the desquamated cells of the intestinal mucosa: as much as 400 mg cholesterol per day has been estimated to originate from these cells (Cheng and Stanley, 1959). Thirdly, bile juices contain mixtures of cholesterol, lecithin, phospholipids and bile salts. The cholesterol from these secretions can be as much as 1 g per day in an adult male.

Three sources of cholesterol are present for absorption from the small intestine, and together they contribute approximately 2.4 g per day (Borgström, 1960).

(ii) Mechanism for absorption

The absorption of cholesterol from the upper small intestine is dependent on the cholesterol being in an un-esterified form. The free alcohol is formed by the action of pancreatic and intestinal cholesterol esterases in the presence of bile salts (Swell, Field and Treadwell, 1953), and once formed it is readily incorporated into micelles. The cholesterol is absorbed into the intestinal mucosa, at the proximal end of the small intestine, and the bile salts of the micelles are re-absorbed into the latter part of the small intestine (Boyd and Eastwood, 1968). The bile acids are returned to the liver via the hepatic-portal vein.

Free cholesterol is esterified in the mucosal cells, usually with linoleic acid, and the ester is transported in a lipoprotein complex via the lacteals and lymphatic system to the thoracic lymph duct. The chylomicrons pass into the systemic circulation approximately 12 hours after absorption.

(iii) Limiting of absorption

Human small intestine has a limited ability to absorb dietary cholesterol (Connor , Hodges and Bleiler, 1961), although it seems that the cholesterol derived from endogenous sources are more readily absorbed. This is presumably because the endogenous cholesterol is more readily incorporated into micelles formation (Borgström, 1960). Steady state isotopic studies of Wilson and Lindsey (1965) suggest that there is little variation in the proportion of neutral sterols in the faeces

derived from the endogenous cholesterol sources when either low or high cholesterol diets are fed.

The capacity for dietary cholesterol to be absorbed from the small intestinal lumen has been calculated by Kaplan et al (1963) to be independent of the amount of cholesterol in the diet over a minimum of 0.5 g/day. The maximum absorption was calculated to be 250 mg/day for an adult human male, or approximately 25% of the daily intake. Further support was given by Wilson and Lindsey's figure of 300 mg/day in volunteers given a dietary intake of 3 g/day.

Man has the ability, therefore, to regulate his plasma cholesterol concentration by maintaining an upper limit of exogenous cholesterol absorbed from the small intestine. The small intestines of rats and rabbits do not work so efficiently in regulating cholesterol absorption. Although the feeding of high cholesterol diets to rats and rabbits has markedly different effects, both species show a large capacity for absorption of exogenous cholesterol from their small intestine.

b) Excretion of cholesterol

The majority of body cholesterol is excreted in the form of bile acids or neutral sterols (Cook et al, 1956). The capacity for humans to increase either bile acid or neutral sterol output when cholesterol is introduced into the diet has been studied by Wilson and Lindsey (1965), who concluded that man is unable to compensate for an increased cholesterol absorption by increasing the bile acid or neutral sterol output. The introduction of further dietary changes will tend to alter sterol output as was demonstrated by Grundy and Ahrens (1966), who observed an increased excretion of bile acids and

neutral sterols in the faeces of patients fed diets higher in poly-unsaturated fatty acids than saturated fatty acids. A more extensive account of factors affecting cholesterol concentrations and faecal sterol elimination will be made later in this chapter.

The rat will absorb large quantities of cholesterol but only a small elevation in plasma cholesterol concentration can be measured. This is due to the efficient conversion of cholesterol to bile acids in the liver. Wilson (1964) noted that when rats were fed diets high in cholesterol,the bile acid output was five times higher than low cholesterol control rats. The neutral sterol output in the faeces showed little change.

The quoted values for bile acid excretion in rats varies not only with the methodology employed but also with the diet fed to the rats. Values of as little as 2.2 mg/day from a 100 g rat to 7.3 mg/day have been quoted (Van Belle, 1965). Portmann and Murphy (1958) found that animals fed semisynthetic diets had lower bile acid excretion values than rats fed a mixed cereal diet.

In man, the amount of cholesterol excreted is about 1 g/day and this is compensated for by an absorption and synthesis of 1 g of cholesterol per day. The total amount of bile acid excreted is approximately the same as the endogenous neutral sterols, although bile acid excretion can be elevated in a number of situations. The feeding of a hydrophilic colloid (Forman et al, 1968), unsaturated fatty acids (Avigan et al, 1962) and high residue diet (Portmann et al, 1958) have all been shown to increase bile acid excretion and often lower serum cholesterol concentrations.

c) Control of cholesterol synthesis

(i) Biosynthesis

Acetyl-CoA is used as the source of all the carbon atoms in the synthesis of cholesterol. There are several major stages in the synthesis of cholesterol, the first being the formation of mevalonate, a six carbon compound. Two pathways have been proposed for the formation of mevalonate; one being by HMG-CoA and the other via an HMG-S-enzyme complex (Brodie et al, 1964). Quantitatively, the HMG-CoA pathway is more important. The enzyme responsible for converting HMG-CoA to mevalonate, HMG-CoA reductase, is suppressed by excess cholesterol and a negative feedback control mechanism is therefore in operation (Siperstein, 1960; Siperstein and Guest, 1960).

An isoprenoid unit is formed from mevalonate with the loss of CO_2, and six of these units together form the steroid skeleton of lanasterol via squalene. Cholesterol is formed as the product of several further reactions from lanosterol.

Hotta and Chaikoff (1955) suggested that hepatic cholesterol biosynthesis was responsible for the majority of the endogenous cholesterol in the body. More recent evidence (Chevalier, 1967, Wilson and Reinke, 1968; Lindsey and Wilson, 1965) suggested that the role of liver in endogenous cholesterol production was also shared by the small intestine.

(ii) Small intestinal synthesis

Animals that have been fed radioactive cholesterol for several weeks, therefore causing a direct feedback inhibition of hepatic cholesterol synthesis, show a plateau in their plasma cholesterol specific activity which is below the specific activity of the dietary cholesterol (Frantz

and Schneider, 1954). This implied that there was significant endogenous cholesterol synthesis entering the exchangeable body pools which was not of hepatic origin. The small intestine was shown to be the major extrahepatic source of newly synthesised cholesterol (Wilson, 1968).

The contribution of the extrahepatic tissues has been shown to be 10-18% in rats (Morris, 1957), 9% in dogs (Taylor et al, 1960) and 20-40% in squirrel monkeys (Wilson and Dietschy, 1960) of the total plasma cholesterol. Human studies on Caucasians (Kaplan et al, 1963) showed only 25% of the plasma cholesterol to have been derived from dietary sources, and up to 75% could be from extrahepatic sources. Further studies by Ho and Taylor (1968) on the Masai tribe of Kenya found a 50% suppression of endogenous cholesterol synthesis and correspondingly, despite a high absorption of dietary cholesterol, these people have a low serum cholesterol and negligible incidence of atherosclerosis (Mann et al, 1964). It is evident that the quantitative importance of the control of hepatic synthesis is therefore species dependent. In most species, the control of hepatic and extrahepatic synthesis is different.

Dietschy and Siperstein (1965) observed a high rate of cholesterol synthesis in the ileum but a low rate in the jejunum and they concluded that the synthesis took place in the crypt cells of the intestine. Biliary diversion caused an increase in the rate of cholesterol synthesis throughout the small intestine, and when infusions of bile salts were made, this increase was diminished. It was suggested (Dietschy, 1967) that the concentration of conjugated bile salts in the small intestine might regulate the synthesis of

this extrahepatic cholesterol by acting as a non-competitive inhibitor at the HMG-CoA reductase site.

McIntyre and Isselbacher (1973) postulate that the synthesis of cholesterol within the gut is related to the absorption of cholesterol from the lumen, and the cholesterol itself may be an inhibitory factor. This hypothesis considers that the intestinal synthesis is not in fact linked to the exchangeable cholesterol pool but is geared to the provision of cholesterol for epithelial cell turnover. The precise method of control is still undecided, but whether bile salts or cholesterol itself are acting as inhibitory factors, the small intestine still has a significant role as a cholesterol synthesising organ.

(iii) Hepatic cholesterol synthesis

Rat liver shows marked diurnal variations in cholesterol synthesis, being greatest at midnight and at its lowest between 8 a.m. and noon, (Bach et al, 1969). The diurnal variations are probably caused by changes in the synthesis and degradation of microsomal HMG-CoA reductase. The levels of this rate-limiting enzyme, and consequently the diurnal rhythm can be altered by changing the time of feeding (Edwards et al, 1972).

The hepatic synthesis of cholesterol is depressed when rats are fed hypercholesterolaemic diets (Franz et al, 1954). This is due to the negative feedback mechanism at the HMG-CoA reductase step (Dietschy and Siperstein, 1967; Siperstein, 1963). Harry et al (1973) postulated that the decrease in cholesterol synthesis, brought about by cholesterol feeding, might be mediated by increased liver microsomal cholesterol ester fractions. The effect of cholesterol feeding can be overcome by biliary obstruction, whereby a corres-

ponding increase in synthesis occurs. This is not due to the inhibition, via the cholesterol ester, being lifted but is caused by an increased enzyme synthesis.

Two regulatory mechanisms therefore exist in the liver: firstly a negative feedback inhibition when dietary cholesterol is absorbed and resulting in temporary increases in body cholesterol, and secondly variations in HMG-CoA reductase synthesis, thus altering the levels of this rate-limiting enzyme.

d) Tissue storage of cholesterol

Excretion and degradation can contribute largely towards the rate of removal of cholesterol from the plasma, but the uptake and storage of cholesterol by different tissues is of major importance when dynamic aspects of cholesterol metabolism are studied. Two aspects of tissue cholesterol storage will be considered: firstly the rate of uptake of plasma cholesterol by the tissues and secondly the reversibility of the tissue cholesterol with plasma.

(i) Rate of equilibration

Intravenous injection of C^{14}-cholesterol into humans, and the subsequent measurement of tissue specific activity, suggests that the cholesterol of red blood corpuscles and liver are in rapid equilibrium after 3 days (Chobanian et al, 1962). Human fat deposits and aortic tissue do not reach equilibrium for about 100 days. Apart from the brain, most tissues in the body attain equilibrium with plasma cholesterol within 20 days, and once equilibrium has been reached, the specific activity of the plasma is similar to the specific activity of tissue cholesterol.

Avigan et al (1962) found that some rat tissues, notably the heart, lung, kidney and muscle, displayed a specific activity above plasma when injected with radioactive cholesterol. The liver and small intestine cholesterol had a specific activity similar to that of plasma.

Two categories of cholesterol turnover could be calculated for rats; a fast pool whereby an equilibrium with plasma cholesterol can result within a few days, and a slow pool where equilibrium only resulted after a number of weeks. Goodman and Noble (1968) suggested that the turnover of plasma cholesterol in humans could also be explained by a two-pool model.

(ii) Reversibility of tissue cholesterol stores

Elevation of plasma cholesterol concentration may be lowered by the transfer of cholesterol to other tissues and likewise, a steady concentration of plasma cholesterol can be maintained by the continuous exchange between tissue and plasma pools. Rabbits which were fed high cholesterol diets, displayed a reversible tissue storage effect. The plasms cholesterol concentration was elevated and a rapid uptake of cholesterol into the aorta, skin and liver was found. Placing the rabbits on low cholesterol diets resulted in a very slow rate of disappearance of skin and aortic cholesterol, and a rapid loss of liver cholesterol.

The rabbit responds to high dietary cholesterol by elevating its plasma cholesterol and the animal will tend to counteract these elevations by storing excess cholesterol in its tissues, including the aorta. The reversibility of these tissue pools is generally high, although the skin and aorta tend to retain much of their accumulated cholesterol. Fig.1.1 summarises the partial reversibility of tissue cholesterol storage in the rabbit.

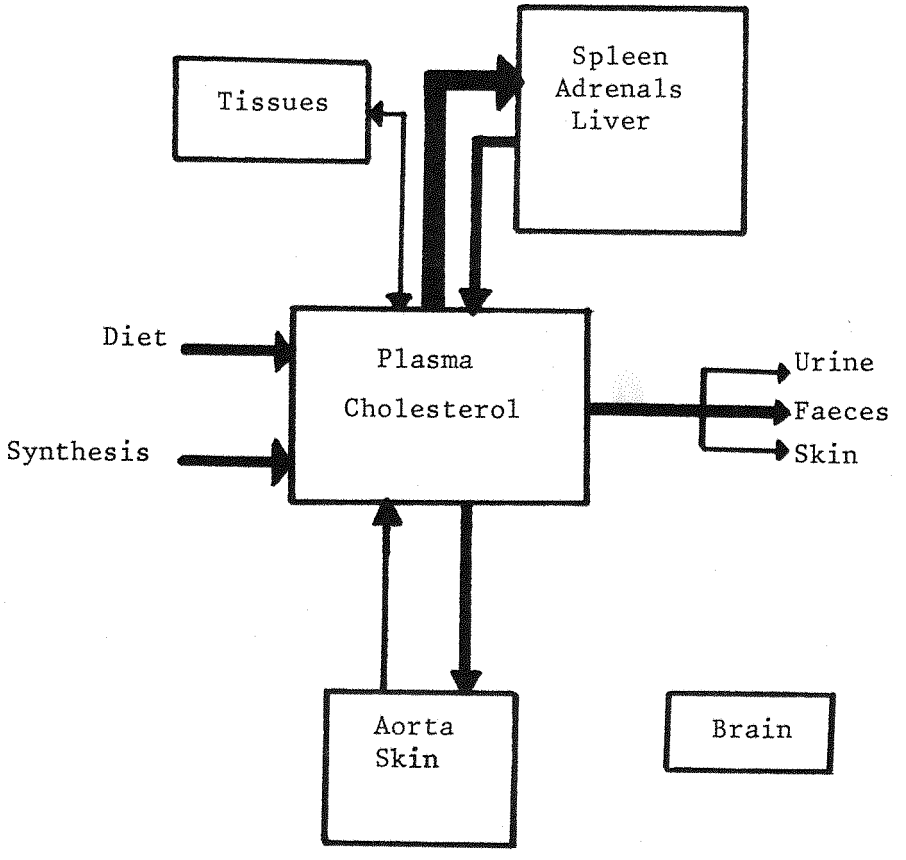


Fig. 1.1 - Partially reversible tissue cholesterol storage in the rabbit

Rats and dogs do not respond to cholesterol feeding in the same way as rabbits because they regulate their plasma cholesterol by increasing bile acid production and suppressing hepatic cholesterol synthesis. In man, the accumulation of cholesterol within tissues is known to occur, including the aorta, but the quantitative significance of this in the role of cholesterol homeostasis is not decided. The rate of removal of cholesterol from human aortic tissue is slow.

Summary

Four ways by which cholesterol may be regulated have been presented. Man has the ability to limit his intake of dietary cholesterol, but he does not have the ability to increase faecal bile acid or neutral sterol output when dietary cholesterol intake is increased. Man will depress body synthesis by only 25% when cholesterol concentrations are raised, and his ability to direct excessive cholesterol to reversible tissue pools may not be a significant factor in cholesterol homeostasis.

Control of cholesterol concentrations in rats is brought about by increasing the faecal excretion of sterols (mostly as bile acids). A 90% suppression of body cholesterol synthesis occurs when high cholesterol diets are introduced. The rat, therefore, has two efficient control mechanisms for regulating plasma cholesterol, and the feeding of high cholesterol diets will have little effect on plasma cholesterol.

The rabbit will depress its body cholesterol synthesis by only a slight amount (10-20%) and its main control of cholesterol concentration is by reversible tissue storage. Cholesterol feeding in

rabbits will show large increases in their plasma cholesterol concentrations and because they form fatty layers in their aortas, they are often used as an experimental model in atherosclerotic studies.

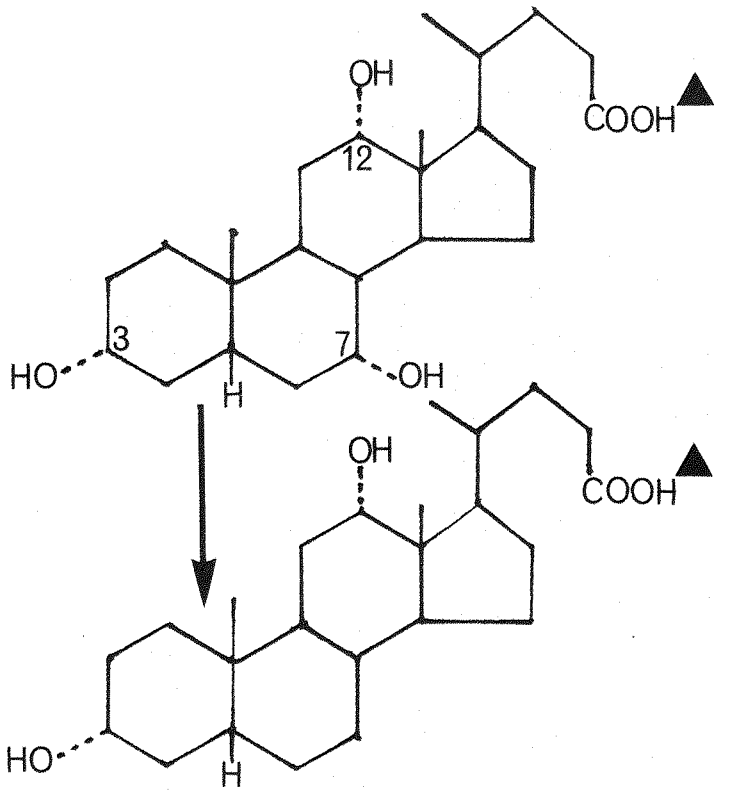
B. BILE ACID METABOLISM

Bile acids are formed in the liver as major products of cholesterol catabolism. They are conjugated with amino compounds (glycine or taurine) and stored in the gall bladder for release into the jejunum. Rats and horses do not possess a gall bladder and, as in the rabbit and guinea pig whose gall bladders are unable to concentrate bile, there is a continuous secretion of bile juices from the liver (Davenport, 1966). Animals with the ability to store bile juices release the bile mixture into the jejunum on stimulation via the hormone, cholecystokinin.

The function of bile salts is two fold in the small intestine: firstly they are required for the efficient lipolytic activities of pancreatic lipase and secondly they form micelles with hydrolysed triglycides and cholesterol therefore aiding their absorption from the proximal small intestine. Bile salts also play other roles which will be discussed in more detail; their synthesis, circulation and absorption from the intestines are to be considered.

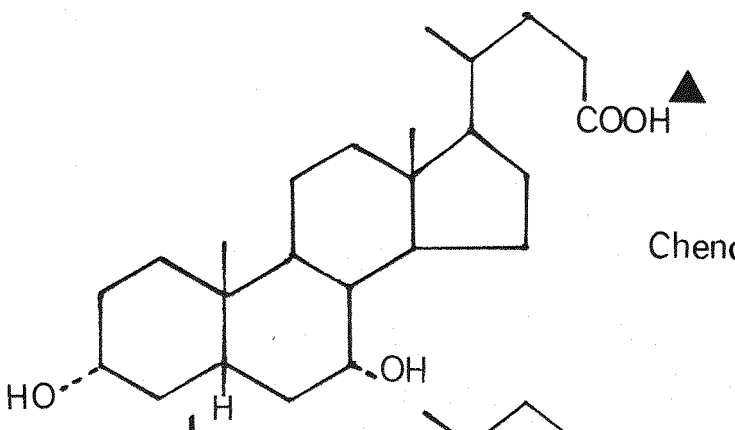
a) Hepatic synthesis of bile acids

The two main bile acids, formed from cholesterol, are cholic acid and chenodeoxycholic acid (Fig.1.2).These two bile acids are termed primary bile acids and are conjugated to glycine or taurine at their carboxyl end. In man, the two bile acids are present in similar amounts, with the glycine conjugate being three times more prevalent than the

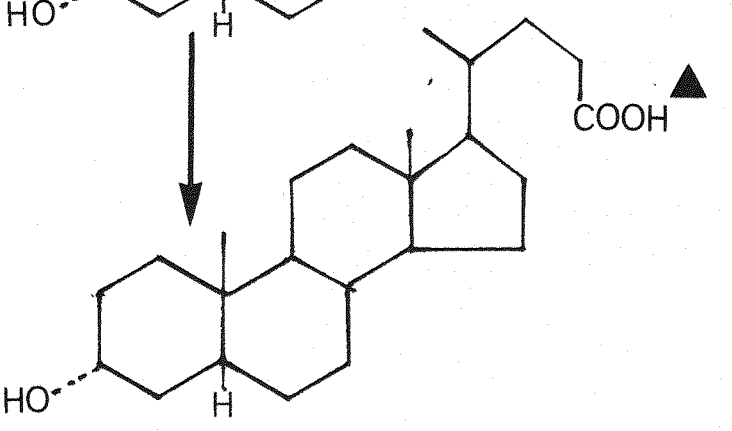


Cholic Acid

Deoxycholic Acid



Chenodeoxycholic Acid



Lithocholic Acid

▲ Position for conjugation.

Fig. 1.2 - Products of cholesterol catabolism

taurine derivative. The rat has more cholate than chenodeoxycholate, and the bile acids are almost invariably conjugated with taurine. The rabbit has all glyco-conjugates and because its liver does not have the ability to re-hydroxylate deoxycholate, a large proportion of its bile occurs as the deoxycholate salt. Table 1.1 summarises some of the species variations in bile acid metabolism.

Table 1.1 - Species variation of cholesterol and bile acid metabolism

	Human	Rabbit	Rat
Serum cholesterol (mg/l)	2200	500	800
Serum bile acids (mg/l)	10	12	0.8
Ratio cholesterol:bile acids	220:1	41:1	1000:1
Bile acids:			
Pool size (mg/kg)	50	250	95
Daily excretion (mg/kg)	5	26	30
Half-life time (days)	2.8	6.8	2.3
Bile composition:			
Tauro:glyco	1:3.2	1:100	100:1
Cholic:cheno:deoxy	1.1:1.0:0.6	5:1:95	8:2:0
7α-hydroxylation of deoxy	-	-	+++

(After Van Belle, 1965).

Danielson (1963) summarised the reaction sequences for the formation of cholic and chenodeoxycholic acids from cholesterol. Cholesterol initially undergoes a hydroxylation in the C-7 position, followed by the inversion of the 3-α hydroxy to the 3-β position. The saturation of the bond in the steroid nucleus between C-4 and C-5 is made, and a third hydroxyl in the C-12 position, is added to the steroid nucleus. The side chain is cleaved thereby leaving cholic acid.

Conjugation has been found to occur in microsomal preparations of mouse, rat and guinea pig liver. The specificity for the bile acid is not great and the formation of the conjugate involves a reaction using ATP and coenzyme-A.

Control of bile acid biosynthesis is concerted at the first microsomal enzyme in the pathway, 7-α hydroxylase (Danielsson and Sjövall, 1975). A 90% inhibition occurs when a rat's portal vein is infused with taurocholate (Shefer and Mosbach, 1967), although this inhibition only occurs with physiologically high concentrations of bile salt (33 mg per kg of rat per hour).

Bile acids not only inhibit their own synthesis by feedback inhibition, but they also suppress hepatic cholesterol biosynthesis at the enzyme HMG-CoA reductase. Furthermore, they are required for cholesterol hydrolysis in the small intestine, and for the efficient absorption into the intestinal mucosa therefore enabling them to limit the amount of cholesterol absorbed into the body. Conjugated bile salts are responsible for the inhibition of extrahepatic cholesterol synthesis (Dietschy, 1967) notably in the small intestine.

b) The entero-hepatic circulation of bile acids

The discharge of bile salts into the small intestine has been described previously. Fats, glycerides and cholesterol are absorbed in the upper parts of the small intestine whilst the remaining bile acids pass through the ileum to be absorbed at the proximal portion of the small intestine (Boyd and Eastwood, 1968). The absorbed bile acids are bound to albumin, and other plasma proteins, to be transported back to the liver via the hepatic portal vein. About one thousand times more bile acids are transported by this route than by the lymph vessels (Ho and Taylor, 1971). The proportion of bile acids in various organs has been summarised in Table 1.2

Table 1.2 - Distribution of bile acids in the entero-hepatic circulation

Total body pool (man)	2.8 g/person
Liver	1.66 g
Blood	0.002 g
Gall bladder	1.14 g
Gastro-intestinal tract	9.4 g/day*
Excreted	0.56 g/day

*Estimated for three circulations of entero-hepatic system

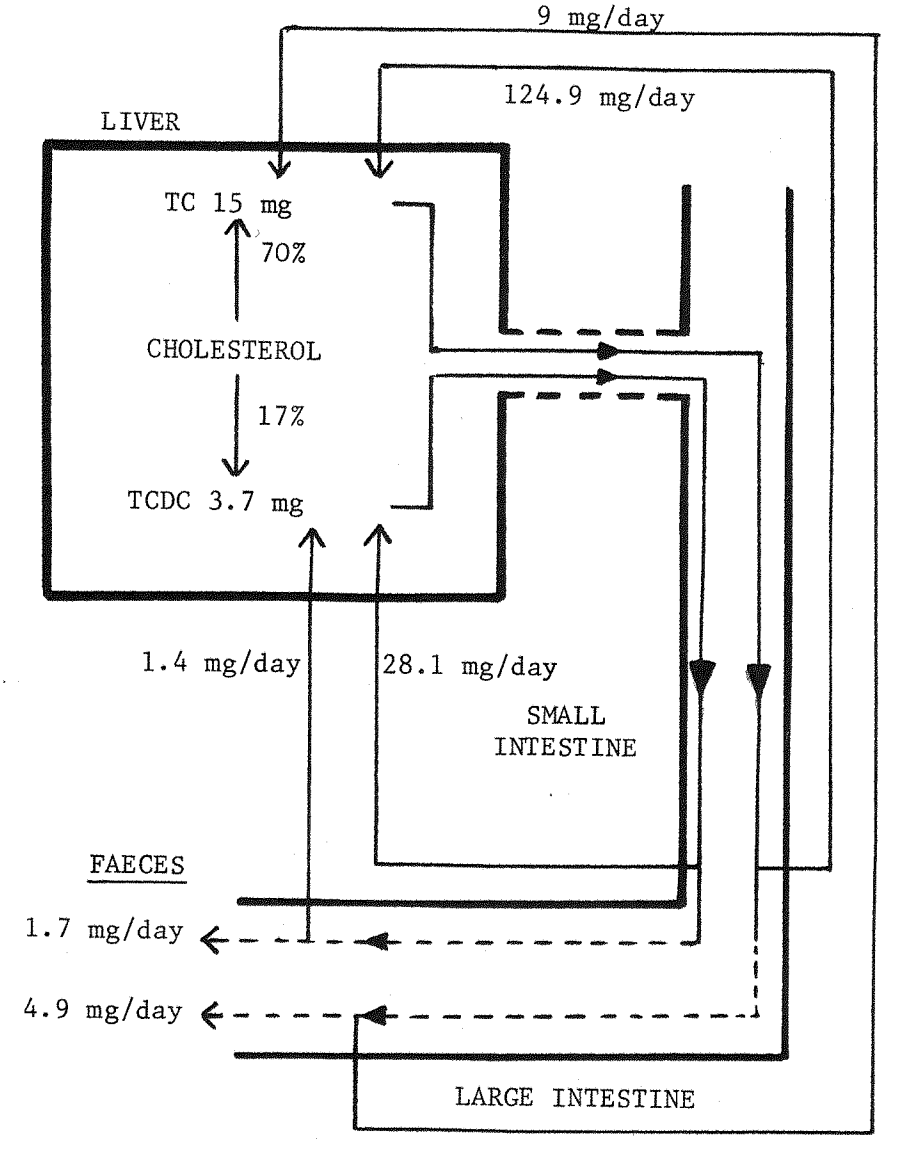
Norman and Sjövall (1958) showed that most of the bile acids (88%) of the rat were in the small and large intestine, and the faeces. Unlike humans, whose bile acids circulate three times per day, the rat's bile acids circulate between ten and twelve times

per day. Portman and Murphy (1958) calculated a cholic acid pool of 9.9 mg/100 mg live weight of rat when fed on Purina Chow. The calculated amount of bile acids, in the form of cholic acid, are summarised in Fig.1.3.The amount of chenodeoxycholic acid in this Figure are calculated on the assumption that chenodeoxycholate is absorbed to the same extent as cholate in the small intestine, and only 45% of chenodeoxycholate and 60% cholate are reabsorbed in the large intestine. The ratios of cholate:chenodeoxycholate often alter in response to dietary changes and therefore, as with most bile acid measurements, the figures quoted are determined by diet, animal age and weight, and methodology (Van Belle, 1965).

Rabbits form very little chenodeoxycholate because their cholesterol is catabolised mainly to cholic acid. The main bacterial product of cholic acid is the secondary bile acid, deoxycholic acid. The entero-hepatic circulation passes reabsorbed bile acids back to the liver and because of the excessive bacterial metabolism of bile acids by the rabbit's intestinal flora, up to 95% of rabbit bile salts are in the form of deoxycholate. Human bile contains up to 25% deoxycholate, but there is little deoxycholate in rat bile. Rat liver has the ability to hydroxylate the deoxycholate at the C-7 position, thereby producing cholic acid. Rabbits and humans cannot re-hydroxylate in the C-7 position. Further species differences were summarised in Table 1.1.

c) Intestinal absorption of bile acids

Dietschy (1968) has suggested four mechanisms for transporting bile acids across the intestinal wall:



TC = Taurocholate pool

TCDC = Taurochenodeoxycholate pool

Fig. 1.3 - Daily formation, circulation and excretion of bile acids in 200 g rat (Van Belle, 1965)

(i) Active transport

Lack and Weiner (1961) studied the transport of bile acids in the inverted gut sac preparation of Wilson and Wiseman (1954) and found an active uptake of the acids against a concentration gradient. The ileal sac preparation from the distal small intestine showed a far greater rate of uptake than sacs from the proximal small intestine which was consistent with the observations that the majority of bile acids are absorbed in the lower small intestine (Boyd and Eastwood, 1968). There seems to be only one carrier site for the transfer of bile acids from the mucosal to the serosal side of the gut. This site shows preference to conjugated bile acids with a negative charge, and no hydrolysis of C-24 peptide linkage occurs within the intestinal cells (Playhoust and Isselbacker, 1964).

(ii) Passive ionic transport

Two factors are in favour of a passive transport system: firstly, there is a greater concentration of bile acids in the intestinal lumen than the portal vein's blood, and secondly the mucosa is negatively charged in relation to the serosa thus providing an electrochemical gradient for the bile acids to pass from the lumen into the serosa (Dietschy, 1968). The pH of the small intestine causes most of the conjugated bile acids to be in ionic states, (pKa for glyco-conjugates is 4 and for tauro-conjugates pKa is 2). The negative charge of these bile acids allow them to pass slowly across the intestinal wall, down an electrochemical gradient, to the serosal side of the gut wall. This is true for all of the small intestine.

(iii) Passive micellar diffusion

As the bile acids move down the ileum they become more concentrated. The increased concentration will approach the critical micellar concentration (CMC)which is the point where polymers of bile acids occur to form micelles. Micellar bile acids will pass slowly across the small intestinal wall.

(iv) Passive non-ionic diffusion

The conjugated bile acids are usually in ionic form at the acidity of the intestine, but unconjugated acids, which are predominant in the colon, are in an unionised form (pKa for unconjugated bile acids is approximately 6). Significant amounts of unconjugated acids are absorbed from the gut in the unionised form by simple diffusion.

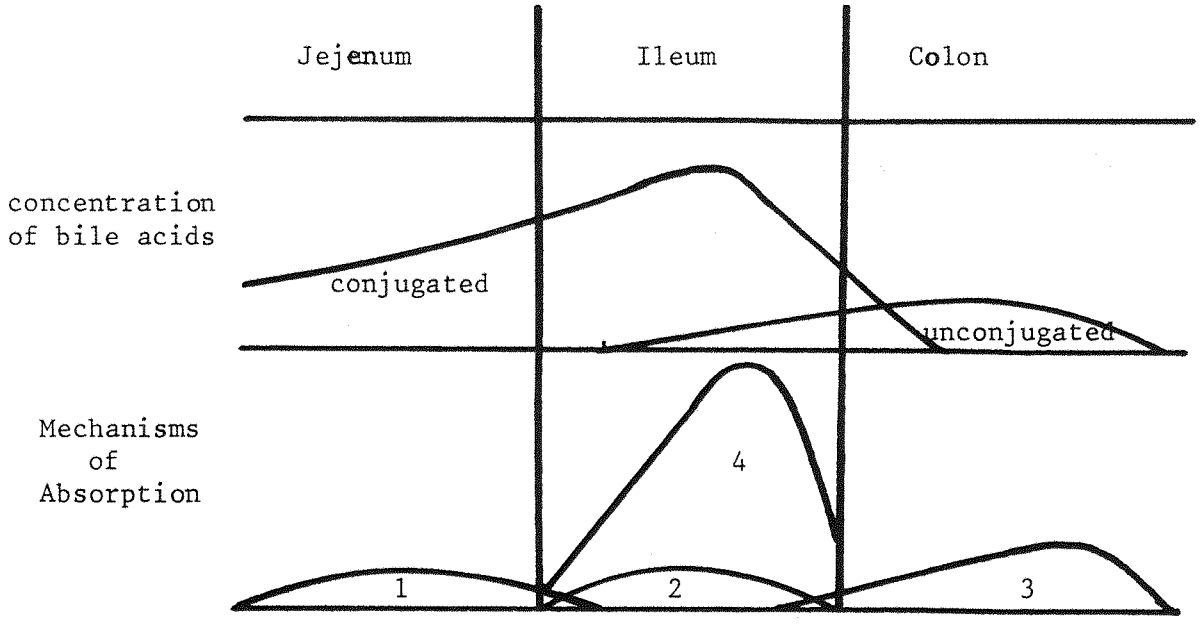
Figure 1.4 summarises the absorption of bile acids from the intestine and indicates the relative contribution of each of the four main mechanisms of absorption.

The inter-relationship between bile acids and cholesterol, and the ways in which cholesterol concentrations might be affected are portrayed in the diagram (Fig.1.5).

1.2 ATHEROSCLEROSIS, CHOLESTEROL AND BILE ACIDS

A. DEVELOPMENT OF ATHEROSCLEROSIS

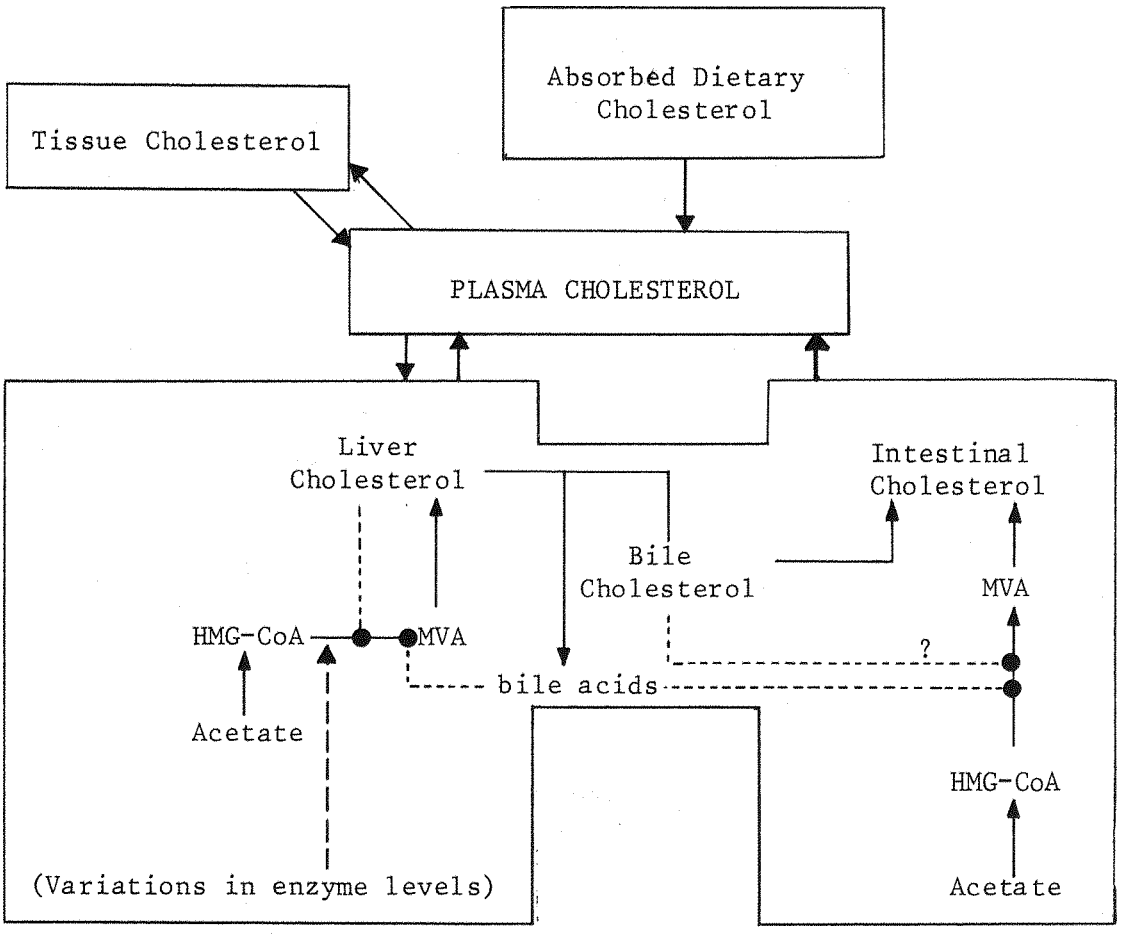
Atherosclerotic injury is evident by its disruption and disintegration of the innermost lamellae of the large arteries. The injured surfaces of the artery may become embedded by amorphous materials and coated with collagen surfaces, thereby rendering them less elastic (Constantinides, 1965). The first signs of atherosclerosis are mani-



1. Passive ionic diffusion
2. Passive micellar diffusion
3. Passive non-ionic diffusion
4. Active Transport

(From: Ho and Taylor in Treatment of the Hyperlipidaemic State. Ed. Casdorph (1975))

Fig 1.4 - Mechanisms for the absorption of bile acids



HMG-CoA β-hydroxy-β -methyl-glutaryl CoA

MVA Mevalonic acid

----● Inhibition of HMG-CoA reductase

Fig. 1.5 - Factors influencing Plasma Cholesterol Concentration

fested in the form of small scars along the aortic or major arterial wall. There is no great concentration of lipids in these early stages and this is correspondingly termed the pre-lipid stage. Sections which have been cut from an aorta after the pre-lipid stage show signs of small vacuoles of lipid within the wall. The number of lipid-containing cells becomes progressively greater under the small collagen scars until fatty streaks become visible to the naked eye. It has been suggested that the formation of these lesions takes place in the first decade of life (Adams, 1973).

The early lesions can develop into advanced lesions as more lipid is laid down in the cells of the intima and so much fat may be deposited that the old cells die off. The disintegration of these cells is accompanied by a release of their fat droplets, and the lesions will assume the consistency of a 'gruel' or soft paste. The major lipid in this 'gruel' is cholesterol, with phospholipid and triglyceride also present in minor quantities. The fatty gruel acts as a foreign body in the vascular system and consequently a reactive growth around the lesion engulfs the fatty outgrowth. The encapsulated cells become more fibrous with age and a fibrous cap or capsule results; the lesion is now referred to as a pearly or fibrous plaque. The lipid of the fibrous plaque is different to that found in the fatty streaks, being mainly cholesterol ester.

Analysis of the atheromatous fat shows that the predominant lipid is cholesterol, especially in its esterified form. Lecithin-cholesterol acyl transferase (LCAT) is present in the arterial wall, but a different esterifying enzyme the acyl-CoA dependent system, is thought to be responsible for cholesterol esterification

(Caro, 1973). Oleic acid is favoured as the fatty acid in the esterification, a fact of interest because it has been shown to be far more 'sclerogenic' than other fatty acid esters in tissue culture studies (Stein and Stein, 1973).

Atherosclerosis will gradually cause occlusion of the coronary arteries, aorta or other major arteries and in the case of the coronary arteries, the inability to supply the myocardium with blood will eventually occur, resulting in ischaemic heart disease (Davidson, Passmore, Brook and Truswell, 1975).

A number of theories have been postulated to explain the development of atherosclerosis. Two of the more favoured theories are presented:

(i) Filtration theory

Plasma proteins will diffuse through the intima wall of the arteries and the low density lipoproteins (LDL) which contain most of the cholesterol, can become trapped within the intima while the lipoproteins pass through. Glycosaminoglycans, a form of mucopolysaccharide found in plaques, have been suggested to be responsible for retarding the passage of LDL.

(ii) Thrombogenic theory

A minute lesion on the endothelial lining might result in platelets aggregating on this site. The aggregation will therefore cause a small thrombus to be formed, from which the thrombin can become embedded in the arterial wall. The atherosclerotic lipid originates from the thrombi. Fibrolysis will normally remove the fibrin formed in the aggregation but a decreased fibrinolysis will result in an increased tendency for atheroma to form.

B. FACTORS ALTERING PLASMA CHOLESTEROL CONCENTRATION AND BILE ACID EXCRETION

The relationship between IHD and diet is now widely accepted and has been subject to several reviews (Keys, 1970; Fidanza, 1972; Shaper, 1972 and Reisser, 1973). Certain risk factors have been identified which increase the risk of IHD in a cumulative manner, i.e. the more risk factors, the greater the danger of IHD occurring.

Three types of risk factors are evident: those which are themselves not classed as abnormalities such as male sex and increasing age; secondly, there are some factors which are associated with conditions such as diabetes mellitus, obesity, hypertension and hyperlipidaemia. A third group of risk factors are environmental in origin, of which smoking, stress, lack of exercise and dietary habits are examples (Brusis and McGandy, 1971).

Plasma cholesterol concentrations are lower in populations with low incidence of IHD whereas they are elevated in people with higher risk of suffering from IHD (Keys, 1970). It is generally accepted that a low plasma cholesterol is desirable and some of the factors which affect cholesterol concentrations are discussed below:

a) Dietary factors

(i) Fats

Polyunsaturated fatty acids possess hypocholesterolaemic properties, and their mode of action could be manifested in three ways: 1) Moore (1968) found humans fed high levels of polyunsaturated fats in their diets increase their excretion of bile acid in the faeces. 2) Polyunsaturated fatty acids hinder the absorption of cholesterol and may

also cause changes in the gut flora (see later). 3) An initial drop in plasma cholesterol, which occurs when high levels of poly-unsaturated fat are eaten, has been explained by the mobilisation of cholesterol from one tissue compartment to another (Sprintz et al, 1965). Unsaturated fatty acid cholesterol esters are thought to be metabolised more rapidly than the saturated esters.

The effect of saturated fats will increase the concentration of plasma cholesterol. The chain length of saturated fatty acids is important in this hypercholesterolaemic action. Palmitic (C-16) and myristic (C-14) acids have pronounced effects on plasma cholesterol concentration whilst C-18:0 and C-12:0 seem to be the upper and lower limits of the acyl chain length which will cause any increment. A relationship whereby the hypercholesterolaemic action of saturated fatty acids is double the hypocholesterolaemic activity of unsaturated fatty acids has been postulated (Keys et al, 1958).

(ii) Dietary sterols

Dietary variations in cholesterol are often accompanied by corresponding changes in saturated fatty acid intake: those foods rich in cholesterol also have high saturated fat content. The amount of dietary cholesterol will effect plasma cholesterol concentrations and Keys (1965) postulated a formula to summarise the change in plasma cholesterol when fat and cholesterol are varied in the human diet (Fig.1.6). Mattson et al (1972) showed that for an increase in dietary cholesterol of 100 mg/1000 Kcal of diet, an increase of 12 mg/100 ml plasma occurred, and the relationship between dietary and plasma cholesterol showed a linear correlation.

$$\Delta chol = 1.2 \ (2\Delta S^{1} - \Delta P) + 1.5 \ \Delta Z$$

where: $\Delta chol$ is the predicted change in the concentration of serum cholesterol in man (mg/100 ml)

S^1 is the percentage of total calories provided by glycerol esters of saturated fatty acids, excluding stearic acid

P is the percentage of total calories provided by glycerol esters of unsaturated fatty acids

Z^2 is mg dietary cholesterol per 1000 Cal

Fig. 1.6 - Relationship between dietary fat intake and serum cholesterol in man

Diets rich in polyunsaturated fatty acids often contain significant amounts of phytosterols. A common plant sterol, β-sitosterol, will lower plasma cholesterol in man when administered orally in large doses (12 g/day). This depression of circulating cholesterol is only maintained whilst the phytosterol is being administered (Farquhar, 1958). The site of action of β-sitosterol is thought to be in the mucosal cells of the intestine where the cholesterol esterification step is inhibited and cholesterol absorption is therefore depressed.

(iii) Vitamins

Pharmacological doses of nicotinic acid (3 g/day) have the ability to reduce serum cholesterol in certain hyperlipidaemias (notably Fredrickson Type II and Type IV where LDL values are increased). The mode of action is probably manifested in a decrease of cholesterol synthesis and increase in oxidation of cholesterol (Kritchevsky and Tepper, 1964).

Ascorbic acid, administered orally, has been shown to have varying effects in human subjects. The majority of workers claim no effect in either hypercholesterolaemic (Samuel and Shalchi, 1964 and Peterson et al, 1975) or normocholesterolaemic patients (Anderson et al, 1972). A hypocholesterolaemic action in guinea pigs has been reported and there was a corresponding increased catabolism of cholesterol to bile acids in the animals with ascorbate incorporated into their diets (Ginter, 1973).

(iv) Dietary fibre

The indigestible portion of the diet has been shown to have significant effects on plasma and liver cholesterol concentrations

et al, 1974; De Groot, 1963; Vijayagopolan, 1973a; Moore, 1967 and Owen et al,1975a). Sterol excretion, (Vijayagopolan and Kurup, 1973b; Owen et al,1975c and Morgan et al, 1974) and gastro-intestinal function (Kirwan et al, 1974; Burkitt, 1971 and 1973; Bing, 1974, Heaton, 1973; and Painter and Burkitt, 1971). A detailed account of the importance of maintaining high dietary fibre intakes is presented later in this chapter.

(v) Hydrophilic colloids

Certain colloid forming materials, one of which is derived from psyllium seeds, will lower serum cholesterol in humans (Garvin et al, 1965). An increase in bile acid excreted, together with an increased water content and lowered transit times have been implicated (Forman et al, 1968).

(vi) Minerals

Soft water has been suggested as being a risk factor in IHD (Crawford, 1972). Intakes of other minerals have been shown to have a good correlation with IHD mortality, notably magnesium intake (Seeling and Heggtell, 1974) and high zinc:copper ratios (Klevay, 1973; and 1975). Klevay has suggested that one of the mechanisms of action of dietary fibre was to lower zinc availability and absorption from the diet (1974).

(vii) Protein

Anderson et al (1971) found that the quantity of protein eaten had no effect on plasma cholesterol concentrations in short-term human studies, provided the minimum protein requirements were met. Magee and Fragola (1959) found protein source had an effect on cholesterol values: rat plasma cholesterol concentrations were lowered by feeding casein based diets compared to most other protein sources.

b) Cholestyramine

Cholestyramine is a quaternary ammonium anion exchange resin with a polystyrene skeleton. In the intestinal lumen chloride ions are exchanged for bile acids on the resin's surface, therefore forming an insoluble and unabsorbable complex. The majority of bile acids secreted into the intestine are reabsorbed (Bergström, 1961), but entero-hepatic cycle is partially disrupted when cholestyramine is administered and an increased excretion of bile acids via the faeces occurs.

Sevenfold increases in the hepatic conversion of cholesterol to bile acids has been reported (Beher et al, 1966) and because the controlling factors of cholesterol biosynthesis have been removed there is a corresponding increase in the cholesterol synthesis of both the intestine and liver. The rate of cholesterol synthesis seems to be less than the rate at which hepatic cholesterol is oxidised to bile acids and a net lowering of hepatic and plasma cholesterol results (Questran booklet, Bristol Laboratories, 1972).

The affinity of cholestyramine for bile acids has been demonstrated in vitro using sodium cholate and in vivo with rats (Whiteside et al, 1966). Increased bile acid excretion was observed by Huff et al (1963) in rats although no corresponding decrease in plasma cholesterol was noticeable. The cholesterol concentrations of chicken, dog and man are lowered by the administration of cholestyramine (Tennent et al, 1959). Mice and pigs, as with rats, have the ability to compensate for the increased excretion of bile acids and have no lowering of plasma cholesterol when administered cholestyramine.

The removal of micellar bile acids in the intestinal lumen leads

to a less efficient absorption of cholesterol. Studies with rats (Huan et al, 1963) showed a reduced in vivo uptake of C^{14}-cholesterol by rats administered cholestyramine. A secondary effect of the ion exchange resin is therefore to decrease the amount of sterol absorbed from the small intestine.

c) Gastro-intestinal flora

Bile acids and unabsorbed cholesterol passing into the large intestine are extensively metabolised by bacteria. The primary bile acids, cholate and chenodeoxycholate, are deconjugated and metabolised to their respective secondary bile acids, deoxycholate and lithocholate (Fig. 3). Cholesterol is metabolised to coprostanol.

Changes in the serum cholesterol concentration of man, together with changes in the pool size and turnover rate of cholesterol, induced by different carbohydrate sources has been related to changes in the intestinal microflora (Kitchevsky et al, 1959; Portman, 1960). Danielson and Gustafsson (1959) showed an elevation of serum cholesterol in rats when fed antibiotics in their diets. This elevation was accompanied by an increased rate of turnover of bile acids. Germ-free rats have higher serum cholesterol, lower faecal bile acid output and three times the half-life of cholic acid compared to conventional rats.

The type of diet has a pronounced effect on cholic acid turnover and gut flora (Portman and Murphy, 1958). Rats fed Purina Chow had a biological half-life for cholic acid of 2.0 days, whereas a standard, low residue diet using sucrose as a source of carbohydrate gave a half-life of 4.2 days. Corresponding increases in excretion of cholate were noted in the Purina Chow fed rats.

Studies by Hill and Aries (1972) suggest that Western diets will favour the increased degradation of bile acids by the gut bacteria to form possible carcinogenic compounds. A high residue diet will encourage a more rapid transit time, reduce the amount of intestinal metabolism of the sterols and thus reduce the chance of carcinogen production (Burkitt, 1971). Deoxycholic acid, itself a product of intestinal bacterial action on a primary bile acid, is weakly carcinogenic and might be important considering the amount of deoxycholate the bowel is exposed to over a number of years. Clostridium paraputrificum may be of importance since it has been shown to modify bile acids towards more carcinogenic compounds (Finegold et al, 1974).

Finally, the administration of antibiotics which will modify the gut flora has the effect of decreasing cholic acid turnover and increasing serum cholesterol in rats (Lindstedt and Norman, 1956). In man, however, the oral administration of antibiotics such as neomycin will decrease the serum cholesterol; this is probably due to the disruption of intestinal absorption overriding the effect of changed gut flora (Powell et al, 1962).

d) Species variations

Experimentally induced atheroma has been studied widely in animals. Spontaneous atheromatous lesions have been noticed in a number of animals such as pigs, dogs and cats, and both the rabbit and chicken are also susceptible to fatty deposition in the aorta when hyperlipidaemia is induced. The lesions produced in rabbits and man have a similar chemical composition, but only when rabbits are subjected to an antibody immune response, coupled with hyperlipidaemia could plaques be produced similar in structure to those found in humans

(Hardin et al, 1973). Hyperlipidaemia can readily be produced in rabbits and chickens, and it is for this reason that they are often used as experimental animals. Rabbits do not possess the ability to regulate exogenous cholesterol absorption or increase sterol excretion and they will only partially stop endogenous synthesis by the liver when a high concentration of cholesterol is present.

The rat is not a very suitable animal to study atheromatous formation because it resists the formation of fatty deposits within its aorta and its cholesterol levels are difficult to elevate. Increased plasma and hepatic cholesterol is efficiently metabolised to bile acids, and these bile acids are excreted in the faeces. The addition of bile salts to the diet will only partially inhibit cholesterol synthesis, but will inhibit cholesterol catabolism. Cholic acid raises liver cholesterol, but has no effect on serum cholesterol, and if cholesterol and cholic acid are added together, a large increase in both hepatic and plasma cholesterol concentrations is observed. Despite a raising of blood and liver cholesterol, atheroma are not found until such severe conditions as hypothyroidism (by the addition of uracil) or renal hypertension (by viosterol addition, or a very high protein diet) are also encountered. The effect of additions of different bile salts alone on cholesterol metabolism is shown in Table 1.3.

Hamsters are very resistant to formation of atheroma (Bernick et al, 1962) and despite the additions of cholesterol and sodium cholate to their diets no signs of a fatty deposition in the aortas are visible.Altschul(1950) however, claimed to have found traces of atheroma in hamsters fed high cholesterol diets. The feeding of cholic

Table 1.3 - Effects of dietary bile acids on plasma and liver cholseterol and the biosynthesis of cholesterol

(Adapted from Van Belle, 1965)

Species	Bile acid	Cholesterol		
		Serum	Liver	Biosynthesis
Rat	Basal	1 (= 870 mg/l)	1 (=2.6 mg/g)	1.0
	+ cholate	1.0	1.2	0.4
	+ hyodeoxycholate	1.2	1.1	1.3
	+ lithocholate	1.6	1.1	1.0
	+ chenodeoxycholate	1.2	1.1	0.5
Mouse	Basal	1 (=1070 mg/l)	1 (=3.1 mg/g)	1.0
	+ cholate	1.3	1.9	0.03
	+ hyodeoxycholate	0.8	0.9	1.6
	+ lithocholate	1.0	1.0	1.4
	+ chenodeoxycholate	1.0	0.8	0.15
Hamster	Basal	1 (=1350 mg/l)	1 (=2.4 mg/g)	1.0
	+ cholate	1.7	1.4	0.06
	+ hyodeoxycholate	1.2	1.0	1.7
	+ lithocholate	1.9	1.5	0.9
	+ chenodeoxycholate	1.6	1.3	0.7

acid supplements alone to hamsters will raise plasma cholesterol by 70% and hepatic cholesterol will increase by 40%; cholesterol biosynthesis was, however, almost completely suppressed (Beher et al, 1963). The hamster thus seems more sensitive to cholate feeding than the rat (Table 1.3).

The homeostatic mechanisms for cholesterol regulation have been summarised in Table 1.4 for man, rabbit and rat.

1.3 DIETARY FIBRE

The non-nutritive portions of food have often been considered an insignificant part of our diet. Much epidemiological evidence exists relating low dietary fibre intakes to many common diseases such as diverticulosis, colonic cancer, appendicitis, gall bladder diseases and IHD. The fibrous part of our diet possesses chemical and physical properties which are required for the normal functioning of the gastrointestinal tract (Cummings, 1973; Eastwood, 1972).

A. CHEMICAL BASIS OF DIETARY FIBRE

a) Quantitative estimations

The majority of polysaccharides of the plant cell wall are not available as an energy source to non-ruminants: these animals lack the enzymes which are necessary for the hydrolysis of the β-glycoside linkages in the large structural polysaccharides.

Quantitative estimation of the indigestible residue is difficult because of analytical problems involved and also the ambiguity of the term 'fibre'.

"Crude fibre" is often used as an indication of the least

Table 1.4 - Mechanisms of cholesterol homeostasis in rat, rabbit and man

Mechanism	Rat	Rabbit	Man
Limitation of absorption	-	-	++
Suppression of synthesis	++	±	±
Increase of excretion	++	-	-
Reversible tissue storage	-	+++	?

+ ++ +++	Degrees of efficiency of mechanism
-	No existence
±	Not significant
?	Not determined

digestible fraction of food based on the assumption that the residue remaining after sequential acid and alkali extraction represents the indigestible matter. This assumption is incorrect because it is found that about 50 - 90% of the pectin, and 80% of the hemicelluloses and pentosans are lost in the crude fibre preparation (Van Soest, 1973).

The so-called 'Weende' method for crude fibre estimation is still the basis for many analyses despite its inaccuracies (A.O.A.C., 1970). Other techniques have been developed, including enzyme methods (Weinstock and Benham, 1951) and the extensive fractionation procedure developed by Southgate (1969). The merits of some of the more commonly used preparations have been discussed elsewhere (McConnell and Eastwood, 1974).

A method of 'fibre' estimation which has been recently favoured relies on the use of detergents in place of the detrimental alkali-boiling stage (Van Soest, 1963; Griffith and Jones, 1963). Van Soest compared anionic, cationic and nonionic detergents, buffered at various pH values, in their ability to yield fibrous residues with low nitrogen content. Cetyltrimethylammonium bromide (CTAB) in strong acid and sodium lauryl sulphate in neutral solution were found to be most effective. The methods yielded acid detergent fibre (ADF), a lignocellulose residue, and neutral detergent fibre (NDF) a residue of the plant cell wall less its pectin. A lignocellulose residue remaining from the use of Teepol in acid solution has been termed normal acid-detergent fibre (Griffith and Jones, 1963) and lower nitrogen values could be attained by pre-incubating the substrate in a pepsin solution (Griffith and Jones, 1966).

b) Chemical constituents

'Crude fibre' gives an indication of the amount of cellulose and lignin present in food and forages. McCance and Lawrence (1929) described the non-hydrolysed portion of man's food as 'unavailable carbohydrate'. The term unavailable carbohydrate covers a variety of compounds shown in Table 1.5. Lignin is not a polysaccharide but has an amorphous structure based on substituted phenylpropane units. The term 'dietary fibre' was therefore adopted in order to include the non-polysaccharide portion of food (Trowell, 1972). Dietary fibre avoids the incorrect usage of 'carbohydrate' and distinguishes itself from crude fibre by giving a more accurate value of the indigestible part of the plant cell wall when applied to non-ruminants.

The plant cell wall consists of a primary wall of cellulose and pectin with a secondary wall of microfibrils running through the structureless matrix (Eastwood, 1973). The secondary cell wall develops later in growth and has cellulose microfibrils interspaced with lignin, pentosans and hemicelluloses to provide strength within the wall.

Lignin is found closely associated with hemicelluloses. The lignin of the plant cell renders plasticity to its wall, and it is also responsible for providing rigidity and strength to the plant. The composition of the cell wall has great bearing not only on its physical properties but also on its biological degradation. Highly lignified diets are less digestible by ruminants than diets with a low lignin content (Van Soest, 1973). The encrusting effect, whereby the digestible contents of the plant are encapsulated by an impervious coating, may explain why there is a loss of protein in the faeces of animals fed highly fibrous diets (McQueen and Van Soest, 1973).

Table 1.5 - Components of dietary fibre

Cellulose
Lignin
Pectin
Hemicellulose
Gums
Mucilages

A good correlation exists between the digestibility of the cell wall, in ruminants, and the lignin:cellulose ratio. Caution must be exercised when correlating the values of 'fibre content' of foods with their digestibility. McCance and Walsham (1948) found that about 20% of wholemeal wheat fibre was digested by the intestinal flora in humans. Southgate and Durnin (1970) using more varied diets, found that 13-15% of cellulose ingested was absent from the faeces, whereas up to 95% of the pentosans were lost.

The products of cellulose degradation in monogastric animals is probably volatile organic acids. Under anaerobic conditions and depending on pH and temperature, 80 - 90% of the carbon in cellulose can be fermented *in vitro* to acetate, butyrate, lactate and ethanol (Hall, 1965). Tang *et al* (1968) found that rats fed a cereal diet supplemented with up to 10% cellulose would produce increased quantities of volatile fatty acids (mainly butyrate) in the caecum. Cellulose had digestibilities ranging from 31 - 43% in rats with intact caeca whilst in caecectomised rats, the digestibility fell to about 20%. The volatile fatty acids could make a significant contribution to the energy intake of the rat when they are absorbed from the large intestine (Elsden *et al*, 1946) or when the animal practices coprophagy to permit the acids to be absorbed from the upper intestinal tract.

Cellulose has been shown to lack any hypocholesteraemic activity (Kiriyama *et al*, 1969) in fact it may slightly increase plasma and liver concentrations in cholesterol-fed rats (Ershoff and Wells, 1962). Purified wood cellulose (Solka floc) was used as a control indigestible fibre source throughout all the experiments in the subsequent chapters.

The structure of lignin has not been convincingly elucidated, mainly because its preparation and isolation involves strong oxidising conditions. Lichens, mosses and fungi are devoid of lignin, but lignification occurs in ferns and other higher members of the plant kingdom. The basic monomer for the lignin molecule is the substituted phenylpropane unit. Three classes of lignin exist in the plant kingdom: that of the Gymnosperms (softwoods); the Dicotyledonous angiosperms (hardwoods); and finally, the Monocotyledonous angiosperms (grasses and annual plants). It is the last group which provides the most interest to the nutritionist and the basic monomeric unit is 4-hydroxyphenylpropane (Pearl, 1967).

An established view of cellulose is that it is a crystalline chain of glucose, linked by β,1-4 linkages. Most of the cellulose in the parenchymous cells of vegetables are in a hydrated gel form. Isolation of pure cellulose from such cells is difficult due to the closely related pectins and the encrusting materials of hemicelluloses and lignin (Eastwood, 1973).

The hemicelluloses are a heterogenous mixture of linear and highly branched polysaccharides often bound covalently to lignin Pearl (1967). They are complex polymers of xylose, arabinose, glucuronic acid, galactose, mannose and uronic acid, thought to yield plasticity to the plant cell wall.

Pectins are found in the primary cell wall and in the intercellular layers of plants. In growing cells this polyuronic acid polymer is branched, but with age it assumes a more structural role and becomes less branched with a gel-like consistency. Pectic substances are hydrophilic and pectin will change from an insoluble material to a more water soluble substances as the plant or fruit ages (Worth, 1967).

c) Composition of wheat bran

The bulk of the indigestible matter in a wheat grain is derived from the outer coats: the endosperm is almost entirely digestible and provides only 4% of the total crude fibre of the grain. A diagrammatic longitudinal section of a wheat grain is portrayed in Fig. 1.7.

Bran constitutes 14% of the weight of the grain and provides 93% of the total crude fibre. The germ is removed with the bran when the grain is milled and the remaining 3% of the grain's crude fibre is derived from this germ. Wheat has an overall crude fibre value of only 2% whilst the outer layer of the grain, the bran layers, has an acid detergent fibre content of 14.8% of the dried weight (Canadian Red Spring Wheat) (McConnell and Eastwood, 1974). The composition of commercial wheat bran is shown in Table 1.6 the commercial bran usually contains about 16% of the endosperm.

The relatively high amounts of hemicelluloses found in bran has been reported to contain 64% xylose, 32.7% arabinose and 3.2% uronic acids. The consequences of these hemicelluloses will be discussed later.

Bran is not simply a source of dietary fibre. The aleurone layer, which is almost half the weight of bran, contains 73% of the pyridoxine, 42% of the riboflavin, 33% of the thiamine and 50% of the pantothenic acid in the wheat grain (Fisher, 1974).

The presence of phytic acid in bran has been reported to possibly hinder the intestinal absorption of calcium, zinc and iron. There may be a connection between the high incidence of rickets and osteomyelitis in immigrant communities of Britain and their high intakes of phytic acid in the diet (Ford et al, 1972).

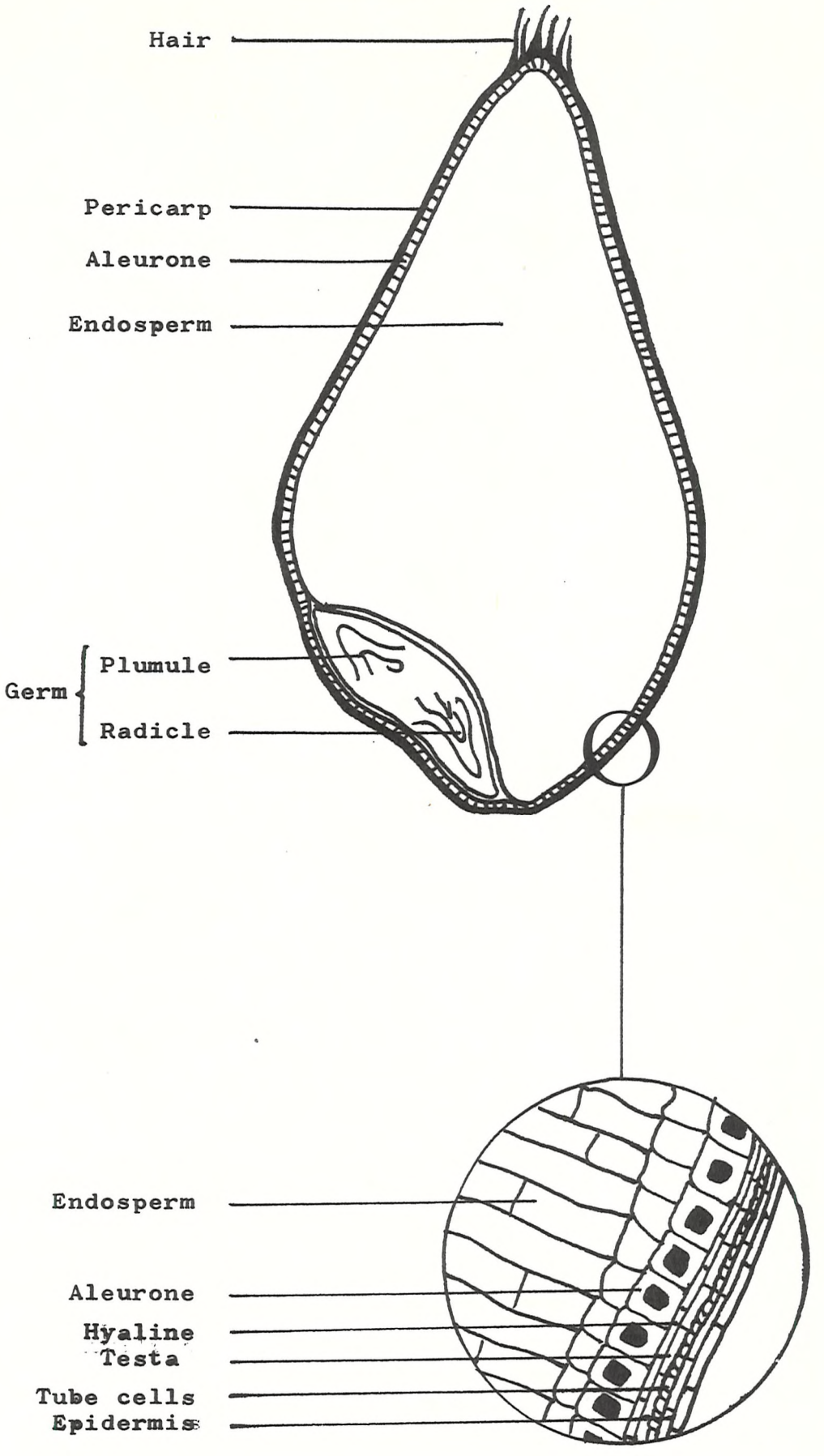


Fig. 1.7 - Longitudinal section of a wheat grain

Table 1.6 - Approximate composition of wheat bran (Fisher, 1973)

	Percent
Cellulose + lignin	22.0
Hemicellulose	25.0
Protein	9.5
Starch	8.5
Phytin (organic part)	2.0
Lipid	5.2
Sugars	5.0
Ash	5.7
Moisture	14.0

The lower cholesterol concentrations encountered in populations with high fibre intakes (Trowell, 1974) has been attributed in part to the binding capacity of phytic acid in the diet with zinc (Klevaney, 1975). Reinhold (1975) has found evidence suggesting that dietary fibre largely determines the availability of bivalent metal ions from bread and that phytates are of only minor importance in this respect. The net effect of bran-rich diets seem to be a decrease in intestinal absorption of bivalent metal ions such as iron (II), zinc and calcium, whether by the binding of fibre, phytic acid or both together.

B. CHANGES IN THE CONSUMPTION OF DIETARY FIBRE

Low intakes of dietary fibre have been linked to many of the common diseases of civilisation (Painter and Burkitt, 1971; Trowell, 1972; Burkitt, 1969, 1971a and 1972). Difficulty arises in calculating dietary fibre intakes because crude fibre figures from food tables are not an accurate guide. Southgate (1969) noted that crude fibre of wholemeal flour was 2-3% whereas the unavailable carbohydrate was, in fact, 11.2%.

The modern Western diet is composed of a starchy carbohydrate diet in a far more refined form than the staple diet of Africans; furthermore, the proportion of calories obtained from fat sources is greater in the Western diet, and has been seen to increase dramatically over the last half century. The typical African living in rural areas eats large quantities of millet and corn,

each of which has a high fibre content after grinding. In contrast to Africans who adopt a Western pattern of living, the incidence of IHD is negligible in the rural populations (Trowell, 1972).

It is not clear whether a high serum cholesterol is the cause of IHD, but it is believed to be a major contributory factor in the development of the disease. A good correlation between serum cholesterol and crude fibre intake has been shown by Trowell (1972a), in North Americans. The American vegetarian has a mean serum cholesterol of 2000 mg/l, whereas the standard American male has a value of 2800 mg/l. It has been noted by Trowell (1972) that a low dietary fibre intake is not necessarily the sole cause of IHD, but a high fat intake together with differing amounts of stress may also be implicated.

Hardinge, Cooks and Stone (1962) suggested that a better correlation exists between crude fibre intake and serum cholesterol than for fat intake and cholesterol. The case was highlighted by Italian descendants in America who eat an amount of fat similar to that of the native American (approximately 38% of the total calories), yet have a lower serum cholesterol. The crude fibre intakes of the Italian descendants was higher than the native American.

The crude fibre intake in Britain is about 4-8 g/day of which Trowell (1972) estimates that only 0.1 g is derived from a cereal source. The intake in America is approximately 8-11 g/day, whereas the rural South African will consume an average of 24.8 g/day (Cummings, 1973).

The role of dietary fibre in the prevention of some of the common diseases originates from the dramatic changes in dietary

patterns which occurred about one hundred years ago. Firstly, the quantity of cereal fibre consumed fell and secondly the source of dietary fibre changed from cereals to vegetables and fruit. At the same time, sugar consumption rose and the proportion of fat used as an energy source increased.

The purpose of milling flour is to obtain the maximum yield of white endosperm with a minimum of "contamination" by the brown outer layers, or bran. Prior to the introduction of the present day 'roller mill' method of separation in 1880, flour was stone-milled which resulted in a product with a higher crude fibre content.

An 80% extracted flour from the stone-milling method had a crude fibre content of 0.5%, whereas the crude fibre content of a 70% extracted flour is less than 0.1%. The crude fibre content of wholemeal flour is 2%. The impact of the change to roller milling is claimed to be overemphasised by Fisher (1973) because comparisons are usually made between wholemeal flour (fibre content 2%) and present day white flour (fibre content less than 0.1%). A more accurate situation is reflected in a comparison between 80% and 70% extracted flours (Jones, 1958).

Changes in dietary habits have caused large increases in the intake of fibre from fruit and vegetables over the past one hundred years. The consumption of fruit rose from 66.4 kg/head/year to 124.1 kg/head/year between 1909 and 1970, and the average yearly consumption of potatoes fell from 134.6 kg/head/year in 1880 to 100 kg/head/year in 1970 (Robertson, 1972; Cummings, 1973). The overall consumption of flour as an energy source decreased from

163 kg/head/year in 1835 to 127 kg/head/year in 1880, and then a figure of 66 kg/head/year was recorded in 1970. The total amount of cereal fibre therefore fell for two reasons. The amount of fibre contained in the flour decreased due to a mill milling procedure, and then the amount of flour consumed dropped at the expense of sugar, fat and fruit as new energy sources (Hollingsworth and Greaves, 1967).

Robertson (1972) suggested that although the amount of dietary fibre derived from cereals is now only 13% of our total fibre intake, the overall amount of fibre consumed has not changed.

C. BIOLOGICAL EFFECTS OF DIETARY FIBRE

a) Faecal bulk and transit time

A property of dietary fibre which has many clinical aspects is its ability to absorb water, increase intestinal mobility and cause a greater faecal bulk. Williams and Olmsted (1936) noted that many highly fibrous additions to the diet including bran and carrots caused increased faecal output. Eastwood *et al* (1972) recorded the wet weights of stools from patients fed bran or cellulose supplements and they found that the water content was much higher in patients on high fibre intakes than low intakes. McConnell *et al* (1974) measured the water holding capacities of various foodstuffs. 100 g Wheat bran had the ability to retain 447 g of water, whilst potatoes only 49 g, carrots 208 g and sprouts 168 g of water per 100 g of dried vegetable. Fruit showed little ability to retain water with the apple (177 g water per 100 g fruit) being superior to other fruits tested. The

correlation between the cationic exchange properties and the water adsorption properties of the fruit and vegetables was good, and Eastwood (1972) suggests that most fibrous foods will act as a gel within the gut so that adsorption, gel filtration and ion exchange may occur. Particle size had an effect on the water holding capacity of fibre and its effect on colonic function (Nutrition Reviews, March 1975); the smaller the size, then the less water is absorbed. Cooking had little effect.

Burkitt (1971) has recorded faecal outputs of 470 g faeces per day in rural Africans whose diets are highly fibrous. The suggestion that the low incidence of colonic cancer might be the result of such high fibre intakes has been made. Painter (1965) stated that a soft, swiftly passing stool subjects the sigmoid colon to less strain and therefore the development of diverticula is not favoured. In areas where high fibre intakes are common, the incidence of diverticular disease is negligible.

The time required for unabsorbed material to pass through the gut is influenced by the amount of dietary fibre eaten. Burkitt (1972) found that transit times were greater in Englishmen (77 hours) than Africans (35 hours). The introduction of bran to patients with colonic 'stasis' decreased transit times by almost half. Treatment of diverticular disease with bran supplements has proved successful, and it seems that for 'normal' colonic function to occur, the faecal mass must be fibrous with large amounts of water and have a more rapid transit time. Rats fed high fibre diets (Gustaffson and Norman, 1969) of stock pellets had shorter transit time than a semi-synthetic diet. The lower transit time

was associated with higher faecal bile acid excretion and less copophagy in both germ-free and conventional rats.

Faecal bulk and transit times might be related to the production of volatile fatty acids (VFA) produced by the gut flora. The majority of the anions in the faeces are derived from the bacterial metabolism of unabsorbed carbohydrate. Feeding dietary fibre has been seen to increase stool VFA output (Williams and Olmsted, 1936). Approximately 38% of the cellulose and 56% of hemicelluloses are metabolised in the human colon by bacteria. At the pH of the lower gut, most of the acids are unionised and are not, therefore, very lipid soluble. These unionised acids will act as osmotic cathartics (Fordtran, 1971).

Williams and Olmsted (1936) noted that feeding of one gram of 'fibre' caused an increase in faecal output by twelve grams in humans. There are three possible ways in which faecal weight and bulk could be increased.

Firstly, VFA can be produced by the metabolism of dietary fibre in the gut by bacteria. Secondly, sequestered bile acids from the small intestine could be metabolised to form ionised osmotic cathartics. Thirdly, the dietary fibre itself will absorb water (McConnell et al, 1974).

The "bulk-water" is the water which is trapped between the fibre particles whilst the water which is actually adsorbed to the surface of the fibre is termed the "water of hydration". In cases where the capacity of fibre to retain its bulk water is exceeded, such as diarrhoea, the bile acid retaining capacity of the fibre remains unchanged (Eastwood, 1973). The increase in stool weight due to the fibre's water holding capacity will reduce

the reabsorption of electrolytes and bile acids in the large intestine by increasing the bulk and transit time of the stool (Eastwood et al, 1973).

The significance of the ability for dietary fibre to take up and retain water is reflected in the action of pectin and other hydrophilic polysaccharides to lower serum cholesterol and increase bile acid excretion. Pectin will decrease cholesterol in man after 3 weeks (Keys et al, 1959) and cholesterol fed rats (Wells and Ershoff, 1961) by causing a decreased cholesterol absorption within the small intestine and an increased faecal bile acid elimination (Leverille and Sauberlich, 1966).

b) Hypocholesterolaemia induced by dietary fibres

The serum cholesterol concentrations of rabbits fell when they were fed Purina Chow (containing a high level of cereal fibre); when rabbits were placed on a semi-synthetic diet with low 'natural' fibre content, their plasma cholesterol concentration rose (Kritchevsky and Tepper, 1968). Moore (1967) found a protective effect against atheroma formation when wheat straw or peat were used as a source of fibre in butterfat-fed rabbits. The rabbits on a high fat diet with shredded cellophane as a source of indigestible roughage had elevated plasma cholesterol concentrations and were more prone to atheroma formation. Oats (Fisher, 1967) have a cholesterol lowering action in chickens and certain mucilaginous polysaccharides have also been shown to possess hypocholesterolaemic activity (Fahrenbach et al, 1966).

Alfalfa will lower plasma cholesterol concentration of rabbits, although its action is not confined entirely to its fibrous content

(Kritchevsky and Story, 1974). Cholesterol is precipitated by certain saponins within alfalfa thereby causing a net lowering of sterol absorption from the intestine; the fibrous portion of alfalfa will sequester bile acids and could lower cholesterol values in this way (Cookson, 1967).

Rats fed standard chow pellets, containing a high cereal content, showed increased excretion of faecal sterols, increased turnover of cholic acid and shorter transit times in both conventional and germ-free rats when compared to a semi-synthetic diet (Gustaffson and Norman, 1969). Portman (1960) noticed an increased bile acid excretion in rats fed commercial stock diet and Kritchevsky et al (1972) found a decreased cholesterol absorption coupled with increases in sterol excretion in rats fed highly fibrous diets. Bagasse, the fibre from sugar pulp, had no effect on rat plasma cholesterol values when diets were not fortified with cholesterol. The use of bagasse as a source of fibre did, however, cause increases in bile acid excretion and the hepatic conversion of acetate to cholesterol (Morgan et al, 1974). Balmer and Zilversmit (1974) showed that additions of soybran to rats increased the sterol excretion and turnover of cholesterol with a decreased absorption of dietary cholesterol resulted from the small intestine. The plasma cholesterol concentrations of their rats were lower in rats fed commercial diets, or diets with additions of soybran, than in semi-synthetic, low fibre diets.

Crude konjac mannan has a hypocholesterolaemic effect in rats fed hypercholesterolaemic diets (Kiryama et al, 1969). The ability of the crude mannan to lower cholesterol values of rats

was restricted to granules with a molecular weight of 9×10^5 and a radius of gyration of 1000 Å (Kiryama et al, 1972). The mannan had to be in a water soluble and non-crystalline state to have any action. In vitro experiments suggest that the gel-like mannan can inhibit the intestinal transport of bile acids, whilst in vivo the mannan inhibited cholesterol absorption in rats (Kiryama et al, 1974). The mode of action of konjac mannan seems to be similar to pectins (Leverille and Sauberlich, 1966).

A number of commonly eaten Indian pulses have been tested for hypolipidaemic activity in rats. Blackgram, (Phaseolus mungo) was shown to prevent any increase in plasma, liver and aortic cholesterol when incorporated into hypercholesterolaemic (2% cholesterol and 0.5% sodium cholate) diets (Devi and Kurup, 1970). The hypolipidaemic properties of this pulse were found to be contained within three fractions: a mild lowering of raised plasma cholesterol could be observed by the free fatty acids of the blackgram; the polysaccharide fraction (containing glucose, galactose, mannose, uronic acids, fibre and protein) when fed in a 56% oral dosage, overcame the effect of cholesterol feeding. A third fraction had the highest hypocholesterolaemic action; this fraction was a globin preparation and the effect of cholesterol and fat feeding was overcome by doses of only 5% of this globin fraction (Devi and Kurup, 1972 and 1973).

Prema and Kurup (1973) found that two other pulses, Redgram (Cajanus cojan) and Horsegram (Dolichus biflorus), had the ability to counteract the additions of cholesterol and sodium cholate to the diets of rats. The active component of these two pulses was found to be in their globin fractions and the hypocholesterolaemic action of redgram was greater than horsegram.

Not all Indian pulses were found to possess hypolipidaemic properties. Prema and Kurup (1973) could find no effect with Greengram (Phaseolus radiatios) and Bengalgram (Cicer arietinum). The action of Bengalgram in man has, however, been confirmed in the long term studies of Mathur, Khan and Sharma (1968) who found that the pulse would lower the serum cholesterol concentrations of human volunteers after feeding for twenty-two weeks. The volunteers were maintained on a high fat intake of butterfat (156 g per day) and the reason for the long period required for the hyperlipidaemic action to become apparent is still uncertain. The drop in serum cholesterol corresponded with a corresponding increase in faecal bile acid elimination via the faeces.

The effect of various starches on rat serum cholesterol has been studied by Vijayagopolan and Kurup (1970). They found that the plasma and liver cholesterol concentrations were higher in those rats fed diets high in purified ground rice than rats fed diets with ground whole paddy. The active component of the paddy was later reported to be the bran and husk (Vijayagopolan and Kurup, 1972). Further studies (Vijayagopolan and Kurup, 1973a and 1973b), showed a polysaccharide portion from both the bran and husk was extremely effective in lowering rat cholesterol when incorporated into diets containing cholesterol and cholate. An oral dose of only 15 mg per rat per day would overcome the feeding of a diet containing 2% cholesterol and 0.5% sodium cholate. The action of the polysaccharide fraction was to increase the conversion of cholesterol to bile acids and no change in the intestinal absorption of cholesterol could be observed.

The feeding of a 5% supplement of ground dried mushrooms to rats maintained on high cholesterol diets for ten weeks, resulted in a decrease in plasma and liver cholesterol concentrations (Tokuda and Kaneda, 1966). The active component on the mushroom (Lentinus edodes) was isolated in an aqueous extract and a 30% aqueous ethanol extract. Further investigations isolated the individual compounds responsible for the hypocholesterolaemic action: the compounds were termed Lentysine (Rokiyo et al, 1970), and Lentinacin (Chibata, Okumura, Takeyama and Kotera, 1969).

The action of wheat bran in human subjects has not been fully elucidated. The majority of studies undertaken have failed to show a lowering of cholesterol when bran was incorporated into diets (Truswell and Kay, 1975; Eastwood, Kirkpatrick, Mitchell, Bone and Hamilton, 1975; Eastwood, 1969 and Durrington, Wick and Heaton, 1975). The studies so far undertaken have been of a short duration and it may be that for any lowering of serum cholesterol to be observed, the concentrations have to be originally elevated. The action of leguminous fibre (Mathur, Khan and Sharma, 1968) did not become evident until twenty weeks had elapsed, and high dietary fat intakes were administered both before and during the experimental feeding. The feeding of bran to rats fed a high cholesterol diet containing 1% cholesterol was to lower liver cholesterol, but not plasma (Ranhortra, 1973). Owen, Munday, Taylor and Turner (1975a) found that rats tended to resist elevations of plasma and liver cholesterol when bran was added to hypercholesterolaemic (1% cholesterol and 0.25% sodium cholate) diets. The addition of bran to rats on low cholesterol diets has no effect on cholesterol

values, although increased bile acid excretion was found to occur (Owen, Munday, Taylor, Turner, 1975c).

The hypocholesterolaemic constituent of vegetable fibre has not been established, although polysaccharide and protein fractions have been evaluated for Indian pulses and mushrooms. Bile acids and their metabolites are adsorbed onto the fibre and bacteria of the faeces (Gustaffson and Norman, 1968). Norman and Short (1962) found that after injecting labelled cholate into rats, the labelled bile acids became closely associated with the solid portion of the intestinal contents as the label passed through the gut. In the large intestine, 44% of the label was associated with the bacterial solids as lithocholic acid, whilst deoxycholate predominated within the liquid phase. Germ-free rats had all their bile acids in the liquid phase, and the bile acid excretion for germ-free rats was calculated to be 1.9 - 2.2 mg per rat per day whereas for conventional rats the figure was 5.1 mg per day (Gustaffson, Norman and Sjovall, 1960).

Measurements of bile acid excretion in human volunteers fed high and low fibre diets suggests that there is an increased excretion of bile acids when a high fibre intake is maintained (Shurpulehar, 1971). Eastwood (1972) could find no overall increase in excretion from human volunteers.

Carroll (1964) showed that rats fed a commercial diet, high in fibre had an increased cholesterol synthesis from acetate. This effect was partly due to the higher levels of unsaturated fatty acid in the diet, but Morgan et al (1974) confirmed that high fibre diets did increase cholesterol synthesis. Fibre may be absorbing bile acids in the gut and therefore removing them from the normal

entero-hepatic circulation. The amount of bacterial metabolism may influence the extent of binding because the quantity of bile acids bound to faecal solids is greater in conventional rats than in germ-free rats (Gustaffson and Norman, 1968).

The majority of the bile acids in the rat occur in the small intestine (approximately 75%), whereas only 5% occur in the liver. The remaining 20% are mostly in the large intestine (Norman and Sjovall, 1958). The bile salts in the rat small intestine are in a micellar formation with lipids, and there is probably little adsorption of the bile acids onto fibre. In the colon, free bile acids are strongly adsorbed into the fibrous residue and it seems likely that the large intestinal bacteria form secondary bile acid metabolites which have an altered physical state (Eastwood and Hamilton, 1968; Norman, 1964). A further effect of high fibre diets on rats was noted by Eastwood and Boyd (1967) when additions of carboxymethylcellulose and bran to diets caused an enhanced secretion of bile acids into the intestine of rats. This is undoubtedly due to the 'dilution' of the intestinal contents by the fibre and may account for previous observations that acetate is incorporated more readily into cholesterol when rats are fed high fibre diets.

The binding of bile acids to the indigestible portions of the diet has been studied in vitro by Eastwood and Hamilton, 1968; Kritchevsky and Story, 1974, Story and Kritchevsky, 1975, Owen, Munday, Taylor and Turner, 1975b; and Balmer and Zilversmit, 1974). Eastwood and Hamilton (1968) using a heterogenous mixture of maize and barley demonstrated a specific binding for bile acids which

increased inversely with the pH of the incubation medium. Bile acids were bound more strongly than their corresponding bile salts. Balmer and Zilversmit (1974) found that cholesterol was precipitated out of micellar formation when a micellar solution of sodium taurocholate and cholesterol, was incubated with soya bran. The action of fibre could therefore be both as a mild ionic exchange resin for bile acids and as a substance which decreases the amount of cholesterol absorbed from micellar solution.

Eastwood and Hamilton (1968) suggest that lignin has a high affinity for bile acids, and this was confirmed by Story and Kritchevsky (1976). The mechanism of binding is probably hydrophobic in nature and together with the bacteria of the faeces, a large proportion of bile acids are associated with the faecal solids. Methylated lignin had a very strong affinity for binding for bile acids.

Morgan et al (1974) found that lignin had very little affinity for bile acids. Studies by McConnell and Eastwood (1974) showed that little correlation existed between acid detergent fibre content or lignin content of various vegetable and cereal sources and their capacity to act as cationic exchange resins. In vivo action of lignin could not be equated with the effects of cholestyramine in humans (Barnard and Heaton, 1973). Heaton and Barry (1972) showed no lowering of cholesterol concentration in patients fed lignified diets. Thiffault et al (1970) did find a lowering of cholesterol in patients with Fredrickson type II hyperlipidaemia, but further studies by Linder and Möller (1973) failed to confirm this action in hyperlipidaemic patients. The action of lignin is

therefore still open to confirmation, whereas a true bile acid sequestering agent such as cholestyramine, is well known to both lower cholesterol and increase faecal bile acid excretion (Carey, 1961).

Summary

Dietary fibre passing through the gut could act by several mechanisms (Fig. 1.8),whereby cholesterol metabolism, cholesterol turnover and sterol excretion might be altered:

(i) Bile acids may be sequestered from the small to the large intestine. A binding can occur, in the colon, between deoxycholate and related bile acids, and the indigestible fibre. Lithocholic acid, however, has been reported as being strongly bound to the bacteria and not the fibrous portions of the faeces (Midtvedt and Norman, 1972).

(ii) Dietary fibre will absorb water and increase intestinal transit time. The increased transit time causes a "washing-through" of bile acids in the intestine and will render them less available for reabsorption from the colon. A significant amount of reabsorption of bile acids occurs from the rat colon. The uptake of water will cause a 'gel' to be produced whereby bile acids and cholesterol will become trapped in its structure. Micelle formation might be interrupted and sterol absorption within the upper small intestine therefore hindered.

(iii) Bacteria will digest fibre and some of the sequestered bile acids. The production of volatile fatty acids from bacterial metabolism will cause an increase in the water content of the faeces, therefore

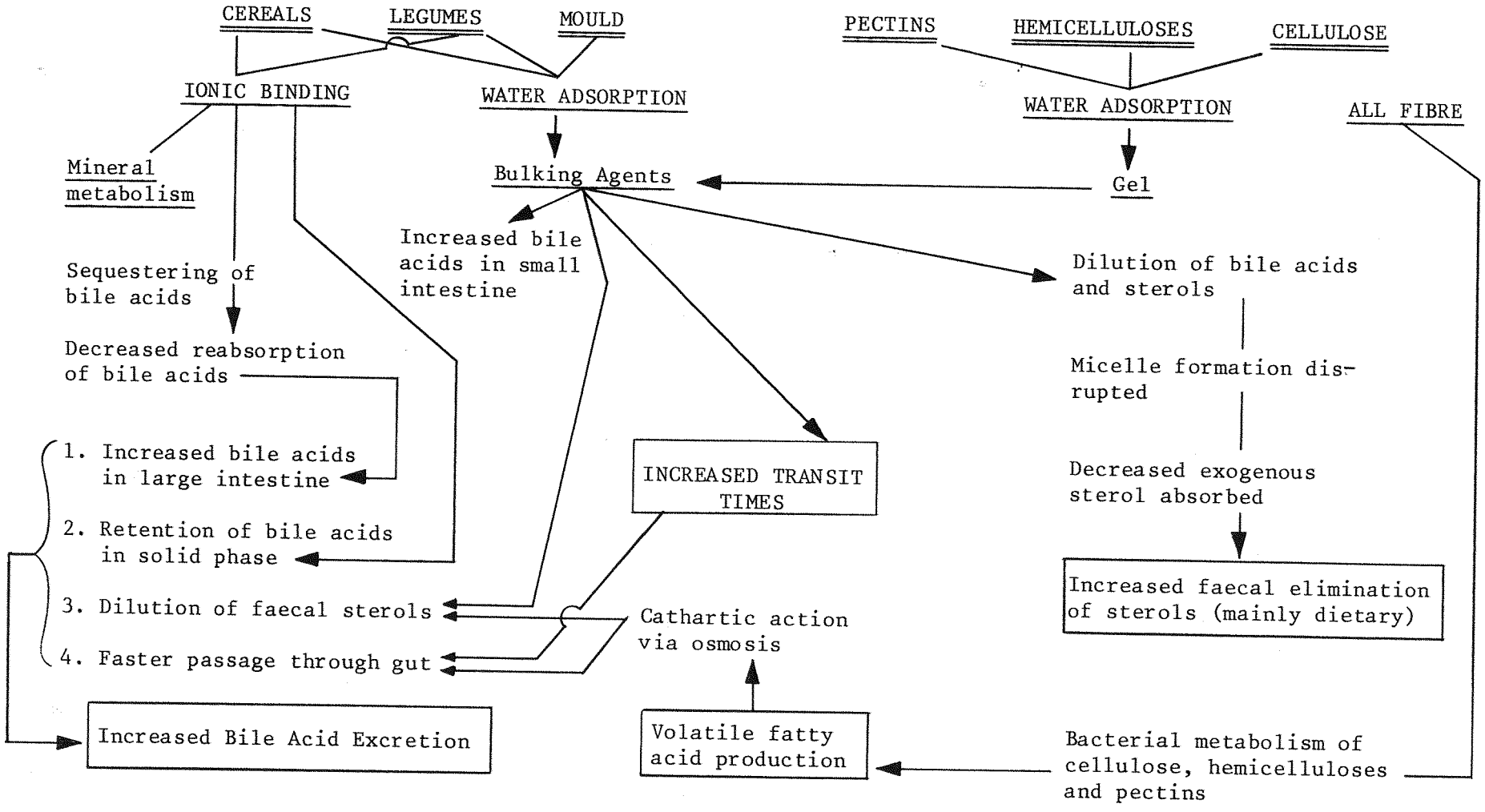


Fig. 1.8 - Dietary fibre and sterol excretion

increasing faecal bulk and causing a more rapid elimination of the bile acids in the faeces.

(iv) Hepatic function might be altered when highly fibrous diets are eaten. Carroll (1964) and Morgan et al (1974) have observed changes in hepatic cholesterol-genesis of rats. Vijayagopolan and Kurup (1973a and 1973b) have suggested than an isolated polysaccharide prepared from the husk and bran of paddy will increase hepatic bile acid production. Boyd and Eastwood (1969) noted an increased secretion of bile salts into the small intestine of rats fed high fibre diets.

The effects of dietary fibre on sterol metabolism are summarised in Fig.1.8.

CHAPTER 2

METHODS AND MATERIALS

2.1 MATERIALS AND ANIMALS

A. REAGENTS

All solutions were prepared in glass-distilled water except where otherwise stated.

Chemicals were purchased from British Drug Houses, Poole, Dorset; Sigma Chemicals, London and Koch-Light Laboratories, Colnbrook, England.

Reagent	Source
Radiochemicals	
Cholesterol-^{3}H (G) (33 mCi/mg)	Radiochemical Centre, Amersham, Bucks.
Anaesthetic	
Nembutal (pentobarbitone sodium)	Abbott Laboratories, Queenborough, Kent.
Chemicals	
Scintillation grade naphthalene	Aldrich Chemicals Co. Inc.
β-Sitosterol	Aldrich Chemicals Co. Inc.

B. DIETARY CONSTITUENTS

Solka Floc (Wood cellulose)	Johnsen, Jorgensen & Wettre Ltd. London.
Casein	Prideaux Milk Food Ltd., Evercreech, Somerset.
Maize starch	C.P.C. (UK) Ltd., Trafford Park, Manchester.
Maize oil (refined, deodorised)	Van Den Berghs & Jurgens Ltd., Purfleet, Essex.
Wheat bran	Christopher Hill Group Ltd., Poole, Dorset.
PRD Stock diet	Christopher Hill Group Ltd., Poole, Dorset.
PMD Stock diet	Christopher Hill Group Ltd., Poole, Dorset.
Microfungal protein (Code A3/5)*	Rank, Hovis, McDougall Ltd., High Wycombe, Bucks.

*See Appendix

C. ANIMALS AND DIETS

Rats used for experiment were male albino Wistars from the University of Southampton colony, maintained in the Animal House, Department of Physiology and Biochemistry, Southampton. The rats were weaned onto PRD stock diet one week prior to feeding and the PRD in the Animal House was sterilised by autoclaving. No detrimental effects to the growth response or litter sizes were noticed throughout a two year period when rats were maintained on this autoclaved diet despite the possibility of damage to proteins (Ford, 1976).

The rats were selected for experiment on a weight basis (scatter 15-25 g). Unless otherwise stated, the rats were divided into experimental groups of six animals per group and housed three per cage.

Golden Syrian hamsters (Mesocricetus auratus) from the University of Southampton colony were weaned onto PMD stock diet one week before feeding of experimental rations. The animals were selected for experiment on the basis of similar weight. Five animals per treatment group were used and each group was housed in a wire cage.

Weekly records of food intake and body weights were kept for all animals. Humidity was maintained at above 50% by twice daily washings of the animal room floor. Temperature of the rooms was maintained at 21^{o} and lighting followed a 12 hour cycle.

All animals were deprived of food overnight prior to the collection of plasma and liver samples on the following day; otherwise they were allowed free access to food throughout the experiment. Animals were allowed free access to water throughout the experiment.

Male animals were used for all experiments.

Semisynthetic diets were fed in either a powder or pellet form. Initial experiments in which diets were presented as a powder, resulted in high food spillage and palatability problems. Pelleted semisynthetic diets were later fed and only a minimal amount of spillage could be measured and there was an increased feed conversion.

Powdered diets had 7-8% water added and the wet mixture was passed through a mincing attachment (8 mm dye) of a Hobart mixer. The strings of wet pellets were collected in shallow metal trays lined with adsorbant paper and immediately dried in a forced draught of warm air. The strings of pellets were manually broken into 1-2 cm lengths and stored in a cool, well ventilated room.

Diet formulations for each experiment will be presented in subsequent Chapters. All diets contained vitamin and mineral supplements. The supplements were added to the diets in the form of premixes, the details of which are given in Tables 2.1 and 2.2.

Table 2.1 - Vitamin mixture

Vitamin	g/kg mix
Biotin	0.0039
Folic acid	0.039
Inositol	3.900
Nicotinic acid	0.975
Calcium pantothenate	1.160
Pyridoxine HCl	0.039
Riboflavin	0.193
Thiamin HCl	0.058
p-aminobenzoic acid	11.000
Cyanocobalamin	0.001(1)
Menaphthone-K	0.200
Choline chloride	11.000(2)
Rovimix A + D 500/100	5.000
Rovimix E_{50}	5.000
Maize starch	955.800

(1) Dissolved in a small amount of absolute ethanol.

(2) Dissolved in 10 ml 70% ethanol: added last.

Table 2.2 - Mineral mixture

Salt	g/kg mix
Cobalt chloride ($6H_2O$)	0.0214
Cupric sulphate ($5H_2O$)	0.4484
Ferrous sulphate ($7H_2O$)	25.4113
Magnesium sulphate ($7H_2O$)	110.6690
Manganese sulphate ($4H_2O$)	5.0059
Potassium dihydrogen orthophosphate	366.6227
Potassium iodide	0.7452
Sodium chloride	131.3215
Zinc carbonate	0.2252
Calcium carbonate	359.5291

Mineral and vitamin mixes were stored in black polythene bags at 5°C until required.

2.2 METHODS

This section will outline the techniques which were used for several experiments. Methods employed for only one experiment will be detailed in the subsequent Experimental Chapter.

A. COLLECTION OF SAMPLES

a) Plasma

Animals were killed by exposure to diethylether, unless otherwise stated. Immediately an animal was seen to cease breathing, the thorax was opened to expose the heart. Blood was collected into a syringe by direct cardiac puncture using lithium heparin as an anticoagulant.

Plasma was prepared by spinning the blood in a bench centrifuge for 10 minutes at 2600 rpm. The plasma samples were stored in a deep freeze until required for cholesterol and triglyceride analyses.

b) Liver

Liver samples were collected following the exsanguisation of the animals. The whole liver was removed, (gall bladders of hamsters were dissected from the liver), dipped in distilled water and blotted dry with tissue paper. The whole liver was weighed, wrapped in aluminium foil and stored in a deep freeze.

c) Faecal samples

The faeces of animals housed in metabolism cages were collected every 12 hours. The samples were weighed, dried in a vaccum oven for 10 hours and reweighed. Dried samples were stored in a deep freeze for short periods (1-2 days) until required for analyses.

B. ANALYSIS OF SAMPLES

a) Plasma analyses

(i) Total cholesterol

Total cholesterol was determined either manually or by an automated procedure. Both methods are described.

Manual method

The method of Zlatkis, Zak and Boyle (1953) was employed for the manual determination of plasma total cholesterol. The method estimated cholesterol colorimetrically and is claimed to be 4 or 5 times more sensitive than the original Liebermann-Burchard colour reaction.

Plasma (0.1 ml) was added to 3.0 ml glacial acetic acid in a test tube. 2.0 ml colour reagent (0.5 ml saturated ferric chloride solution in 50 ml concentrated sulphuric acid) was added carefully and the contents of the tube mixed on a vortex shaker. The test tubes and their contents were left at room temperature for 25-30 minutes.

The contents of the tubes were read on a Pye-Unicam SP-500 spectrophotometer at 560 nm wavelength in glass cuvettes of 1 cm light path. Standard curves were constructed using 100, 200, 300, 400 and 500 µl of standard cholesterol solution (1000 mg/l) in glacial acetic acid. All spectrophotometric readings were made against a distilled water (0.1 ml water, 2.9 ml acetic acid and 2.0 ml colour reagent) blank.

Automated method

An isopropanol extract of plasma was used to determine total cholesterol by an automated procedure, (Technicon Clinical Methods, No. 16A, September, 1972). The isopropanol extract was added to a segmented stream of Liebermann-Burchard colour reagent and heated to

60°C. The resulting colour development was read at 630 nm.

Samples for the autoanalyser (Technicon Instrument Corp., Tarrytown, New York) were prepared by pipetting plasma (0.1 ml) into 1.9 ml 99% isopropanol in a disposable plastic 2.5 ml test tube. The tube was stoppered, shaken and spun at 2500 rpm for five minutes. The supernatant was decanted into a stoppered polyethylene vial for analysis.

(ii) Free and esterified cholesterol

Cholesterol and cholesterol esters were absorbed onto alumina and eluted first with petroleum ether and then with benzene, thereby removing the esters and free cholesterol respectively (Kerr and Bauld, 1953).

A slurry of 200 mg activated alumina (activated by heating in an oven at 150°C) in petroleum ether (40-60°C fraction) was packed into a 100 mm long x 6 mm diameter glass column with a glass wool plug at the base. 0.2 ml plasma were added to 2 ml acetone:ethanol solution (1:1 v/v) in a stoppered tube. The tube was shaken, centrifuged at approximately 2500 rpm, and the supernatant transferred to a glass test tube. The solvent was evaporated off in a hot water bath and the lipid redissolved in 2 ml petroleum ether (40-60°C boiling range). The petroleum ether solution was applied to the column followed by two 0.5 ml washouts with petroleum ether.

The esters were eluted by running two 10 ml aliquots of petroleum ether through the column, whilst free cholesterol was eluted with two 10 ml portions of benzene. Each fraction was collected in a 30 ml glass boiling tube and the respective solvents evaporated.

Cholesterol and its esters were remaining in the tubes were measured by the ferric chloride - acetic acid method of Zlatkis, Zak and Boyle (1953) as previously described.

(iii) Triglycerides

Plasma triglycerides were measured either manually or by an automated method.

Manual method

The method described by Van Handel (1960) was adopted for the manual determination of plasma triglyceride. The method is a modification of the procedure of Van Handel and Zilversmit (1957) which removes the phospholipids from a chloroform extract of plasma by incubating with zeolite (hydrated magnesium silicate). The triglycerides are hydrolysed to yield free glycerol which is subsequently oxidised to formaldehyde; chromotropic acid is used to estimate the concentration of formaldehyde. 0.5 g of activated zeolite (Cheng and Zilversmit, 1960) was dispersed in 5 ml chloroform in a thick-walled test tube, 250 µl plasma were added, and the contents of the stoppered tube shaken periodically for four hours. The tube was centrifuged at 2500 g for 3 minutes and a 2.5 ml aliquit of the supernatant transferred to a test tube. The chloroform was evaporated in a warm (40°C) water bath. At this point, a standard of 0.01 to 0.05 mg glycerol trioleate and a reagent blank containing no lipid were introduced. All tubes had 0.5 ml 0.07 N (0.4%), ethanolic potassium hydroxide added and were heated in a water bath at 60°C for 20 minutes. After saponification, 500 µl 0.2 N sulphuric acid were added to each tube and the tubes heated for a further 20 minutes in a boiling water bath after which they were left to cool to room temperature. 50 µl 0.025 M sodium periodate were added

to the tubes which were shaken and allowed to stand at roon temperature for 10 minutes. 50 µl of 0.5 M sodium arsenite followed by 5 ml 0.2% chromotropic acid in 60% sulphuric acid were added. The contents of the tubes were shaken, heated at 105°C in a heating block (Grant Instruments) for 30 minutes and centrifuged when cool. The optical densities of the supernatants were measured using disposable 1 cm light path plastic cuvettes in a Pye-Unicam SP-500 spectrophotometer at 570 nm.

Automated method

Isopropanol extracts of triglyceride were pumped into an air segmented stream of ethanol and potassium hydroxide. Saponification of the triglycerides was performed in a 50°C heating bath and the glycerol was oxidised and condensed in a second 50°C water bath. The stream of samples passed through a flouronephelometer where a fluorophore was activated in a flowmeter of 2 mm internal diameter (Technicon Automated Clinical Methods, No. 24, March, 1972).

100 µl aliquots of plasma were pipetted into 2.5 ml disposable test tubes containing 1.9 ml isopropanol and 0.4 g ze lite. The tubes were stoppered, shaken on a vortex mixer and placed horizontally on a circular stand which was allowed to rotate at 10 rpm for 45 minutes. The tubes were then centrifuged at 2500 g for 3 minutes and the supernatant decanted into a stoppered vial for analysis. It was possible to perform simultaneous cholesterol and triglyceride analyses on these samples.

b) Liver samples

(i) Total cholesterol

A known weight of fresh liver (approximately 1 g) was homogenised in a glass homogenising tube containing distilled water to provide a 10% liver homogenate. Aliquots of the homogenate were assayed for total cholesterol by a manual or automated procedure.

Manual method

1 ml Aliquots of homogenised liver were added to 5 ml freshly prepared alcoholic potassium hydroxide (6 ml 33% KOH in 94 ml absolute ethanol) in a glass universal bottle. The bottle was stoppered and shaken at 37°C for 60 minutes. After cooling, 10 ml petroleum ether (60-80°C boiling range) were pipetted into the bottle followed by 2 ml distilled water. The contents were shaken vigorously for 90 seconds and centrifuged at 2000 rpm for 5 minutes. Duplicate 2.5 ml samples of the upper layer were pipetted into test tubes, evaporated to dryness in a hot water bath, and the sediment assayed for cholesterol with a Liebermann-Burchard reagent (Abbell, Levy, Brodie and Kendall, 1952).

A standard curve was constructed using aliquots (100-500 µl) of standard cholesterol solution (100 mg dissolved in 100 ml petroleum ether) evaporated to dryness in a test tube. All test and standard samples of dried lipid had 6 ml of a modified Liebermann-Burchard colour reagent added, (0.5 ml concentrated sulphuric acid in 100 ml ice-cold acetetic anhydride followed by 50 ml glacial acetic acid). All tubes were stoppered, incubated and shaken at 37°C for 30 minutes. When cooled, the absorbance of the solutions were read on a Pye SP-500 spectrophotometer at 620 nm wave length and a 1 cm light path.

Automated method

100 µl liver homogenate (10%) were pipetted into 1.9 ml isopropanol in a 2 ml test tube. The tube was stoppered, shaken and centrifuged at 2500 rpm for 5 minutes. The supernatant was assayed for total cholesterol on a Technicon Autoanalyser II as previously described.

(ii) Triglycerides

100 µl of liver homogenate were pippetted into a 2.5 ml test tube containing 1.9 ml isopropanol and 400 mg zeolite. The tube was stoppered and its contents gently mixed for 45 minutes after which it was centrifuged at 2500 rpm for 3 minutes. Estimation of triglyceride in the supernatant was performed with the aid of a Technicon Autoanalyser (Technicon Automated Clinical Methods, No. 24, March, 1972).

C. STATISTICAL ANALYSIS OF RESULTS

Mean values ($\bar{x}$) were calculated as: $\bar{x} = \frac{1}{n} \Sigma x$,

where x is the sum of all the individual observations and n is the number of observations.

The standard error of the mean for each treatment group was calculated from the formula:

$$\text{Standard Error (SE)} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n (n - 1)}}$$

where x is the observation

$\bar{x}$ is the mean of the observations in the group

n is the number of observations

The statistical difference between two treatment groups (1 and 2) was evaluated by the Student 't' test.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the mean values of all the observations in treatment groups 1 and 2 respectively and SE_1 and SE_2 are the standard errors of treatment groups 1 and 2 respectively.

The probability that two groups of observations were statistically different was determined from the appropriate tables. A 'p' value of less than 0.05 was considered statistically significant, i.e. there was less than a 5% probability that the difference observed was to random error.

Analysis of variance

The variance ratios (F ratio) of the different components and the variation within each group (error) were calculated as described by Bailey (1974). The probability that the difference observed was a result of experimental conditions ('p' value) was deduced from F-tables.

CHAPTER 3

THE HYPOCHOLESTEROLAEMIC ACTION OF WHEAT BRAN AND A MOULD* IN RATS

*See Appendix

3.1 INTRODUCTION

The intake of dietary fibre has been related to the incidence of atherosclerosis and coronary heart disease: low intakes might lead to raised serum cholesterol and a higher risk of atherosclerosis (Trowell, 1972). A variety of gums, grains and pectin have been shown to possess hypocholesterolaemic activity when used as a source of fibre in rat diets (Ershoff and Wells, 1962; Vijayagopolan and Kurup, 1970). Ranhotra (1973) found little hypocholesterolaemic activity in cereal products when incorporated into rat diets containing cholesterol, although when wheat bran was used as a source of fibre a decrease in liver cholesterol concentrations did occur.

Two experiments were undertaken to evaluate the hypocholesterolaemic activity of wheat bran and a mould. Experiment 1 involves the feeding of diets containing cholesterol and sodium cholate in which either cellulose (control diet) wheat bran or the mould acted as sources of fibre. A second control diet containing cellulose without cholesterol and cholate was used to assess the potency of any hypocholesterolaemic activity.

Experiment 2 investigates the action of bran and the mould on the sterol output and the plasma and liver cholesterol concentrations of rats receiving diets without cholesterol and cholate additions. A control diet containing 10% cellulose was used in this experiment.

3.2 EXPERIMENTAL

A. DIETS

The composition of the diets is shown in Tables 3.1 and 3.2. Microcrystalline cellulose (Solka Floc) was used as a source of indigestible fibre for the control diets and fibre was supplied by either wheat bran or a mould in the experimental diets.

Table 3.1 - Composition of diets for Experiment 1 (g/kg)

Dietary component	Diet Numbers			
	1	2	3	4
Casein	200	200	200	50
Vitamin mix	10	10	10	10
Mineral mix	40	40	40	40
Mould	-	-	-	350
Cellulose	100	100	-	-
Wheat bran	-	-	100	-
Corn oil	100	100	100	100
Cholesterol	0	10	10	10
Sodium cholate	-	2.5	2.5	2.5
Maize starch	550	537.5	537.5	437.5

Each diet was fed in powder form to six 150 g male Wistar rats housed collectively in wire cages. The animals were sacrificed after 6 weeks of feeding for collection of liver and blood samples. Food intakes and body weights were measured weekly.

Table 3.2 - Composition of diets for experiment 2 (g/kg)

Dietary component	Diet Number		
	1	2	3
Casein	200	189	-
Mould	-	-	400
Cellulose	100	-	-
Wheat Bran	-	100	-
Sucrose	150	150	150
Vitamin mix	10	10	10
Mineral mix	40	40	40
Corn oil	100	100	100
Maize starch	400	411	300

The diets were calculated to be isonitrogenous, each diet containing 18.5% crude protein (N x 6.25).

Each diet was fed to a group of 6 male Wistar rats individually housed in wire mesh cages for five weeks. Faecal samples were collected daily during the final week. After five weeks, the animals were killed for plasma and liver sample collection.

The diets were fed in pellet form.

B. ANALYTICAL METHODS

The animals were killed after an overnight fast.

a) Cholesterol analyses

In Experiment 1 plasma and liver total cholesterol were determined by the manual method of Zlatkis, Zak and Boyle (1953) as described in Chapter 2, section 2.2.

In Experiment 2 total cholesterol concentrations of plasma and liver samples were measured by an automated technique (Chapter 2, section 2.2).

b) Triglycerides

The concentrations of triglycerides were measured in the plasma and liver samples from animals in Experiment 2 by an automated procedure (Chapter 2, section 2.2).

c) Moisture content of faeces

The moisture content of individual samples of faeces collected every 12 hours from animals of Experiment 2 was measured one week prior to killing. The samples were weighed immediately after collection, loosely wrapped in aluminium foil and placed in a vacuum oven (60-70^{o}C) for 9-10 hours. The samples were cooled in a disiccator over phosphorus pentoxide and reweighed. The loss of weight after drying represented the moisture content of the faecal samples.

d) Colorimetric determination of faecal sterols

Acetone:ethanol (1:1 v/v) extracts of faeces collected from rats of Experiment 2 were prepared from the samples used for moisture analysis. The dried samples were ground into powder using a Moulinex coffee

grinder and a known weight of the powder (1-2 g) representing a 24 hour collection period, was placed into a 200 ml round bottom flask. The powdered samples were refluxed for 24 hours with 50 ml acetone: ethanol (1:1 v/v) solution. The contents of the flask were filtered hot through a presoaked Whatman No. 54 filter paper and collected in 100 ml volumetric flasks. Three washes with 10 ml hot acetone: ethanol were made to ensure all the extract was transferred and the final volume was made up to 100 ml with acetone:ethanol solution.

(i) Bile acids

Acetone:ethanol extracts of rat faeces were analysed for total di- and trihydroxycholanic acids (Singer and Fitchen, 1961).

0.25 ml of a barium hydroxide reagent (a suspension of 5 g barium hydroxide and 0.4 g barium acetate in 100 ml distilled water which was boiled, cooled and filtered) was added to 5 ml of the faecal extract in a test tube. The tube was immersed into a hot water bath (80-90^{o}C) for 5 minutes and cooled. The volume was made up to 5 ml with ethanol, and the contents of the tube filtered through a Whatman No. 54 filter paper using two subsequent washings with 2 ml portions of ethanol. The volume of the filtrate was adjusted to 10 ml by the addition of ethanol.

Duplicate 2 ml aliquots of the filtrate were evaporated to dryness in a glass centrifuge tube and 4 ml calcium oxide reagent added (100 mg powdered calcium oxide suspended in 100 ml fresh ethyl acetate containing 2 g sodium hydroxide). The tube was placed in a water bath at 90^{o}C for 2 minutes, cooled and centrifuged at 2500 rpm for 15 minutes. The supernatant was decanted off and 5 ml Minibeck reagent (1 volume glacial acetic acid in 9 volumes of concentrated sulphuric acid) added. Standards of deoxycholic acid (0.4 mg) and cholic acid (0.2 mg) were introduced and all tubes were shaken at 40^{o}C for 90 minutes. The absorption of the solutions were read against a Minibeck reagent blank at 310 nm and 390 nm on a Pye SP 500 spectrophotometer using 1 cm light path and silica cuvettes.

The concentrations of di- and trihydroxycholanic acids were calculated from the following equations:

$$\text{Concentration of dihydroxy acids } (\mu g/ml) = A \times O.D.^{310} - B \times O.D.^{390}$$

$$\text{Concentration of trihydroxy acids } (\mu g/ml) = C \times O.D.^{310} - D \times \text{concentration dihydroxy acid}$$

where:

$$A = \frac{E_t^{390}}{E_t^{390} \times E_d^{310} - E_t^{310} \times E_d^{390}}$$

$$B = \frac{E_t^{310}}{E_t^{390} \times E_d^{310} - E_t^{310} \times E_d^{390}}$$

$$C = \frac{1}{E_t^{310}}$$

$$D = \frac{E_d^{310}}{E_t^{310}}$$

E_t is the extinction coefficient ($\mu g/ml$) of the trihydroxycholanic acid and E_d the extinction coefficient of the dihydroxycholanic acid at the respective wavelengths.

Total faecal acid sterols

1.0 ml of an acetone:ethanol (1:1 v/v) extract from rat faeces was pipetted into a test tube and the solvent evaporated. The residue was redissolved in 2 ml 99% isopropanol to be analysed for Liebermann-Burchard-positive compounds on an autoanalyser programmed for cholesterol determinations.

e) Total plasma Ca^{2+} and Mg^{2+}

Plasma obtained from animals in Experiment 2 was assayed for total calcium and magnesium.

100 µl plasma were pipetted into 5 ml lanthanium chloride solution (50 mM HCl + 10 mM $LaCl_3$). The concentrations of calcium and magnesium ions in the lanthanium chloride solution were measured on a Pye SP 90A atomic adsorption spectrophotometer. Standards of calcium (0.2 to 0.3 mmol/l), and magnesium (0.33 to 0.51 mmol/l), were made up in the lanthanium chloride solution. All glassware was washed in concentrated hydrochloric acid and rinsed in deionised water.

3.3 RESULTS

Experiment 1

The addition of cholesterol and sodium cholate to the cellulose diet resulted in a 63% increase in the plasma cholesterol level and a 97% increase in the concentration of cholesterol in the liver (Table 3.3). The group fed the bran diet had plasma and liver concentrations of cholesterol significantly lower than a group fed the hypercholesterolaemic cellulose diet, diet 2, (36 and 42% lower respectively). The diet containing mould as a source of protein and dietary fibre also had significantly lower plasma and liver cholesterol levels than the diet 2 cellulose group (28 and 39% respectively). The rats fed bran and mould diets had plasma cholesterol concentrations significantly higher than the negative control group fed a cellulose diet without additions of cholesterol and cholate. The rats fed on the mould diet also had liver cholesterol levels higher than the negative controls, whereas in the rats fed a bran diet the liver cholesterol concentration was not significantly higher.

Table 3.3 - Mean values + SEM for 6 observations per group

Diet	Dietary components (g/kg)		Added cholesterol + cholate	Cholesterol	
	Casein	Fibre		Plasma (mmol/l)	Liver μmol/g)
1	200	Cellulose 100	-	3.75 ± 1.00+	6.99 ± 0.35+
2	200	Cellulose 100	+	6.10 ± 0.20***	13.80 ± 0.63***
3	200	Bran 100	+	4.16 ± 0.14*+	8.06 ± 0.40+
4	50	Mould 400	+	4.38 ± 0.15**+	8.38 ± 0.20**+

Significance of difference from diet 1:

* P <0.05, ** P <0.01, *** P <0.001.

Significance of difference from diet 2:

+ P <0.01.

Table 3.4 - Food intake and body weight gains

(Mean values for 6 observations)

Diet	Initial body weight (g)	Final body weight (g)	Weight gain (g/rat)	Food intake g/rat/6 weeks	F.C.E.*
1	136	369	233	858	.271
2	135	370	235	862	.272
3	135	367	237	834	.284
4	136	369	233	852	.273

* $\frac{\text{Total weight gain (g)}}{\text{Total food eaten}}$

Experiment 2 (Table 3.5)

No significant differences were observed in the plasma and liver concentrations of cholesterol of rats fed cellulose, bran or mould diets. Plasma triglycerides were similar throughout the treatments but rats fed bran and mould diets showed increased liver triglyceride concentrations compared with the cellulose group.

Food intakes were similar for all groups over the five week experimental period. The final body weights of the bran and mould fed rats were higher than for the cellulose group and the weight of fresh livers in the bran fed rats was significantly higher than in the cellulose rats but not the mould-fed group. Expressing the liver weights for each group as g/kg body weight shows there were no significant group differences after adjustment for the differences in final body weights.

The faecal excretion of bile acids (mg/rat/24 hours) was significantly raised in the animals receiving bran (19.1 $\pm$ 0.6) and mould diets (10.3 $\pm$ 1.0) compared with the cellulose group (6.2 $\pm$ 0.3). The daily excretion of bile acids in rats of the bran group was 84% greater than the rats fed a mould diet. Faecal sterol excretion (mg/rat/24 hours) was significantly raised in animals receiving the mould diet (28.8 $\pm$ 1.5) compared with rats fed cellulose and bran diets (17.6 $\pm$ 1.5 and 16.0 $\pm$ 1.0 respectively) (Table 3.6).

Rats fed cellulose as a source of indigestible fibre produced 47% more faeces than rats fed bran and 42% more than the mould group (wet weight determination). Both bran and mould diets caused an increased water content of the faeces compared to a cellulose diet: the faeces of rats receiving the mould diet contained 24% more moisture than rats on the bran diet and 74% more than the rats receiving the cellulose diet.

Table 3.5 - Results of Experiment 2

(Mean values ± SEM of six animals per treatment)

	Cellulose	Bran	Mould
Plasma cholesterol (mmol/l)	1.84 ± 0.07	1.91 ± 0.11	2.00 ± 0.06
Plasma triglyceride (mg/l)	435 ± 46	530 ± 46	541 ± 37
Liver cholesterol (μmol/g fresh wt)	7.44 ± 0.22	8.08 ± 0.60	8.14 ± 0.53
Liver triglyceride (μg/g fresh wt)	5656 ± 467	8973 ± 143**	8894 ± 830*
Fresh liver weight (g)	7.86 ± 0.25	9.31 ± 0.58*	8.88 ± 0.45
Liver weight (g)/kg rat body weight)	28.61 ± 0.47	30.90 ± 1.07	29.53 ± 1.15
Mean food intake (g/rat/week)[1]	161.5 ± 3.3	159.9 ± 3.1	160.7 ± 5.0
Mean final body weight (g)[2]	274.0 ± 6.1	299.2 ± 9.5*	300.0 ± 7.9*

Significance of difference (Students 't' test) from cellulose group:

* P <0.05
***P <0.01

[1]Mean value for last weeks of 5 week period

[2]Prior to killing

Table 3.6 - Faecal analyses of Experiment 2

(Mean values ± SEM for 6 observations)

	Cellulose	Bran	Mould
Faecal wet weight (g/24 hours)	4.36 ± 0.18	2.96 ± 0.14***	3.08 ± 0.29***
Faecal dry weight (g/24 hours)	3.14 ± 0.13	1.82 ± 0.09***	1.56 ± 0.15***
Moisture (%)	27.1	38.5***	47.8***+++
Colour of faeces	Grey	Dark Brown	Light Brown
Consistency	Firm	Firm	Soft
Bile acid excretion:			
mg/rat/24 hours	6.2 ± 0.32	19.11 ± 0.63***	10.35 ± 0.97***+++
mg/g faecal dry weight	2.11 ± 0.11	11.14 ± 0.37***	6.81 ± 0.64*+
'Sterol' excretion			
mg/rat/24 hours	17.65 ± 1.52	16.03 ± 1.02	28.78 ± 1.47***+++
mg/g faecal dry weight	5.97 ± 0.49	9.39 ± 0.63**	18.93 ± 0.52***+++

Significance of difference from cellulose diet: * P <0.05; ** P <0.01; *** P <0.001.

Significance of difference from bran diet: + P <0.05; +++ P <0.001.

Table 3.7 - Plasma Ca^{2+} and Mg^{2+} concentrations

	Cellulose	Bran	Mould
Calcium (mmol/l)	3.17 ± 0.07	3.20 ± 0.03	3.19 ± 0.04
Magnesium (mmol/l)	0.97 ± 0.03	0.92 + 0.02	0.91 ± 0.03

No significant differences between treatments in the concentration of plasma calcium or magnesium were observed.

3.4 DISCUSSION

Tsai (1973) reported that additions of cholesterol and cholic acid to rat diets caused large increases in liver and plasma cholesterol concentrations: cholesterol or cholic acid alone only produced small elevations. In the present work rats fed for 6 weeks on a cellulose control diet supplemented with cholesterol (1%) and sodium cholate (0.25%) had increased liver and plasma cholesterol concentrations compared to animals receiving an unsupplemented diet (Table 3.3). Replacement of cellulose by wheat bran or the mould in a hypercholesterolaemic diet resulted in decreased plasma and liver cholesterol values. The mould may resemble other edible fungi which possess cholesterol-lowering properties (Kaneda and Tokuda, 1966) and further experiments have been performed to study their similarities. Wheat bran has not previously been shown to possess hypocholesterolaemic activity in rats although Ranhotra (1973) did comment that bran would maintain low liver cholesterol concentrations in rats fed on diets supplemented with cholesterol. Tsai (1976) maintained rats on diets containing 0.5% cholesterol for 32 days and found that neither pectin, cellulose, gum arabic, caragheen nor wheat bran could reduce serum cholesterol levels but it was stated that only wheat bran had the ability to maintain low body cholesterol. At 42 days, only pectin and caragheen displayed hypocholesterolaemic activity.

Cholesterol and sodium cholate were used to produce large elevations in the concentration of cholesterol in liver and plasma of animals in Experiment 1. Neither Ranhotra nor Tsai *et al* used a bile salt supplement and consequently the concentrations of cholesterol in their experimental animals were not raised much above values found in control rats. It would therefore seem that hypocholesterolaemia can only be produced in rats with raised cholesterol levels and neither wheat bran or the mould could completely overcome the effect of cholesterol and cholate supplements because their plasma and liver cholesterol concentrations were still greater than rats receiving a non-supplemented control diet.

Wheat bran has little effect on serum cholesterol levels in humans (Truswell and Kay, 1975) but it will reduce serum triglycerides and calcium (Heaton and Pomare, 1974). Neither wheat bran nor the mould produced changes in plasma triglycerides or calcium in rats compared to cellulose controls: there was, however, an increase of liver triglycerides in rats receiving bran and mould diets (Table 3.5). A lack of change in plasma calcium might have been expected due to the high inclusion of calcium carbonate in the rat mineral mixture. Plasma magnesium concentrations have been implicated as a possible risk factor in IHD (Seeling and Heggtell, 1974) but analyses of plasma from rats fed diets containing bran or mould failed to show any differences in magnesium levels compared to cellulose controls (Table 3.7).

The concentrations of plasma and liver cholesterol were similar in rats receiving unsupplemented diets containing cellulose, wheat bran or the mould for 5 weeks. Similar results were obtained by Morgan et al (1974) using bagasse fibre. Diets with wheat bran or mould resulted in rats producing a decreased weight of faeces compared to the cellulose group although the water contents were increased. Bran and mould increased bile acid excretion, with the rate of excretion in the bran group being twice that of the mould group (Table 3.6). Faecal neutral sterols output was increased in those animals receiving a mould diet and only part of this increase could be attributed to the extra dietary sterols present in the mould. It therefore appears that despite the lack of change in plasma and liver cholesterol, alterations in sterol metabolism do occur when bran or the mould replace cellulose in the diet of rats.

The rate of cholesterol catabolism is maintained in a repressed state by the cholesterol and bile acids returning to the liver (Dietschy and Wilson, 1966). An increase in faecal bile acid excretion leads to a decrease in bile acids recycling via the entero-hepatic circulation and also in a decreased cholesterol absorption; compensatory increases in cholesterol

synthesis and bile acid formation will presumably occur. Man cannot compensate sufficiently for changes in bile acid excretion by synthesising cholesterol, for example when cholestyramine is administered, and there is therefore a decrease in the total body pool of cholesterol. Rats possess a more efficient compensatory mechanism and little change in total body cholesterol is observed when bile acid excretion is increased. Cholestyramine therefore has a hypocholesterolaemic action in man, but not in the rat (Tennent et al, 1960; Gallo et al, 1966).

The rat is relatively resistant to changes in plasma and liver cholesterol and it is likely that for any hypocholesterolaemic activity to occur, the plasma and liver concentrations of cholesterol have first to be elevated.

CHAPTER 4

EFFECTS OF A MOULD* ON THE CONCENTRATION OF PLASMA AND LIVER CHOLESTEROL IN THE GOLDEN SYRIAN HAMSTER

*See Appendix

4.1 INTRODUCTION

Rabbits and chickens develop atheromas when fed diets supplemented with cholesterol: rats are more resistant to changes in plasma cholesterol and rarely form atheromatous lesions (Marx et al 1951). The use of hamsters as experimental models for atherosclerotic research has been reported (Goldman, 1950; Altschul, 1950) and it seems that they may be more susceptible to changes in plasma and liver cholesterol concentrations than the rat, especially when fed diets containing cholesterol and/or bile acids (Marx, 1951; Van Belle, 1965).

Three experiments are described in this chapter: the first experiment investigates the hypocholesterolaemic activity of the mould in hamsters fed on diets without cholesterol and sodium cholate supplementation. A second experiment was undertaken with animals fed a basal diet containing fractions of the mould to identify which constituent of the mould was responsible for its hypocholesterolaemic activity. A third experiment involved the feeding of diets containing cellulose or wheat bran both with and without added cholesterol and sodium cholate: the experiment was designed to evaluate the ability of bran to lower cholesterol concentrations in hamsters fed normal and high cholesterol diets, thereby providing a comparison between hamsters and rats.

4.2 EXPERIMENTAL

A. DIETS

Experiment 1: The hypocholesterolaemic action of a mould in hamsters

Four experimental diets were fed to groups of 5 hamsters with one cage per group for 11, 26, 36, 45, and 50 days. Plasma and liver samples were collected for total cholesterol analysis after each period of feeding. The composition of the diets is shown in Table 4.1.

Table 4.1 - Composition of Diets for Experiment 1 (g/kg)

Dietary component	Treatment Number			
	1	2	3	4
Mould A3/5	400	200	100	-
Casein	-	100	150	200
Sucrose	150	150	150	150
Vitamin mix	20	20	20	20
Mineral mix	40	40	40	40
Corn oil	100	100	100	100
Solka floc	-	50	75	100
Maize starch	290	340	365	390
Dietary protein supplied by mould (g/kg)	180	90	45	0

Animals of groups 1, 2 and 3 were fed diets containing 40, 20 and 10% mould respectively. The diets were formulated to provide equal concentrations of protein, energy and fibre. Animals in group 4 had casein as a sole source of protein. A fifth group of animals was fed commercial stock diet (Porton Mouse Diet, PMD, Christopher Hill Group Ltd., Poole, Dorset).

All semisynthetic diets were fed in powdered form. Food intakes and body weights were measured weekly.

Experiment 2: The effect of diets containing ether, ethanolic, aqueous and residual fractions of a mould.

Mould was extracted sequentially with diethyl ether, water, 30%, 70% and absolute ethanol. Each extract was added to a basal diet in amounts equivalent to 400 g/kg mould. A sixth fraction consisting of the residue remaining from the fractionation was mixed into the basal diet to replace part of the starch and Solka floc. Two control diets were also fed, one containing 40% untreated mould and the other being the basal diet of casein and cellulose. The diets are shown in Table 4.2.

Eight groups of male hamsters were each fed one of the above diets for 6 weeks. The animals were housed 5 per cage and allowed free access to water and food throughout the experiment. The diets were fed as pellets.

Experiment 3: The influence of cellulose, wheat bran and pectin on the concentration of plasma cholesterol in hamsters.

Hamsters were fed cellulose or wheat bran as a source of indigestible fibre in normo-cholesterolaemic and hypercholesterolaemic diets. A fifth experimental group was fed pectin in a diet without additions of cholesterol and sodium cholate. The diets are shown in Table 4.3.

Each diet was fed to a group of 6 male weanling Golden Syrian hamsters for 5 weeks. Plasma and liver total cholesterol and triglyceride concentrations were measured in all animals. The food was fed in pellet form.

Table 4.2 - Composition of diets for Experiment 2 (g/kg)

Dietary constituent	Treatment Number		
	1	2	3-8* (Basal)
Casein	-	200	200
Mould A3/5	400	-	-
Sucrose	150	150	150
Vitamin mix	20	20	20
Mineral mix	40	40	40
Corn oil	100	100	100
Solka floc	-	100	100
Maize starch	290	390	to 100%

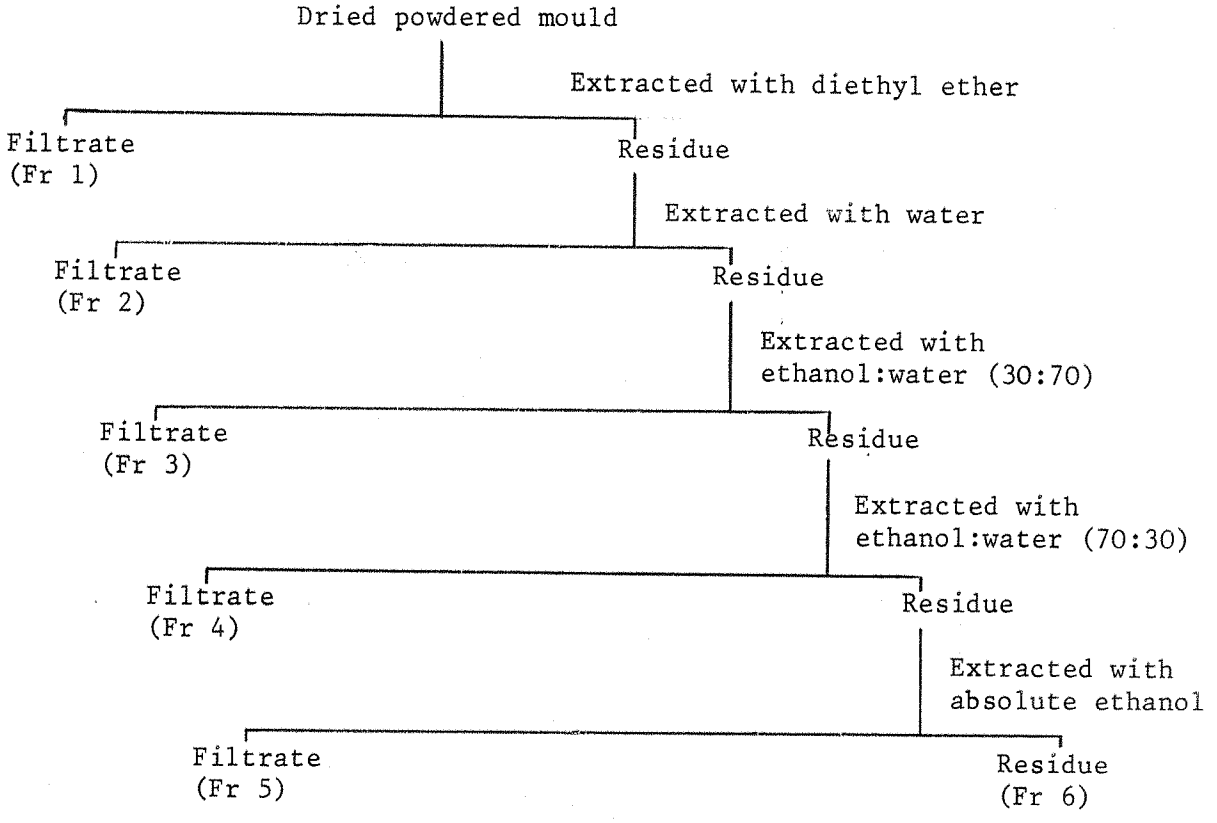
*See section 4.2 B for details of mould fractions in individual diets

Table 4.3 - Composition of diets for Experiment 3 (g/kg)

Dietary constituent	Treatment Number				
	1	2	3	4	5
Casein	200	200	200	200	200
Vitamin mix	20	20	20	20	20
Mineral mix	40	40	40	40	40
Sucrose	150	150	150	150	150
Cellulose	100	100	-	-	50
Wheat bran	-	-	100	100	-
Pectin	-	-	-	-	50
Cholesterol	-	10	-	10	-
Sodium cholate	-	2.5	-	2.5	-
Maize starch	490	477.5	490	477.5	490

B. FRACTIONATION OF MOULD A3/5

An extraction procedure similar to that described by Kaneda and Tokuda (1966) was employed. The extraction procedure was as follows:



Each fraction was prepared by shaking the powdered mould or residue with 200 ml of solvent in a 1 litre stoppered round bottom flask for 5 minutes. The solvent was filtered through a Whatman's No. 54 paper and the residual material was extracted a further two times. The filtrate was rotary evaporated until either a viscous liquid or a solid remained.

The yields of each fraction are shown in Table 4.4.

Table 4.4 - Yields from fractionation experiment

Fraction number	Solvent	% Yield	Diet number
F1	Diethyl ether	3.9	3
F2	Aqueous	29.9	4
F3	Ethanol:water (30:70)	5.6	5
F4	Ethanol:water (70:30)	5.2	6
F5	Absolute ethanol	1.6	7
F6	Residue	53.8	8

C. ANALYTICAL METHODS

Collections of heparinised blood and liver were made following a period of overnight fasting.

Concentrations of plasma and liver total cholesterol in the animals from Experiment 1 were measured by manual methods described in Chapter 2, Section 2.2.

Concentrations of plasma and liver cholesterol in the animals in Experiments 2 and 3 were measured by an automated method outlined in Chapter 2, Section 2.2. Simultaneous triglyceride measurements were performed on samples from the animals of Experiment 3.

4.3 RESULTS

Experiment 1

The animals fed on diets containing three different amounts of mould for 11 days did not show any differences in plasma or liver cholesterol concentrations. Values of plasma cholesterol concentrations in mould fed animals (3.99 - 4.27 mmol/l) were similar to a casein control group (3.82 mmol/l) and a group maintained on stock diet (3.49 mmol/l).

At 26 and 36 days, differences in plasma and liver cholesterol were observed: the concentration of liver cholesterol (8.21 μmol/g) was greatest in the group fed a diet containing 40% mould for 26 days, although the plasma cholesterol was not significantly greater than in the cellulose and casein control diets. Hamsters fed the diet containing 40% mould protein had plasma cholesterol concentrations 33% higher than the casein control animals at 36 days despite liver values being similar.

Hamsters fed for 45 and 50 days on the experimental diets showed similar patterns in their plasma cholesterol levels. The plasma cholesterol was significantly raised in animals fed a casein-cellulose basal ration compared to those animals fed a diet containing 40% mould. At 50 days, all diets containing the mould had lower plasma cholesterol values than the casein control: increasing the amount of mould caused correspondingly greater decreases in plasma cholesterol.

The results are summarised in Table 4.5.

Food intakes and body weights are summarised in Table 4.6. Animals receiving the stock diet had the largest weight gains: these animals ate more and had slightly lower feed conversions than animals fed diets containing the mould. The complete replacement of casein and cellulose by the mould resulted in lower food intakes and raised feed conversion efficiencies which was probably due to the replacement of the bulky cellulose.

Experiment 2

Six fractions were prepared from dried milled mould and each fraction added to a basal diet containing casein and cellulose in quantities equivalent to 400 g mould/kg diet.

Plasma cholesterol concentrations in the group fed untreated mould (1.89 mmol/l) were significantly lower than for a group fed the basal diet (2.39 mmol/l). An ether soluble fraction, F1, produced a similar decrease in plasma cholesterol (1.91 mmol/l) but five other fractions did not show any hypocholesterolaemic activity. One fraction, F4, which was soluble in 70% ethanol resulted in a 37% increase in plasma cholesterol compared with the unsupplemented basal diet.

Plasma triglyceride values were not significantly different throughout the treatment groups with the exception of hamsters fed F3 where a 23% lowering of triglycerides was observed compared with the basal diet.

Table 4.5 - Plasma and liver cholesterol analyses for Experiment 1

(Mean values ± SEM for 5 observations)

Diet		Concentration of cholesterol									
		Day 11		Day 26		Day 36		Day 45		Day 50	
Casein (g/kg)	Mould (g/kg)	Plasma (mmol/l)	Liver (μmol/g)	Plasma (mmol/l)	Liver (μmol/g)	Plasma (mmol/l)	Liver (μmol/g)	Plasma (mmol/l)	Liver (μmol/g)	Plasma (mmol/l)	Liver (μmol/g)
200	0	3.28±0.34	-[1]	4.92±0.25	7.07±0.24	3.71±0.24	7.36±0.15	5.74±0.28	8.11±0.28	5.76±0.29	-[1]
150	100	4.26±0.86	7.28±1.26	4.69±0.32	7.80±0.13*	3.57±0.10	7.07±0.12	5.29±0.21	7.81±0.15	4.72±0.22*	-[1]
100	200	3.60±0.23	7.55±0.80	3.74±0.13**	7.64±0.11	5.07±0.19***	7.67±0.17	4.69±0.34*	7.73±0.16	4.10±0.07***	-[1]
0	400	4.00±0.28	8.08±0.31	5.51±0.54	8.21±0.22**	4.94±0.04***	7.70±0.13	3.63±0.10***	6.68±0.25**	3.65±0.16***	-[1]
Stock Diet		3.49±0.08	7.39±0.10	3.90±0.22	7.44±0.14	3.54±0.18	7.45±0.18	3.56±0.08	7.41±0.12	4.25±0.27	-[1]

-[1] Measurement not performed

Significance of difference (Students 't' test) from Group 1

* P <0.05

** P <0.01

*** P <0.001

Table 4.6 - Food intakes, body weight gains and F.C.E.[2]

Parameter	Period (days)	Dietary group				
		1	2	3	4	5
Food intake[1]	0-11	70.4	63.8	63.5	69.7	78.4
Weight gain	0-11	19.6	17.3	18.1	20.3	20.1
F.C.E.	0-11	.279	.270	.284	.289	.256
Food intake	0-26	115.7	145.8	135.9	146.1	195.0
Weight gain	0-26	30.9	32.6	39.2	41.5	42.6
F.C.E.	0-26	.267	.223	.291	.283	.218
Food intake	0-36	172.8	160.2	166.3	216.0	241.5
Weight gain	0-36	38.0	36.6	35.9	43.2	44.3
F.C.E.	0-36	.219	.228	.216	.200	.183
Food intake	0-45	247.5	217.0	215.5	267.3	331.2
Weight gain	0-45	43.8	38.7	40.1	43.9	54.5
F.C.E.	0-45	.177	.178	.186	.163	.164
Food intake	0-50	265.0	235.0	240.0	285.0	360.0
Weight gain	0-50	44.2	38.1	40.3	45.9	57.2
F.C.E.	0-50	.167	.162	.168	.161	.159

[1] g/hamster. Mean value from a group of 5

[2] Total weight gain (g) divided by total food eaten (g)

Table 4.7 - Plasma cholesterol and triglyceride values in Experiment 2

(Mean values ± SEM for 5 observations)

Diet	Solvent	Cholesterol (mmol/l)	Triglyceride (mg/l)
Untreated mould	-	1.89±0.29*	904±123.1
Basal	-	2.39±0.19	1076±209.0
Basal + F1	Diethyl ether	1.91±0.06*	662± 42.9
Basal + F2	Water	2.63±0.16	1140±126.9
Basal + F3	Water:alcohol (30:70)	2.83±0.18	824± 79.8*
Basal + F4	Water:alcohol (70:30)	3.30±0.11**	1024± 83.2
Basal + F5	Absolute alcohol	2.31±0.15	1086±112.2
Basal + F6	Residue	2.39±0.27	1040±186.7

Significance of the difference from group 2: * $P < 0.05$

** $P < 0.01$

Table 4.8 - Weight gain and food intake (Experiment 2)

Diet	Mean Wt. gain (g) over 6 weeks	Food intake (g/100 g body wt./day)
Untreated mould	45.3	7.8
Basal	39.5	6.8
Basal + F1	37.9	7.2
Basal + F2	44.8	7.0
Basal + F3	36.4	7.1
Basal + F4	35.9	6.9
Basal + F5	33.2	6.9
Basal + F6	38.8	9.1

Liver weights of animals fed the basal diet + F2 were 50% greater than those fed the basal diet only (Table 4.9): the triglyceride content (mg/liver) was also higher in this group. The hepatic content of cholesterol was significantly lower in the group fed basal + F1 diet (6.1 mg/liver) than the basal only group (8.2 mg/liver) although this was not obvious when the difference in liver weights were accounted for. No significant changes in either liver triglycerides or cholesterol were found throughout the groups when values were expressed as μg/g liver fresh weight other than a raised triglyceride value in those animals fed basal + F2 diet.

Experiment 3

Supplementing a diet containing cellulose with 1% cholesterol and 0.25% sodium cholate caused a 69% elevation in the concentration of plasma cholesterol and liver cholesterol concentration was increased twenty-fold (Table 4.10). The liver weight of hamsters fed an unsupplemented cellulose diet (2.3 g) was significantly lower than the weight of supplemented animals (3.5 g) and there were large differences in the total amount of cholesterol stored in the livers, (9.6 mg and 293 mg/liver respectively).

Replacing cellulose with wheat bran led to increased concentrations of plasma and liver cholesterol in unsupplemented diets. Hamsters fed a diet containing wheat bran supplemented with cholesterol and cholate had a higher plasma cholesterol (4.56 mmol/l) than an unsupplemented diet (3.97 mmol/l) but the concentration was lower than in the supplemented cellulose diet (5.24 mmol/l): similar differences were found with the concentrations of cholesterol in livers.

Additions of pectin, partially replacing cellulose, did not significantly alter plasma cholesterol or triglyceride values but a two-fold increase in liver cholesterol concentration was observed compared with animals fed the unsupplemented cellulose diet. The concentration of liver cholesterol in animals fed on pectin (21.93 μmol/g) and on unsupplemented bran diet (22.70 μmol/g) were similar. All animals receiving cholesterol and cholate supplements had large pale livers.

Table 4.9 - Liver cholesterol and triglycerides for Experiment 2

(Mean values ± SEM for 5 observations)

Diet	Liver wt. (g)	Liver cholesterol (μmol/g)	Liver triglyceride (mg/g)
Untreated mould	3.3 ± 0.6	6.25 ± 0.59	14.8 ± 0.7
Basal	3.3 ± 0.2	6.48 ± 0.24	13.4 ± 0.6
Basal + F1	2.7 ± 0.2*	5.87 ± 0.17	10.8 ± 2.6
Basal + F2	3.6 ± 0.2	7.32 ± 0.70	20.2 ± 1.8*
Basal + F3	3.5 ± 0.4	5.79 ± 0.63	10.7 ± 1.3
Basal + F4	3.2 ± 0.3	7.43 ± 0.36	15.7 ± 3.1
Basal + F5	3.5 ± 0.6	5.91 ± 0.42	13.9 ± 1.3
Basal + F6	3.4 ± 0.7	6.30 ± 0.66	15.2 ± 1.8

Significance of difference from group 2: * $P < 0.05$

Table 4.10 - Plasma and liver cholesterol and triglyceride values

(Mean values ± SEM for 6 observations)

Fibre	Cholesterol + cholate supplement	Plasma		Liver		
		Cholesterol (mmol/l)	Triglyceride (mg/l)	Weight (g)	Cholesterol (μmol/g)	Triglyceride (mg/g)
Cellulose	-	3.09±0.12+++	712±84	2.3±0.1+++	10.84±1.03+++	37.6± 4.2
Cellulose	+	5.24±0.25***	614±67	3.5±0.2***	216.96±7.48***	47.5± 5.6
Wheat bran	-	3.97±0.30*++	517±34**+	2.9±0.2**	22.70±1.81**+++	50.2±11.6
Wheat bran	+	4.55±0.16***+	394±34**+	4.4±0.3***+	155.31±2.01***+	25.0±6.5+
Pectin	-	2.99±0.17+++	766±49	2.3±0.1+++	21.93±2.58**+++	22.9±4.3*++

Significance of difference from group 1: * P <0.05; ** P <0.01; *** P <0.001

Significance of difference from group 2: + P <0.05; ++ P <0.01; +++ P <0.001

Table 4.11 - Weight gain and food intake in Experiment 3

	Group				
	1	2	3	4	5
Mean food intake (g/100 g animal/day)	7.7	6.9	7.3	6.9	8.5
Mean weight gain (g/animal/week)	8.6	7.2	7.5	7.1	8.8
F.C.E.[1] (0-5 weeks)	0.216	0.210	0.212	0.200	0.192

[1] $\frac{\text{g food eaten}}{\text{g live wt. gain}}$

4.4 DISCUSSION

Diets containing 10, 20 or 40% of a Fusarium mould did not lower the concentrations of plasma or liver cholesterol of hamsters after feeding for up to 36 days. Hypocholesterolaemic activity was found in diets containing 20 and 40% mould at 45 days, however, and with 10, 20 and 40% mould at 50 days: the hypocholesterolaemic activity was greatest in diets containing the highest level of mould (Table 4.5).

The mould is not unique as a fungal species in possessing cholesterol lowering properties. Kaneda and Tokuda (1966) showed that a range of common edible fungi would suppress the effects of cholesterol feeding in rats. The hypocholesterolaemic activity of the mushroom was attributed to a water soluble extract and two active compounds have been isolated (Chibata et al, 1969; Rokuyo et al, 1970). Extracting the mould by a procedure similar to Kaneda and Tokuda and feeding each fraction to hamsters suggests that a lipid (ether soluble) portion of the mould was the only fraction able to lower plasma and liver cholesterol (Table 4.7 and 4.9) whilst a water soluble fraction had no hypocholesterolaemic activity in hamsters. β-Sitosterol will decrease cholesterol absorption in rats (Best and Duncan, 1957) and the hypocholesterolaemic action of the ether fraction might be due to a decreased reabsorption of endogenous cholesterol.

Differences in the response of rats and hamsters to cholesterol and bile acid feeding have been reported (Van Belle, 1965). The hamster is more susceptible than the rat to elevations in the concentration of plasma and liver cholesterol as a result of feeding cholesterol. The supplementing of a diet with cholesterol and bile salts resulted in a five-fold increase of plasma cholesterol and a forty-fold increase in liver cholesterol (Marx, 1951): similar changes were obtained in hamsters fed a cellulose and casein diet (Table 4.10). Rats did not show such large concentrations of cholesterol in their livers when fed diets supplemented with

cholesterol and cholate (Chapter 3, Table 3.3) and it therefore seems that hamsters are able to accumulate large amounts of cholesterol in their livers compared with rats.

Wheat bran and the mould showed similar hypocholesterolaemic activities in rats fed diets supplemented with cholesterol and sodium cholate, but no activity was found in rats fed unsupplemented diets. The cholesterol lowering action of the mould has been demonstrated in hamsters receiving diets low in cholesterol (Tables 4.5 and 4.7), which may suggest that the hamster is a more suitable experimental animal for the elucidation of hypercholesterolaemic compounds (other advantages include lower food intake and the presence of a gall bladder). The replacement of cellulose by wheat bran did not decrease plasma cholesterol values but tended to increase them. Bran in diets supplemented with cholesterol and sodium cholate did have some cholesterol lowering action compared with a supplemented cellulose group but this was more conclusive in rats (Table 4.10). There is therefore a difference in the response of hamsters to wheat bran and the mould. Hamster diets supplemented with cholesterol and sodium cholate are obligatory for a hypocholesterolaemic action in bran whereas this is not so for mould and there may be differences in the mechanism responsible for lowering cholesterol concentrations.

The concentration of plasma cholesterol in hamsters fed a diet containing pectin was similar to the group receiving a cellulose diet. This difference in response between hamsters fed mould or pectin against cellulose controls suggest that the mode of action of the mould is different to that of pectin.

CHAPTER 5

THE BINDING OF BILE SALTS IN VITRO TO FIBRE FROM WHEAT BRAN AND A MOULD* (FUSARIUM) AND THE EFFECT OF CHEMICAL FIBRE PREPARATIONS ON CONCENTRATIONS OF CHOLESTEROL IN THE RAT

*See Appendix

5.1 INTRODUCTION

Diets with a high content of dietary fibre have been shown to lower cholesterol concentrations: alfalfa will overcome the effect of feeding cholesterol to rabbits (Cookson et al, 1967) and substituting wheat straw for cellophane in diets containing butterfat decreased plasms cholesterol and the incidence of atheromas in rabbits (Moore, 1967). The hypocholesterolaemic action of dietary fibre may be attributed to its ability to alter cholesterol absorption and increasing bile acid excretion. The ion exchange resin cholestyramine will lower serum cholesterol levels in man by increasing bile acid excretion (Hagerman et al, 1973). The increased excretion of bile acids results in a greater hepatic catbolism of endogenous cholesterol and the absorption of exogenous cholesterol in the small intestine is impaired because of the disruption in the formation of micelles.

Eastwood and Boyd (1967) have described the binding of bile salts to a solid phase within the small intestine of the rat. Dry grain has been found to bind bile salts in vitro (Eastwood and Hamilton, 1968) and a lignin preparation of the dry grain had a high affinity for bile salts. The mechanism of binding was suggested to be hydrophobic although this is not entirely supported by Story and Kritchevsky (1976). Morgan et al (1973) found little binding activity in bagasse lignin and studies in human volunteers fed lignin have not demonstrated a lowering of serum lipids (Barnard and Heaton, 1973; Heaton et al, 1972).

A variety of foods have been found to bind bile acids (McConnell et al, 1974) and in order to identify the active components of wheat bran and the mould, different chemical fibre preparations were tested for their ability to bind bile salts in vitro. Furthermore, the differing capacities for chemical fibre preparations to bind bile acids in vitro have been compared to their cholesterol lowering activities in rats.

5.2 METHODS

A. PREPARATION OF CHEMICAL FIBRE

The term 'Dietary Fibre' (Trowell, 1972) distinguishes between the components of food which are indigestible to the enzymes of the intestine and crude fibre (AOAC, 1970) which is the solid remaining after the chemical treatment of food with acid and alkali. The low values obtained for crude fibre analyses can be partly overcome by omission of the alkali boiling stage, thereby increasing the amount of lignin in the residual material. Van Soest (1963) has adopted a procedure for the chemical analysis of foods using an acid and detergent digestion. A similar method has also been devised by Griffith and Jones (1963). Both methods exclude many of the components which constitute dietary fibre (Trowell et al, 1976) such as hemicelluloses and pectins but they do give a more accurate estimation of lignin and cellulose than the conventional AOAC method (McConnell and Eastwood, 1974).

Although analytical methods for the estimation of individual components of dietary fibre are available (Southgate, 1969; Morley et al, 1972), three types of ligno-cellulose residues prepared from wheat bran were used to investigate the bile acid binding capacity of fibre: crude fibre (CF), normal acid fibre (NAF) and acid detergent fibre (ADF).

a) Crude fibre

(Official Methods of Analysis of the Association of Official Analytical Chemists, U.S.A.: (1970) 11th Edition, p. 129).

Dried defatted wheat bran was milled to pass through a 1 mm sieve. 2 g Bran were added to a 1 litre beaker containing 200 ml sulphuric acid (12.5 g/l). The contents of the beaker were rapidly brought to boiling point and gently boiled for 30 min with continual stirring. The contents were filtered hot, washed with distilled water and transferred to a 1 litre beaker. The contents were boiled with 100 ml sodium hydroxide (12.5 g/l) for 30 minutes, after which they

were filtered hot through a sintered glass crucible, washed three times with 50 ml hot water and once with 50 ml hot ethanol. The residue was dried at 100°C in a force-draught oven for 8 hours. The dried residue was cooled in a dessicator over phosphorus pentoxide. For gravimetric analyses, the crude fibre content was calculated as being the loss in weight on ashing.

b) Normal acid fibre

The preparation of normal acid fibre (NAF) has been described by Griffith and Jones (1963).

2 g ground dried wheat bran was extracted with an ethanol:benzene solution (1:1 v/v) for 8 hours. The defatted bran was placed in a 500 ml round bottom flask containing 200 ml 1 N sulphuric acid with 0.2 ml Teepol (proprietory grade) and refluxed for 3 hours.

The contents of the flask were filtered through a sintered glass crucible (porosity 1) washed three times with 50 ml hot distilled water, once with 50 ml ethanol and once with 50 ml diethyl ether. The residue was dried at 105°C for 8 hours.

A modified procedure for the preparation of NAF was introduced by Griffith and Jones in 1966. This method involved a preliminary incubation of the feed in pepsin and the resulting fibre was claimed to have a lower contamination of nitrogenous material.

1 g of defatted bran was added to 50 ml of a pepsin/hydrochloric acid solution (0.2 g pepsin in 100 ml 0.1 N hydrochloric acid) in a 100 ml stoppered Erlenmeyer flask. The contents were gently shaken overnight at 40°C after which they were filtered through a sintered glass crucible, washed with distilled water and a fibre extraction performed as described above.

Lower yields (0.5 to 1.0% lower) were obtained for pepsin-NAF than NAF. It was decided not to use the pepsin incubation in the preparation of NAF from wheat bran because no significant differences in the in vitro binding capacities could be detected between the two fibre preparations.

c) Acid detergent fibre

(Van Soest, 1963)

2 g of wheat bran, milled to pass through a 1 mm sieve, were added to a 250 ml round bottom flask containing 100 ml of cold acid-detergent solution (2 g cetyltrimethylammonium bromide dissolved in 100 ml 1 N sulphuric acid). 2 ml of decalin (decahydronephthalene) were added to prevent excessive foaming. The contents of the flask were rapidly brought to boiling and gently refluxed for 60 minutes. After 60 minutes, the contents were filtered,whilst hot, through a sintered glass crucible (porosity No. 1) and washed twice with 100 ml boiling distilled water. The residue was washed with 100 ml acetone and dried in an oven overnight at 105°C.

B. PREPARATION OF LIGNIN

Lignin was prepared by treatment of acid detergent fibre with 72% (w/w) sulphuric acid (Van Soest, 1963).

50 ml of 72% sulphuric acid were added to a beaker containing 5 g of acid detergent fibre. The contents of the beaker were allowed to stand at room temperature for three hours with stirring every minute . The contents were filtered through a sintered glass crucible and excess acid was washed out with three 100 ml aliquots of hot distilled water. The lignin remaining in the crucible was dried in an oven at 105°C overnight.

C. IN VITRO BINDING STUDIES

The procedure for measuring the binding capacity of bran and mould fibre was a modification of the method of Eastwood and Hamilton (1968). Samples of defatted milled wheat bran and the mould were shaken in hot 85% aqueous methanol to remove any excess sugars and free starchy endosperm (Southgate, 1969). The samples were filtered, washed in hot distilled water and dried in a vacuum oven at 65°C. All test material was ground to a particle size capable of passing through a 1 mm wire mesh sieve.

100 mg of the test material was weighed into a screw top glass universal bottle (capacity 25 ml). 10 ml of either sodium cholate or sodium taurocholate solution (400 mg dissolved in 100 ml 0.1 M phosphate buffer at pH 6, 7 or 8) were added to the bottle and the stoppered bottle was gently shaken at room temperature for 3 hours. Quadruplicate assays were used for each sample of test material. After 3 hours of incubation the bottles were spun at 2500 xg for 10 minutes and duplicate 200 µl aliquots of the bile salt solution were removed for analysis.

The concentrations of sodium taurocholate were determined by a modified Pettenkoffer reaction (Boyd, Eastwood and McLean, 1966) and sodium cholate by a UV absorbance method described by Mosbach et al, (1954).

a) Sodium taurocholate samples, (200 µl), were pipetted into test tubes and brought to dryness in a heating block at 120°C. 5 ml of 70% sulphuric acid was added to each tube and after 5 minutes an addition of 1 ml 0.25% aqueous furfural solution was made. The contents of the tubes were shaken and allowed to stand at room temperature for 60 minutes. The absorbance of the solutions were measured on a Pye Unicam SP 500 spectrophotometer at 510 nm using 1 cm light path glass cuvettes. A standard curve was constructed with a range of 10-100 µg sodium taurocholate. All standards and test solutions were read against a reagent blank containing sulphuric acid and furfural solution.

b) Sodium cholate samples, (200 µl), were added directly to 5 ml of 65% sulphuric acid in a glass test tube. The contents of the tube were heated in a water bath for 15 minutes at 60°C after which they were rapidly cooled to room temperature, transferred to 1 cm light path silica cuvettes and the absorbance at 320 nm read on a Pye Unicam SP 500 spectrophotometer. All test samples were read against a blank of 65% sulphuric acid. Standards of 10-150 µg sodium cholate were used to construct a standard curve.

The binding capacity of test material was expressed as the percentage difference between the concentration of bile salt in controls (buffer and bile salts only) and the concentration in the test incubations after 3 hours.

The binding capacities of cellulose, cholestyramine, lignin, bran, mould and chemical fibre preparations from bran were measured. The influence of pH of the incubation medium was determined with wheat bran fibre and mould ADF.

D. WATER ADSORPTION IN BRAN AND THE MOULD

The water holding capacities of wheat bran and the mould were estimated by incubating 500 mg of ground sample with 15 ml water for 18 hours. Five incubations per sample were carried out in glass universal bottles with gentle shaking at room temperature. After 18 hours, the bottles were centrifuged at 5000 xg for 45 minutes, the excess water discarded, and the hydrated solids weighed. The differences in weight of samples before and after incubation were indicative of their water holding capacities.

E. IN VIVO EVALUATION OF THE BILE ACID SEQUESTERING PROPERTIES OF CHEMICAL FIBRE PREPARATIONS FROM WHEAT BRAN

The total body cholesterol of rats was elevated by including cholesterol and sodium cholate in their diets for 6 weeks, using cellulose (Solka

floc) as a source of indigestible fibre. The cellulose was partly replaced by chemical fibre preparations of wheat bran (crude fibre (CF); normal acid fibre (NAF) and acid detergent fibre (ADF) prepared as described in Section 4.2, A.), on the sixth week of feeding. The ability of chemical fibre to lower plasma cholesterol concentrations in rats with a prolonged intake of a hypercholesterolaemic diet was determined after a further 6 weeks.

Six groups of five 150 g male Wistar rats were housed 5 per cage and fed their respective diets ad libitum for 12 weeks. Animals of Group 1 were maintained on diet A for 12 weeks. Animals of groups 2, 3, 4, 5 and 6 were fed diet B for six weeks after which groups 3, 4, 5 and 6 were fed diets C, D, E and F respectively for the final 6 weeks. Group 2 animals received diet B for a final 6 weeks. All diets were given as a powder (Table 5.2).

At 12 weeks, plasma samples were prepared for analyses of total, free and esterified cholesterol.

Total cholesterol was determined manually by the method of Zlatkis et al (1953) outlined in Chapter 2.

Free cholesterol and cholesterol esters were estimated by the method described by Kerr and Bauld (1953). A slurry of 200 mg activated alumina (activated by heating in an oven at 150°C) in petroleum ether (40-60°C fraction) was packed into a 100 mm long x 6 mm diameter glass column. 0.2 ml plasma was added to 2 ml acetone:ethanol solution (1:1, v/v) in a stoppered tube. The tube was shaken, centrifuged and the supernatant transferred to a glass test tube. The solvent was evaporated in a hot water bath and the solid redissolved in 2 ml petroleum ether (40-60°C range). The solution was applied to the alumina column using two 0.5 ml washouts with petroleum ether.

Cholesterol esters were eluted by running the 10 ml aliquots of petroleum ether through the column, whilst the free cholesterol

Table 5.1 - Composition of diet (g/kg)

Dietary component	Treatment Number					
	A	B	C	D	E	F
Casein	200	200	200	200	200	200
Milled sucrose	150	150	150	150	150	150
Mineral mixture	40	40	40	40	40	40
Vitamin mixture	20	20	20	20	20	20
Corn oil	100	100	100	100	100	100
Cholesterol	-	10	10	10	10	10
Sodium cholate	-	2.5	2.5	2.5	2.5	2.5
Solka floc	100	100	90	90	90	-
Crude fibre	-	-	10	-	-	-
Normal acid fibre	-	-	-	10	-	-
Acid detergent fibre	-	-	-	-	10	-
Milled wheat bran	-	-	-	-	-	100
Maize starch	390	377.5	377.5	377.5	377.5	377.5

Table 5.2 - Feeding regime of experimental diets

Treatment group	Diet	
	0-6 weeks	6-12 weeks
1	A	A
2	B	B
3	B	C
4	B	D
5	B	E
6	B	F

fraction was collected after elution with two 10 ml portions of benzene. Each fraction was collected in a 30 ml boiling tube and the respective solvents evaporated.

Cholesterol and its esters remaining in the boiling tubes were measured by the method of Zlatkis et al (1953).

Plasma triglycerides were estimated by a manual method (Van Handel, 1960) outlined in Chapter 2.

5.3 RESULTS

A. IN VITRO BINDING STUDIES

Cellulose (Solka floc) and the mould had low capacities to bind sodium cholate at pH 7.0 (Table 5.2). Bran was able to bind 14% of the cholate whilst a lignin preparation from wheat bran could only bind 10% of the bile salt. Cholestyramine resin ('Questran', Bristol Laboratories) bound in excess of 80% of the sodium cholate in the incubation medium. All test solids were added to 10 ml of the bile salt incubation solution in 100 mg quantities.

Acid detergent fibre (ADF), normal acid fibre (NAF) and crude fibre (CF) prepared from wheat bran had greater capacities to bind sodium cholate than the equivalent weight of bran (Table 5.3). At pH 6, bran ADF showed a greater affinity for cholate than NAF and CF. ADF prepared from the mould showed little binding capacity.

The amount of sodium cholate bound to fibre prepared from bran was greater than for the taurine conjugate, sodium taurocholate. The pH of the incubation medium influenced the capacity of fibre to bind bile salts in such a way that an increase of pH led to a decrease in binding (by analysis of variance, $P < 0.001$).

Table 5.3 - Binding capacities of bran, mould, cellulose, cholestyramine and lignin to sodium cholate at pH 7.0

(Mean ± SEM for 4 samples)

Solid phase	% Cholate bound
Milled bran	14.2 ± 1.0
Milled mould	6.8 ± 0.9
Cholestyramine	82.6 ± 3.2
Cellulose	3.8 ± 0.2
Lignin	10.4 ± 0.8

Table 5.4 - Binding of sodium cholate to fibre preparations

(Mean ± SEM for 4 observations)

Fibre type	Bile salt bound %		
	pH 6	pH 7	pH 8
Bran crude fibre	34.7 ± 5.7	21.6 ± 2.1	13.3 ± 1.8
Normal acid fibre	39.9 ± 4.9	37.0 ± 5.1	15.2 ± 1.2
Acid detergent fibre	54.1 ± 2.1	30.3 ± 2.9	23.5 ± 4.5
Mould acid detergent fibre	9.7 ± 0.7	10.0 ± 0.9	7.7 ± 0.8

Table 5.5 - Binding of sodium taurocholate to fibre preparations

(Mean ± SEM for 4 observations)

Fibre type	Bile salt bound %		
	pH 6	pH 7	pH 8
Bran crude fibre	20.2 ± 1.8	13.8 ± 0.4	21.9 ± 2.9
Normal acid fibre	23.6 ± 1.3	20.2 ± 1.3	19.5 ± 3.9
Acid detergent fibre	28.5 ± 0.7	24.5 ± 1.2	13.6 ± 2.5
Mould acid detergent fibre	8.5 ± 0.9	6.4 ± 0.7	4.5 ± 0.3

B. WATER HOLDING CAPACITIES

Bran, mould and cellulose were incubated in water, centrifuged and the amount of water (surface, interstitial and free water) held by each sample determined.

Table 5.6 - Retention of water by bran, mould and cellulose

(Mean ± SEM for 6 observations)

	g water/500 mg sample
Bran	1.2 ± 0.1
Mould	3.8 ± 0.3
Cellulose	0.4 ± 0.1

Cellulose had a low capacity to adsorb water whereas the capacity in the mould was high. Wheat bran incubated as a powder had only a third of the mould's capacity to adsorb water.

C. IN VIVO ACTIVITY OF BRAN FIBRE

All animals showed similar weight gains throughout the 12 week period of experimentation. Diets containing CF, NAF and ADF caused no changes in food intake. The replacement of cellulose by wheat bran in diet F gave rise to a seven percent decrease in food intake with little change in growth performance (Table 5.7).

Animals receiving diets containing cholesterol and sodium cholate had pale 'fatty' livers at week 12. One animal from group 2 (cellulose with cholesterol and cholate) was culled at week 8 because of very severe wet sores on its skin.

Table 5.7 - Body weights and food conversion efficiency

	Week	Group					
		A	B	C	D	E	F
Mean body wt. (g)	0	150	151	150	149	151	150
Mean body wt. (g)	6	340	335	338	341	332	338
Mean body wt. (g)	12	480	472[1]	469	473	481	468
FCE	6-12	.201	.186	.188	.174	.195	.198

[1]Mean of 4 observations

The addition of cholesterol and sodium cholate to diets containing cellulose resulted in elevated concentrations of plasma total cholesterol: there was a 19% increase in free cholesterol and a two-fold increase in esterified cholesterol. Neither CF, NAF nor ADF could counteract the effects of a hypercholesterolaemic diet. Replacement of cellulose with bran decreased the total and free cholesterol but no significant changes were observed in esterified cholesterol (probably due to the large standard error in the cellulose group). Animals receiving a diet containing bran had increased concentrations of triglyceride compared to other treatments. The results are summarised in Table 5.8.

5.4 DISCUSSION

The binding of sodium cholate and taurocholate to chemical fibre preparations from wheat bran increased as the pH of the incubation medium decreased. Eastwood and Hamilton (1968) using a residue of maize and barley also found a pH dependent binding and suggested that the binding was hydrophobic in nature. Story and Kritchevsky (1976) using a radioactive method described by Hagermann et al (1973) found a relatively low capacity for wheat bran to bind cholate compared to cholestyramine and this was confirmed in the results of Table 5.4. However, the use of wheat bran as a source of dietary fibre means that an adequate daily intake (10-15 g/day) will compensate for the lower binding capacity and it may still have a bile acid sequestering activity of therapeutic value. Experiments with humans have so far failed to produce a hypocholesterolaemic effect but this may be due to the relatively short period of experimentation and the use of subjects whose cholesterol values are not initially elevated (Durrington et al, 1975; Truswell and Kay, 1975).

Cellulose had very little binding activity, confirming the observations made by Kritchevsky and Story (1974). Chemical fibre preparations of bran possessed greater binding than untreated bran,

Table 5.8 - Plasma cholesterol analyses

Group	Cholesterol + cholate	Fibre	Plasma cholesterol (mmol/l)			Plasma triglyceride (mg/l)
			Total	Free	Esterified	
1	-	Cellulose	2.62±0.24+	1.05±0.12+	1.58±0.14++	735 ± 99
2	+	Cellulose[1]	5.07±0.73**	1.62±0.23*	3.17±0.54*	730 ± 84
3	+	Cellulose + CF	5.05±0.24**	1.63±0.11*	2.90±0.16**	745 ± 33
4	+	Cellulose + NAF	4.66±0.18**	1.53±0.10*	3.00±0.12**	686 ± 49
5	+	Cellulose + ADF	4.82±0.32**	1.49±0.09*	3.10±0.22**	783 ± 127
6	+	Bran	3.63±0.21*+	1.51±0.32*	2.20±0.47	922 ± 39*

All values expressed as mean ± SEM for 5 observations except where otherwise stated.

[1]Mean ± SEM for 4 observations.

Significance of the difference from group 1: * $P < 0.05$; ** $P < 0.01$.

Significance of the difference from group 2: + $P < 0.05$; ++ $P < 0.02$.

on an equal weight basis. Little of the activity of bran was lost in the least severe preparation (ADF) compared to the more severe treatment encountered with CF. The difference in binding between of CF and ADF suggests that either structural damage has lessened the affinity of CF or there is less lignin present (Van Soest, 1977) although Porter and Singleton (1971) have stated that lignin may also be lost in the preparation of ADF.

Eastwood and Hamilton (1968) demonstrated that lignin was the major component of dry grain responsible for binding bile salts. Story and Kritchevsky (1976) agree with this observation although their results do not adhere to the theory of a hydrophobic binding. Morgan et al (1974) found that bagasse lignin had very little affinity for bile salts and the results of Table 5.2 using Klason lignin prepared from bran suggest that lignin may not be the active component of bran. It is likely that the preparation and source of lignin is very important and severe treatment such as using 72% sulphuric acid probably destroys the integral physical structure of the lignin matrix.

The mould and mould ADF did not exhibit a high affinity for either sodium cholate or taurocholate, and its hypocholesterolaemic action may not be attributed to its fibrous material but to a lipid fraction (Owen et al, 1976) as was described in Chapter 4.

The hypocholesterolaemic action of bran (Owen et al, 1975a) detailed in Chapter 3, could be partly due to its ability to bind bile salts, reducing the amount of bile acids circulating in the entero-hepatic circulation, and thus causing an increased faecal excretion (Owen et al, 1975b). Cholestyramine will not influence plasma cholesterol concentrations in normo-cholesterolaemic rats (Gallo et al, 1966) and the in vivo evaluation of chemical fibre prepared from bran was therefore made in rats whose body cholesterol had been previously elevated for 6 weeks. However, replacing one tenth of the cellulose in the rats' diets with chemical fibre

(equivalent to adding 8-10% bran) had no effect on plasma cholesterol. It therefore seems that despite the large *in vitro* capacity for bran fibre to bind bile salts, the *in vivo* results suggest that there are other factors responsible for the overall hypocholesterolaemic properties of wheat bran.

CHAPTER 6

THE HALF-LIFE OF [G-^{3}H]-CHOLESTEROL AND THE FAECAL EXCRETION OF STEROLS IN RATS FED DIETS CONTAINING CELLULOSE, WHEAT BRAN AND A MOULD*

*See Appendix

6.1 INTRODUCTION

The hypocholesterolaemic action of wheat bran and the mould has been established in rats fed on diets containing cholesterol and sodium cholate. A cereal stock diet has been reported to cause an increased excretion of bile acids compared to a semi-synthetic diet (Gustafsson and Norman, 1969) and supplementing a semi-synthetic diet with soya-bran decreased plasma cholesterol and increased faecal bile acid excretion (Balmer and Zilversmit, 1974). Kritchevsky et al (1973) suggested that fibrous diets will decrease the amount of cholesterol absorbed whereas Balmer and Zilversmit (1974) claimed that there was little difference in the absorption of cholesterol between semi-synthetic diets with and without soyabran. It has been suggested that the turnover of cholesterol was altered by adding soyabran to a diet, probably resulting from the binding of bile acids in the gut and their subsequent removal from the enterohepatic circulation. Wheat bran will bind bile salts in vitro (Owen et al, 1975b; Story and Kritchevsky, 1976) and an experiment was therefore designed to measure the turnover rates of cholesterol in rats fed diets containing cellulose, wheat bran or the mould.

6.2 EXPERIMENTAL

A. DIETS (Table 6.1)

Six sets of three litter mates were singly housed in metabolism cages. The rats were male Wistars, 4 weeks of age at the start of the experiment. All animals were maintained on a casein-cellulose diet containing cholesterol (10 g/kg) and sodium cholate (2.5 g/kg) for 32 days. At 32 days, the diet was changed to a low cholesterol diet containing cellulose (diet A), wheat bran (diet B) or the mould (diet C) as a source of dietary fibre. Each animal was injected intraperitoneally with [^{3}H]-cholesterol (50 μCi/100 g body weight, dissolved in Tween) at 35 days and faecal collections were made twice weekly for a further 31 days. The specific activities of plasma and liver cholesterol were determined after 66 days.

Table 6.1 - Diets for cholesterol turnover experiment (g/kg)

Component	Treatment Number			
	High cholesterol diet	A	B	C
Casein	200	200	189	-
Mould	-	-	-	400
Cellulose	100	100	-	-
Wheat bran	-	-	100	-
Maize starch	387.5	400	411	300
Vitamin mix	10	10	10	10
Mineral mix	40	40	40	40
Sucrose	150	150	150	150
Corn oil	100	100	100	100
Cholesterol	10	-	-	-
Sodium cholate	2.5	-	-	-

All diets were fed as pellets.

B. PLASMA AND LIVER MEASUREMENTS

Plasma and liver total cholesterol and triglycerides were determined by the automated procedures described in Chapter 2.

Radioactivity of plasma cholesterol was measured by extracting cholesterol from 0.2 ml plasma into 1.8 ml 99% isopropanol. 0.5 ml of the isopropanol extract was added to 9.5 ml dioxcint II (8 g butyl PBD and 85 g naphthalene in 1 litre dioxan) and the radioactivity of the cholesterol counted on a Phillips scintillation analyser employing a quench correction programme.

Specific activity of hepatic cholesterol: a known fresh weight of liver (0.8 - 1.0 g) was homogenised in distilled water. Duplicate 1 ml aliquots of the homogenate were pipetted into test tubes followed by 2 ml 10% potassium hydroxide in methanol. The tubes were heated at 80°C in a water bath for 20 minutes. 1 ml of distilled water was pipetted into each tube when cooled and cholesterol was extracted with two 5 ml portions of petroleum ether (40-60°C boiling fraction). 1 ml methanol was added to each tube and a further extraction with 5 ml petroleum ether made. Pooled extracts were placed into a 50 ml round bottom flask, 100 mg of cholesterol carrier added, and the contents of the flask evaporated to dryness in a water bath.

The residual solids were redissolved in 1.5 ml diethyl ether and 1 ml of Fiese's reagent added (200 mg anhydrous sodium acetate, 1 ml bromine and 30 ml glacial acetic acid). The flask was stoppered and left on ice for 20 minutes to allow cholesterol dibromide crystals to develop. The dibromide crystals were filtered through a Whatman's Number 54 filter paper, using ice cold methanol for washing, and allowed to dry at room temperature.

The dried crystals were placed into a preweighed scintillation vial, the vial and its contents were then weighed thereby determining the weight of crystals, and 10 ml of toluene/butyl PBD scintillation fluid added to the vial. The disintegrations per minute were measured

with a Phillips liquid scintillation analyser. The weight of cholesterol in 1 ml portions of liver homogenate was calculated on the basis that 100 mg cholesterol yields 141 mg cholesterol dibromide.

C. FAECAL STEROL EXTRACTION

The faeces of animals housed individually in metabolic cages were collected at three 4 or 5 day intervals. The faeces were wrapped in aluminium foil, dried in a vacuum oven at 60°C for 12-18 hours and ground to a fine powder in a Moulinex coffee grinder. The samples of ground faeces were placed in a 100 ml round bottom flask and refluxed for 24 hours with 50 ml ethanol:acetone (1:1 v/v). The contents of the flask were filtered through a Whatman Number 54 filter paper whilst still hot and collected in 100 ml volumetric flasks. Three washes with 10 ml hot ethanol:acetone solution were made to ensure all the extract was transferred and the final volume was made up to 100 ml with ethanol:acetone solution.

Total radioactivity in the faeces was measured by pipetting 500 µl of the final extract into 10 ml toluene/butyl PBD in a glass scintillation vial. Radioactivity was measured as disintegrations per minute on a Phillips liquid scintillation analyser.

Neutral and acid sterols were separated in accordance with the method of Wells and Makita (1962).

20 ml of the faecal extract was transferred to a glass universal bottle and the ethanol:acetone solvent evaporated in a hot water bath. 10 ml 1.25 N aqueous sodium hydroxide solution was added to the residue and the contents of the bottle were well mixed, and autoclaved at 15 p.s.i. for 3 hours. When cool, the contents of the bottle were transferred to a 50 ml separating funnel using three 5 ml washings with distilled water to complete the transfer. Two fractions were prepared:

a) Neutral sterol extract

The contents of the separating funnel were extracted three times with 100 ml aliquots of diethyl ether. The ether extracts were collected in a round bottom flask and the solvent evaporated under vacuum. The solid sterol fraction remaining after evaporation was redissolved in 1 ml dry diethyl ether (dried with 20 Å molecular sieves) with two further washouts performed with 0.5 ml diethyl ether. The neutral sterol fraction was stored at 3°C in a 2.5 ml stoppered vial.

b) Acid sterol extract

The aqueous mixture remaining after ether extraction for neutral sterols was acidified with 1.25 N hydrochloric acid until the solution was at pH 1.0-1.5. The precipitate of acid sterols was extracted with three 10 ml portions of diethyl ether using a 100 ml separating funnel, and the extracts were collected in a round bottom flask. Diethyl ether was evaporated under vacuum, the dry residue redissolved in 1.0 ml diethyl ether and 50 µl absolute ethanol and the sterol extract transferred to a 2.5 ml glass vial using two further 0.5 ml ether washouts. The vial was stoppered and stored at 3°C.

D. GAS LIQUID CHROMATOGRAPHY OF NEUTRAL STEROLS

a) Standards

Cholesterol, coprostanol, ergosterol, β-sitosterol and 5α-cholestane were prepared as standards. Cholesterol was recrystallised twice from methanol. Coprostanol was isolated from rat faeces (Rosenfield et al, 1954) by evaporating on acetone:ethanol (1:1 v/v) extract from 10 g faeces in a rotary evaporator. The residue was refluxed in 200 ml 5% potassium hydroxide (dissolved in 90% ethanol) for 3 hours. Sterols were extracted with three portions of 200 ml petroleum ether (60-80°C fraction). The pooled ether extracts were washed twice with

100 ml 50% aqueous ethanol and dried with anhydrous sodium sulphate. The solvent was evaporated off by rotary evaporation and the remaining solids redissolved in petroleum ether. The ether solution was applied to an alumina column and coprostanol collected as the second fraction to be eluted with a petroleum ether-benzene mixture.

All other standards were run without further purification.

b) Gas liquid chromatography

GLC analyses were performed on a Pye-Unicam 106 Chromatograph using a glass column 2.17 m long and 4.5 mm internal diameter. The column was packed with 3% OV-1 on a support of diatomite CQ, 100-200 mesh. Nitrogen was used as an inert carrier gas (flow rate 50 ml/minute). A flame ionisation detector was employed (air pressure 15 p.s.i., hydrogen 25 p.s.i.). Column oven temperature was 270°C, detector temperature 300°C and injection chamber temperature 220°C: accuracy of the temperature settings was confirmed by thermocouple readings from the respective chambers.

c) Application of samples

Standards and samples were dissolved in diethyl ether. 500 µl of each sample was pipetted in 2 ml glass vials followed by 20 µl internal standard (20 µg/µl methyl behenic acid). The contents of the vial were evaporated and redissolved in 500 µl of diethyl ether. The vials were sealed with polyethylene caps and placed in a rotary tray for GLC analysis. Sample injection was performed with a 10 µl glass Hamilton syringe using a double-barrelled needle. A Pye-Unicam Autoinjector S4 unit was combined with the Pye 106 chromatograph to enable the continuous application and running of samples. An auto-injector programme was set to deliver 2 µl samples every 35 minutes with three washouts of the syringe in diethyl ether between each application. Variability of the injected volume was corrected by incorporation of the internal standard.

Visual recordings of the chromatographed peaks were made and each peak was simultaneously integrated by an Infotronics Autoinjection digital integrator.

The major peaks from GLC analysis had R_f values corresponding to the standards. All peaks were passed into an AEI mass spectrometer after separation on a Pye 106 chromatograph. No major impurities were detected in either the cholesterol or coprostanol peaks.

E. GAS LIQUID CHROMATOGRAPHY OF BILE ACIDS

All bile acids used for GLC analysis were methylated with a solution of diazomethane in diethyl ether.

Preparation of diazomethane: 17.6 g N-methyl-p-toluene sulphony-1-nitrosamide was added to 480 ml anhydrous diethyl ether in a 1 litre round bottom flask. 80 ml of methanolic potassium hydroxide (3.6 g potassium hydroxide in 8 ml distilled water added to 72 ml methanol) was added to the flask. The top layer of liquid within the flask was distilled into a conical flask containing ice cold diethyl ether. Less than half the ether solution was distilled over in order to minimise the possibility of an explosion. Ground glass joints were firmly fitted and no respiratory contact was made with the diazomethane.

Excess diazomethane remaining in the round bottom flask after distillation, was decomposed by adding 200 ml glacial acetic acid.

a) Methylation

500 µl diazomethane solution was added to each sample of faecal bile acids dissolved in 2 ml diethyl ether. The mixture was left overnight in unstoppered vials avoiding strong light. The final volume of solution was made up to 2 ml with diethyl ether.

Methyl esters of standard bile acids were prepared in a similar manner and their purity checked by measuring individual melting points with a Gallenkamp melting point apparatus.

Sample application: 500 µl of methylated bile acid sample was placed in 2 ml vials and the stoppered vials positioned in a rotary sample tray. Cholesterol acetate (400 µg per 500 µl sample) was used as an internal standard. Samples (2 µl) were injected by a Hamilton syringe as described in section 6.2 D.

b) Gas liquid chromatography

Thirty samples per 24 hours were run under conditions outlined in Section 6.2 D. with a column temperature of 245°C, detector temperature of 300°C and an ionisation chamber temperature of 200°C.

6.3 RESULTS

The three groups of animals grew at similar rates and consumed similar quantities of food when maintained on the casein-cellulose diet containing cholesterol and sodium cholate for 35 days (Table 6.2). Food intake and body weight measurements were made for a further 4 weeks during which the three groups were fed low cholesterol diets containing cellulose, bran or the mould as sources of fibre. No significant differences were found in either food intake or weight gain although animals receiving diets containing the mould grew at a greater rate than those animals of the cellulose group.

Animals receiving mould and bran diets had lower plasma cholesterol concentrations (13 and 6% lower respectively) than rats fed a cellulose diet. There were no significant differences in the specific activities of plasma cholesterol. Plasma triglycerides were lower in the mould (365 mg/l) and bran (442 mg/l) groups than the cellulose group (592 mg/l).

No significant differences were observed in hepatic cholesterol and triglycerides or specific activity of liver cholesterol throughout the three treatments (Table 6.3).

Table 6.2 - Body weights and food intakes

	Days	Treatment group		
		A	B	C
Initial mean body wt. (g)		124	126	125
Mean body wt. (g)	35	235	244	232
Mean body wt. (g)	63	347	350	366
Mean food intake[1]	0-35	100.2	102.2	94.9
Mean food intake[1]	35-63	122.2	130.7	131.7
FCE	0-35	.222	.231	.226
FCE	35-63	.229	.202	.254

[1] g/rat/week

Table 6.3 - Plasma and liver analyses

(Mean ± SEM for 6 observations)

	Treatment groups		
	A (cellulose)	B (bran)	C (mould)
Plasma triglyceride (mg/l)	591.7±21.3	441.7±48.9*	365.0±39.3**
Plasma cholesterol (mmol/l)	2.36±0.01	2.22±0.01	2.06±0.01*
Specific activity plasma cholesterol (dpm x 10^4/mg)	5.0 ± 0.4	4.6 ± 0.3	5.4 ± 0.2
Mean fresh liver wt. (g)	11.5 ± 0.5	11.8 ± 0.4	12.3 ± 0.4
Liver triglycerides (mg/g)	19.5 ± 3.1	22.5 ± 4.3	21.7 ± 4.6
Liver cholesterol (μmol/g)	73.56±3.88	69.15±1.37	76.15±1.36
Specific activity liver cholesterol (dpm x 10^5/mg)	6.3 ± 1.8	5.9 ± 0.8	5.5 ± 0.9

Significance of difference from group A: * $P < 0.05$; ** $P < 0.01$.

The faecal elimination of [^{3}H]-cholesterol over a 31 day period was similar in rats receiving diets containing bran and mould. The rates of excretion expressed in terms of faecal weight (Fig. 6.2) were higher in the mould and bran groups than in the cellulose group.

The daily excretion of tritium was plotted against time (days) on semilogarithmic graph paper (Fig. 6.1). The plot for each group could be resolved into two phases: initially a rapid phase was present, probably resulting from the incomplete equilibration of radioactive cholesterol with the endogenous pool and a second slower and more prolonged phase was observed after 10 days. The half-life of the radioactive cholesterol was calculated from the second phase by the following formula:

$$t_{\frac{1}{2}} = \frac{\log_e (2)}{\lambda}$$

where $t_{\frac{1}{2}}$ is the half-life of the tritium

and λ is the rate of excretion $= \dfrac{\log_e (\text{DPM at } 0) - \log_e (\text{DPM at } 31)}{31}$

therefore $$t_{\frac{1}{2}} = \frac{31 \log_e (2)}{\log_e \frac{(\text{DPM at } 0 \text{ days})}{(\text{DPM at } 31 \text{ days})}}$$

The half-life for a cellulose group was calculated to be 20.7 days: for a bran group, 13.0 days and the mould group was 10.3 days. The turnover of cholesterol was therefore greatest in animals receiving a mould diet with the bran group being 26% slower. The slowest rate of turnover was observed in those animals fed a cellulose diet, the rate being only half that of the mould group.

GLC of faecal neutral sterols was performed on samples collected from individual animals over a 27 day period. The typical separation of neutral sterols is shown in Fig. 6.3.

Table 6.4 - Faecal excretion of tritium from injected (T^3)-cholesterol

(Mean ± SEM for 6 observations)

Group		Day of collection								Total
		3	6	10	14	19	24	27	31	
A Cellulose	Faecal dry wt. (g/day)	2.29±0.11	2.11±0.17	2.25±0.08	1.99±1.40	2.21±0.12	2.34±0.10	2.57±0.07	2.76±0.18	18.52
	dpm x 10^5/ day	29.1±6.4	16.3±0.1	9.7±1.0	7.0±0.1	5.5±0.5	4.9±0.5	4.6±0.2	3.9±0.1	91.0
	dpm x 10^6/ 100 g faeces	173.6±37.1	80.8±8.9	45.8±3.8	37.9±6.5	27.4±3.1	20.8±2.3	17.9±1.2	14.4±0.9	418.6
B Bran	Faecal dry wt. (g/day)	1.70±0.01	1.56±0.09	1.56±0.10	1.54±0.05	1.61±0.06	1.68±0.07	2.18±0.1	1.67±0.04	13.5
	dpm x 10^5/ day	35.2±1.2	17.5±0.1	11.5±0.9	8.8±0.1	6.8±0.6	5.2±0.5	4.2±0.2	3.6±0.6	92.8
	dpm x 10^6/ 100 g faeces	202.9±19.7	113.8±7.9	73.7±3.6	58.1±2.6	42.4±3.2	31.2±2.0	19.8±1.2	21.9±1.5	563.8
C Mould	Faecal dry wt. (g/day)	2.10±0.07	1.82±0.14	1.75±0.09	1.61±0.36	1.73±0.08	1.53±0.07	1.43±0.4	3.7±0.5	13.3
	dpm x 10^5/ day	37.2±2.7	22.4±0.2	12.9±1.2	10.2±0.1	8.3±0.9	5.2±0.5	4.6±0.4	3.7±0.5	104.1
	dpm x 10^6/ 100 g faeces	177.4±9.9	124.6±7.1	75.5±10.2	67.4±9.4	43.2±6.5	33.8±4.1	32.4±2.5	23.8±3.5	23.8

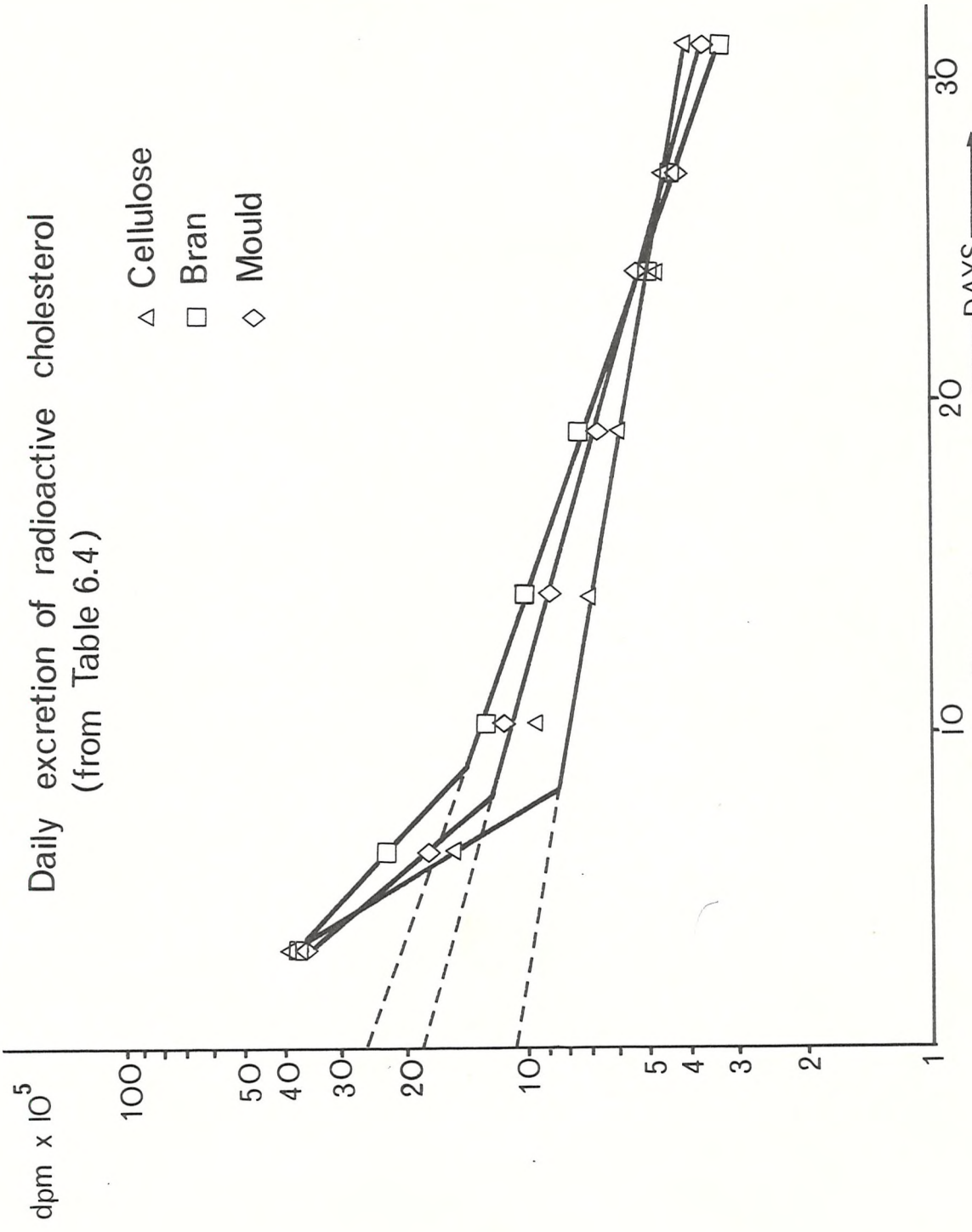

Daily excretion of radioactive cholesterol
(from Table 6.4)
△ Cellulose
□ Bran
◇ Mould
dpm x 10^5
100
50
40
30
20
10
5
4
3
2
1
10
20
30
DAYS

Fig. 6.1

Excretion of radioactive cholesterol per 100g faeces.
(from Table 6.4)

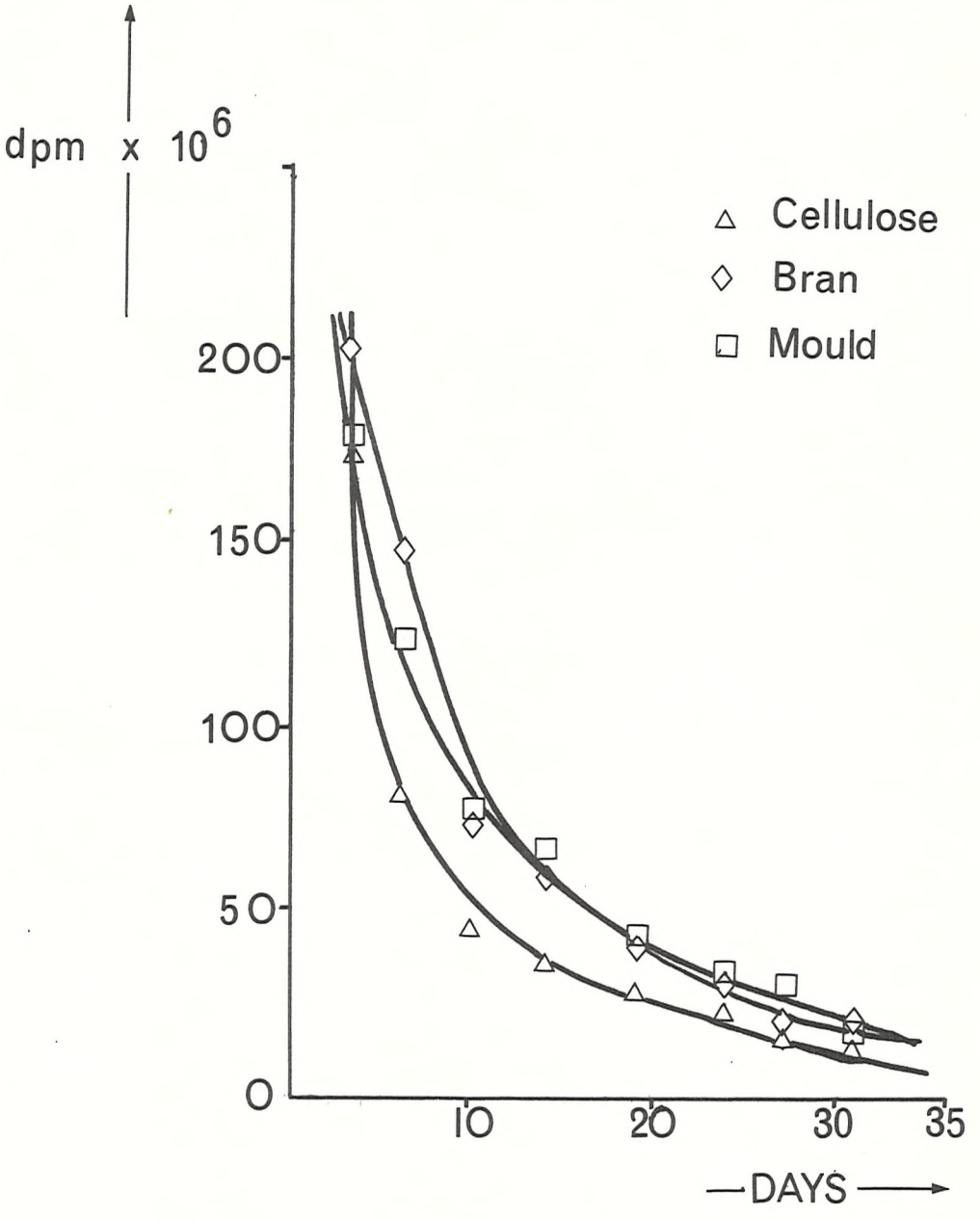


Fig. 6.2

1. Internal standard
2. Coprostanol
3. Cholesterol

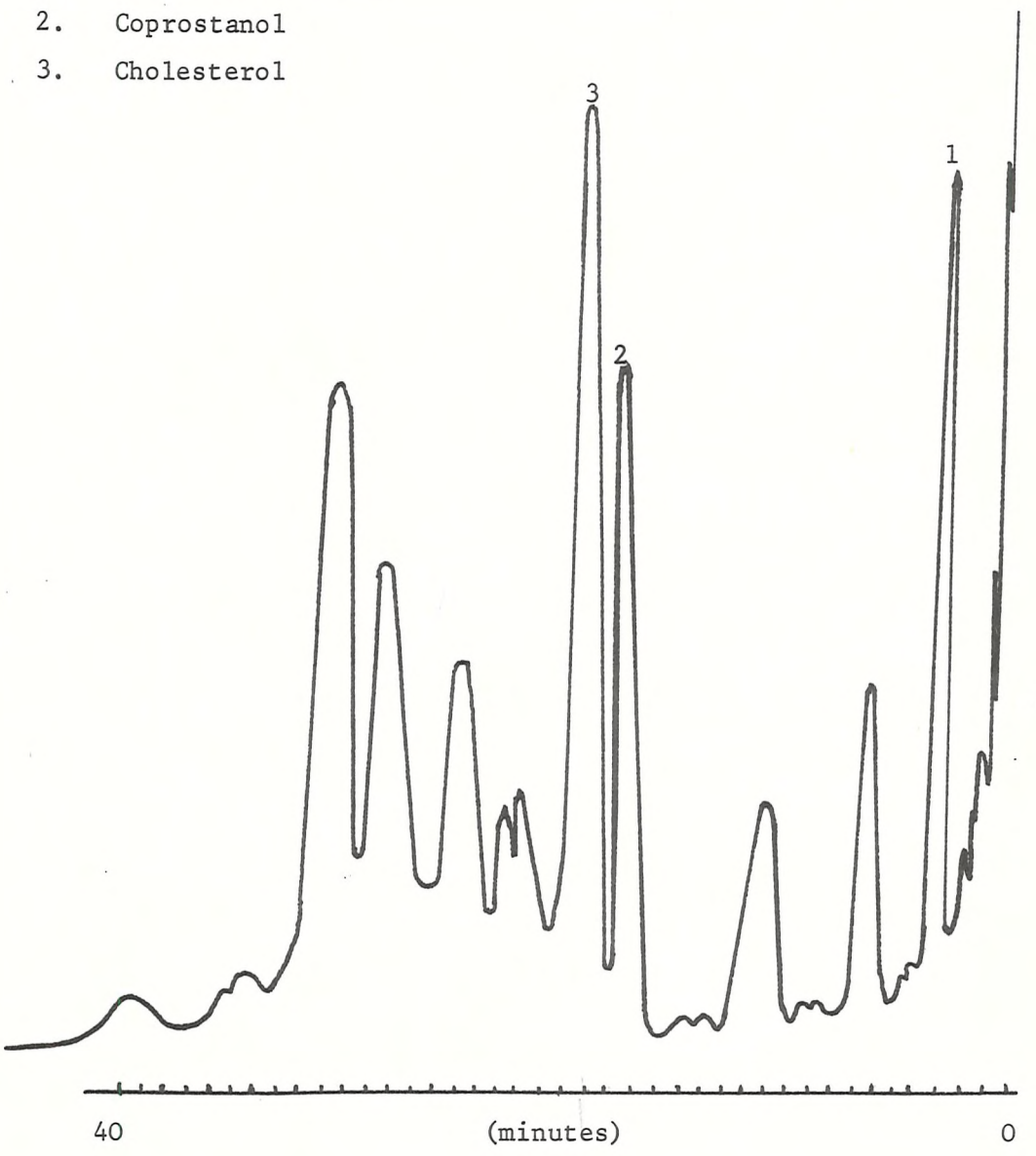


Fig. 6.3 - GLC of faecal neutral sterols

(from Table 6.5)

Peaks were integrated and corrected for sample volume error using an internal standard. The excretion of neutral sterols was expressed as the amount of coprostanol and cholesterol present in the faeces (Table 6.5).

Samples from day 3 could not be analysed due to experimental error. The amount of cholesterol detected in all animals decreased gradually over the 27 day period of collection whereas the amount of coprostanol remained relatively constant. The ratio of cholesterol: coprostanol was similar in rats fed a cellulose diet (0.947) and a mould diet (0.983). Rats receiving a bran diet excreted substantially more coprostanol than cholesterol (ratio = 0.624).

The daily excretion of neutral sterols was greatest in the mould group (5.8 mg/day): the rates of excretion observed in the cellulose and bran groups (3.7 and 4.0 mg/day respectively) were similar.

Seven major peaks were resolved in the GLC of methylated bile acids (Fig. 6.4). A peak corresponding to 3α, 6β, 7β and 3α, 6β, 7α methyl cholanate (β and α-muricholic acids, R_f = 1.66 and 1.68 respectively) was identified on samples allowed to chromatograph for 60 minutes. The calculated total of excreted bile acids did not include the muricholate peaks. Peaks 1 and 2 did not correspond to any standards but because of their positions on the chromatograph relative to deoxycholate it is possible that they were 12-ketomethy-cholanate (R_f = 0.56) and either 6 or 3-ketomethylcholanate (R_f = 0.64 - 0.71) respectively. Peaks 3, 4 and 5 corresponded to the standards of 3α-(lithocholic acid), 3α, 12α-(deoxycholic acid) and 3α,6α-methylcholanate (hyodeoxycholic acid). Two further unidentified peaks were observed with R_f values of 1.21 and 1.33 which may correspond to 3-keto-7α and 3-keto-12α methylcholanate respectively.

The area of each peak was calculated and correcting for sample volume errors using an internal standard: unidentified peaks 1 and 2 were assumed to have a linear molar response similar to lithocholic

Table 6.5 - Daily excretion of cholesterol and coprostanol

(Mean values for 6 observations)

Group	Day of collection	Sterol excretion (mg/day)		
		Cholesterol	Coprostanol	Total
A Cellulose	3	-	-	-
	6	2.60	2.06	4.66
	10	1.94	1.65	3.59
	14	2.15	1.87	4.02
	19	2.13	1.76	3.89
	24	0.84	1.70	2.54
	27	1.04	2.27	3.31
Mean		(1.78)	(1.88)	(3.67)
B Bran	3	-	-	-
	6	2.42	2.65	5.07
	10	1.72	2.20	3.92
	14	2.05	2.43	4.48
	19	0.86	2.11	2.97
	24	0.98	1.69	2.67
	27	0.79	2.95	3.74
Mean		(1.46)	(2.34)	(3.97)
C Mould	3	-	-	-
	6	6.32	4.11	10.43
	10	3.11	3.03	6.14
	14	2.45	2.07	4.52
	19	1.68	2.72	4.40
	24	1.50	3.16	4.66
	27	1.72	2.84	4.56
Mean		(2.79)	(2.99)	(5.78)

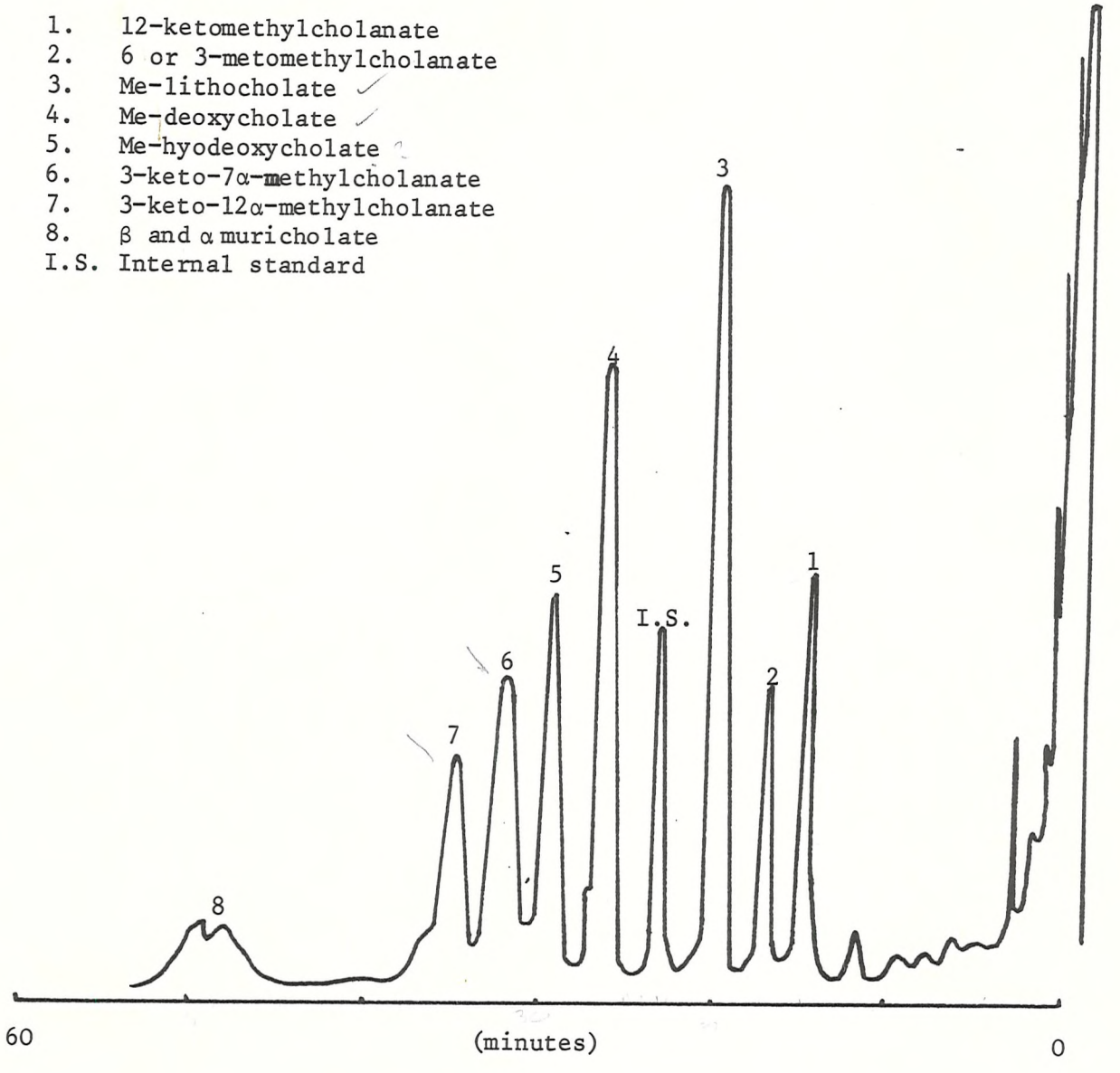


Fig. 6.4 - GLC of methylated bile acids

(from Table 6.6 a-c)

Table 6.6(a) - Daily excretion of bile acids

(Cellulose group)

Day of collection	Peak number (mg/rat/day)[1]								Total
	1	2	3	4	5	6	7	(8)[2]	
3	0.44	0.27	1.13	0.44	1.87	0.17	-	NS	4.32
6	0.48	0.59	1.10	0.50	1.47	0.22	0.18	NS	4.54
10	0.06	-	0.75	0.42	0.88	0.28	0.54	0.99	2.94
14	0.55	0.41	0.54	0.42	0.71	0.20	0.14	0.55	2.97
19	0.32	0.42	0.46	0.41	1.07	-	0.09	0.32	2.77
24	0.38	0.30	0.18	0.43	2.89	0.87	0.06	NS	5.11
27	0.83	0.87	0.91	1.02	1.42	0.47	-	NS	5.52
Mean	0.44	0.47	0.72	0.52	1.47	0.37	0.20		

[1]Mean values for 6 observations

[2]Not included in calculation of total bile acid

NS - Not sampled

Table 6.6(b) - Daily excretion of bile acids

(Bran group)

Day of collection	Peak number (mg/rat/day)[1]								Total
	1	2	3	4	5	6	7	(8)[2]	
3	0.68	1.06	0.54	0.48	5.23	0.37	0.43	NS	8.79
6	0.84	0.37	0.54	1.76	1.21	0.74	0.34	NS	5.81
10	1.46	0.42	0.83	1.54	0.78	0.98	0.21	NS	6.22
14	1.60	0.56	0.73	1.81	1.54	0.29	0.21	1.41	6.75
19	1.16	0.48	0.63	1.60	1.77	0.27	0.43	1.33	6.34
24	1.12	0.48	0.47	1.41	1.04	0.34	0.49	NS	5.39
27	1.36	0.72	0.99	2.25	2.29	0.24	1.10	NS	8.97
Mean	1.17	0.58	0.68	1.55	1.98	0.46	0.46		

[1]Mean values for 6 observations

[2]Not included in calculation of total bile acid

NS - Not sampled

Table 6.6(c) - Daily excretion of bile acids

(Mould group)

Day of collection	Peak number (mg/rat/day)[1]								Total
	1	2	3	4	5	6	7	(8)[2]	
3	0.82	2.31	0.57	0.27	3.68	0.27	0.19	NS	8.11
6	0.80	1.95	0.54	0.73	1.94	0.56	-	NS	6.51
10	1.10	2.62	0.63	0.34	1.59	0.31	0.17	NS	6.77
14	1.73	2.45	0.84	1.31	1.89	0.15	0.20	0.49	8.57
19	2.09	0.82	0.60	2.07	1.73	0.12	0.19	0.68	7.64
24	1.18	0.51	0.34	0.98	0.95	0.12	0.18	NS	4.27
27	0.98	0.73	0.85	0.81	2.34	0.11	0.23	NS	6.05
Mean	1.24	1.63	0.62	0.93	2.02	0.23	0.19		

[1]Mean values for 6 observations

[2]Not included in calculation of total bile acid

NS - Not sampled

Table 6.7 - Mean excretion of faecal bile acids and neutral sterols

Group	Faecal dry wt. (g/day)	Bile acids		Neutral sterols	
		mg/day	mg/100 g faeces	mg/day	mg/100 g faeces
Cellulose	2.25	4.08	181	3.67	163
Bran	1.69	6.89	408	3.97	235
Mould	1.71	6.85	401	5.78	338

acid whilst peaks 6 and 7 were calculated on the assumption that they gave a linear response similar to hyodeoxycholic acid. The daily excretion throughout a 27 day period of individual bile acids are summarised in Table 6.6 (a-c).

Rats fed a diet containing bran had similar mean rates of bile acid excretion (6.89 mg/day) to rats receiving a mould diet (6.85 mg/day) over 27 days. The lowest daily excretion was observed in the cellulose group (4.08 mg/day). Hyodeoxycholic acid was the most prominant metabolite present in the faeces, forming 36, 29 and 30% of the total bile acids in cellulose, bran and mould groups respectively. 24% of the bile acids from the mould group were found to correspond to peak 2, a monoketocholanate, whereas less of this compound was present in bran and mould groups. All animals possessed similar rates of excretion of lithocholic acid, the bacterial metabolite of chenodeoxycholate.

6.4 DISCUSSION

The rate of elimination of the injected radioactive cholesterol from hypercholesterolaemic rats, measured as an indication of the turnover of exchangeable body cholesterol, was greatest in the mould group (half-life = 10.3 days) and slowest in the cellulose group (20.7 days). Bran (13.0 days) tended to resemble the mould rather than the cellulose group with its influence on cholesterol turnover. The increased rate of cholesterol elimination may be attributed to the raised faecal bile acid excretion found in the bran and mould groups. However, the increased bile acid excretion did not result in lowered plasma cholesterol levels in the bran group, and only when there was an increased excretion of both bile acids and neutral sterols (cholesterol and coprostanol, Wilson, 1964) as was observed in animals receiving the mould diets, could a significant decrease in the concentration of plasma cholesterol be detected.

The addition of soyabran to semi-synthetic diets (Balmer and Zilversmit, 1974) and the incorporation of bran and husk from paddy into rat diets (Vijayagopalan and Kurup, 1972) have been shown to increase the faecal elimination of bile acids. The activity of soyabran has been attributed to its capacity of bind bile salts from micellar solutions whereas a hypo-lipidaemic polysaccharide fraction was isolated from the bran of paddy which also increased bile acid excretion. The action of wheat bran resembles that of soyabran in increasing the turnover of cholesterol and increasing bile acid excretion. Chemical fibre preparations of wheat bran bind bile acids in vitro (Chapter 5) but they could not duplicate the hypocholesterolaemic activity of intact wheat bran in rats; therefore, there seems to be other components besides the lignocellulose structure responsible for lowering cholesterol concentrations.

The mould increased both bile acid and neutral sterol excretion. Rats fed a diet containing dried mushrooms showed an increased excretion of neutral sterols but bile acid excretion remained unchanged and these mushrooms were reported to be hypocholesterolaemic in cholesterol fed rats (Kaneda and Tokuda, 1966). The mould has a similar effect on sterol excretion and cholesterol turnover as spinach (Iritani and Nogi, 1971) and the increase in neutral sterol excretion may be due to the suppression of cholesterol reabsorption in the enterohepatic circulation. The increase in bile acid excretion experienced in rats fed diets containing the mould could not be explained by its binding capacity for bile salts (Chapter 5) but may be due to the 'bulking' effect seen in pectin (Leverille et al, 1966) or a chemical potent fraction similar to the polysaccharide component isolated from bran and husk of paddy (Vijayagopalan and Kurup, 1973).

CHAPTER 7

DISCUSSION

The concentrations of plasma and liver cholesterol in rats fed diets supplemented with cholesterol and sodium cholate were lower when either wheat bran or a mould were used as a source of fibre instead of powdered cellulose (Solka floc). No significant differences were observed in plasma and liver cholesterol levels of rats receiving low cholesterol diets containing cellulose, bran or the mould: bran increased the faecal excretion of bile acids and neutral sterols (Chapter 3). Confirmation of these changes in faecal excretion was obtained using gas liquid chromatography techniques (Makita and Wells, 1962; Wells and Makita, 1962) described in Chapter 6.

A number of experiments have been performed on human subjects to establish the cholesterol-lowering properties of wheat bran. Eastwood (1969) was unable to demonstrate any changes in the plasma cholesterol of monks although the experiment was interrupted by Lent. Durrington et al (1975), Truswell and Kay (1975) and Heaton and Pomare (1974) also reported that supplementing human diets with wheat bran produced no lowering of plasma cholesterol: only one report has established a hypocholesterolaemic action of wheat bran (Persson et al, 1975). Truswell and Kay (1976) have summarised the available data for human studies and conclude that wheat bran has little benefit in lowering plasma cholesterol. The lack of activity of bran in humans could be due to two factors: firstly, a lowering of cholesterol was only observed in rats when high cholesterol diets were administered whereas the majority of human subjects consumed diets relatively low in cholesterol. Secondly, hypocholesterolaemia was detected in rats after six weeks on experimental diets, i.e. 5% of their life span, and a longer period of experimentation may therefore be required in humans for an effect to show itself. Mathur, Khan and Sharma (1968) fed Bengalgram to humans whose diet was supplemented with butterfat, and could not demonstrate a hypolipidaemic action until after 22 weeks.

The use of the rat as an experimental model for studying changes in cholesterol concentrations as a result of feeding dietary fibre has not been entirely successful. Kay and Truswell (1975) could not demon-

strate any hypocholesterolaemic activity in rats fed diets containing up to 200 g/kg bran.

Ranhotra (1973) and Tsai et al (1976) failed to lower plasma cholesterol in rats by including wheat bran in diets containing cholesterol. Experiments using legumes (Redgram and Horsegram) lowered the plasma cholesterol of rats fed diets supplemented with cholesterol and sodium cholate. The results of Chapter 2 suggest that diets containing cholesterol (10 g/kg) and sodium cholate (2.5 g/kg) are required for a lowering of plasma cholesterol to be observed and when cholesterol alone is added, the elevation in body cholesterol is not sufficient to emphasize a hypocholesterolaemic response in wheat bran.

The rat has a very efficient cholesterol homeostatic mechanism and excess body cholesterol is rapidly converted to bile acids. The addition of sodium cholate to rat diets has little effect on plasma cholesterol but will inhibit the hepatic conversion of cholesterol to bile acids (Chapter 1, section 1.2). Supplementing diets with cholesterol and sodium cholate will result in the majority of adsorbed exogenous cholesterol remaining in the body: similar effects were observed in hamsters (Marx, 1951).

Changes in sterol metabolism in the rat resulting from the feeding of high fibre diets has been reported by several workers. Portman (1960) noticed an increased bile acid excretion in rats fed a commercial stock diet compared to a semi-synthetic diet. Similar studies have shown that a commercial diet will increase the turnover of cholic acid and cause a three fold increase in bile acid excretion (Gustafsson and Norman, 1969). The effect was not attributed to changes in gut flora because the response was similar in conventional and germ free animals, nor was it caused by the increased 'bulk' of a commercial diet.

Kritchevsky et al (1973) suggested that one of the major factors involved in the lowering of plasma cholesterol by feeding a commercial diet was a decreased absorption of cholesterol from the gut. Gustafsson

and Norman (1969) did not report an increase in neutral sterol excretion but only of bile acids. Balmer and Zilversmit (1974) measured cholesterol absorption by a radioisotope ratio technique (Zilversmit, 1972) and concluded that a diet containing soya bran had little effect on cholesterol absorption but produced a lowering of plasma cholesterol by increasing faecal bile acid excretion. An increased excretion of bile acids was also observed in rats fed a low cholesterol diet containing wheat bran (Chapter 3, Experiment 2) although no changes in plasma or liver cholesterol were observed compared to rats fed a diet containing cellulose. Rats fed diets containing bagasse fibre failed to produce changes in plasma and liver cholesterol despite an increase in daily excretion of bile acids (Morgan et al, 1974).

Soya bran (Balmer and Zilversmit, 1974), bagasse (Morgan et al, 1974) and wheat bran (Chapter 4) have been found to bind bile acids in vitro. Earlier studies with dry grain, a mixture of maize and barley remaining after malting, showed that unconjugated bile acids were bound preferentially to the conjugated acids and maximal absorption was produced when the acidic groups on the fibre were unionised i.e. at acidic pH (Eastwood and Hamilton, 1968). The binding of bile acids or salts within the proximal small intestine could disrupt micelle formation and therefore reduce the efficiency of fat and sterol absorption. Any bile acids associated with the fibre will be less readily available for absorption in the lower small intestine and an increased proportion of the bile acids will appear in the caecum and large intestine.

Primary bile acids are metabolised to secondary bile acids by the bacteria in the large intestine and there is a passive diffusion of the secondary bile acids across the gut wall (Chapter 1). Bile acids and their metabolites are adsorbed onto the solid phase within the large intestine (Gustafsson and Norman, 1968) and when high fibre diets are fed there may be smaller amounts of bile acids available for absorption across the gut wall, partly due to the binding affinity

of dietary fibre and also because of the reduced time available for absorption as a result of the decreased transit times encountered with high fibre diets.

The removal of bile acids from the enterohepatic circulation will stimulate the hepatic catabolism of cholesterol to bile acids and when the rate limiting enzyme, 7α-hydroxylase, was measured in rats (Morgan et al, 1974) a greater activity was found in rats fed a high fibre diet. No corresponding changes in plasma or liver cholesterol were observed probably because of an increased synthesis of cholesterol.

When rats are fed diets supplemented with cholesterol there is an almost complete inhibition of hepatic cholesterol synthesis (Frantz et al, 1954) and it may be possible to observe differences in liver cholesterol between rats fed high and low fibre diets containing cholesterol (Balmer and Zilversmit, 1974). A decreased liver cholesterol will usually result in a lowering of plasma cholesterol because there is a reversible exchange between liver and plasma cholesterol in rats (Avigan et al, 1962).

The excretion of cholesterol in rats which were fed hypercholesterolaemic diets for 5 weeks, injected with radioactive cholesterol and then fed low cholesterol diets for a further 5 weeks showed two distinct phases (Chapter 6). There was an initial fast phase lasting for a few days and a slower, more prolonged phase. Avigan et al (1962) suggested that there were two cholesterol pools in rats: a fast and rapidly equilibrating pool in the plasma and liver where equilibration takes place within a few days, and a second slow pool consisting of organs such as lung, heart, muscle and kidney. It seems likely that the initial rapid rate of radioactive cholesterol excretion was a result of an uptake of unesterified cholesterol from the peritoneal cavity into the liver whilst this is followed by a more gradual excretion reflecting the true rate of cholesterol turnover once the labelled cholesterol had equilibrated with the rapidly exchangeable body pool. A similar

biphasic excretion pattern was seen in rabbits (Iritani and Wells, 1966) and rats (Iritani and Nogi, 1972).

Cholestyramine resin administered to human subjects will cause an increased excretion of bile acids but the rate of cholesterol synthesis is probably insufficient to compensate for the cholesterol used for bile acid synthesis resulting in a net lowering of liver cholesterol. Rats are relatively resistant to cholestyramine (Gallo et al, 1966) especially when fed low cholesterol diets, and there is therefore a similarity in the response of rats to high fibre diets and to cholestyramine. The in vivo evaluation of chemical fibre preparations from wheat bran in hypercholesterolaemic rats failed to reproduce the cholesterol lowering action of bran (Chapter 4), despite the high capacity for bran fibre to bind bile salts in vitro. The binding of endogenous and dietary bile salts to bran fibre may be partly responsible for the increased excretion of bile acids but there may be other factors which are removed in the manufacture of chemical fibre that may contribute to the hypocholesterolaemic nature of wheat bran.

The high binding capacity found in lignin of dry grain (Eastwood and Hamilton, 1968) has not been observed in lignin prepared from wheat bran (Chapter 4) or bagasse (Morgan et al, 1974). The feeding of wood lignin in rat diets produced a lowering of plasma cholesterol accompanied by increased neutral sterols and fat in the faeces, compared to rats fed wood cellulose (Judd, Kay and Truswell, 1976). The therapeutic application of lignin in humans is uncertain. Patients with primary hypercholesterolaemia had reduced plasma cholesterol following the administration of 1-4 g lignin per day (Tiffault et al, 1970), but several other workers have failed to demonstrate any hypocholesterolaemic properties in lignin (Heaton and Barry, 1972; Linder and Möller, 1973; Barnard and Heaton, 1973).

The hypocholesterolaemic action of wheat bran in rats may not be solely due to dietary fibre components binding bile acids. The bran and husk of rice have been shown to lower plasma and liver cholesterol

of rats fed a high fat/high cholesterol diet (Vijayagopolan and Kurup, 1972). A polysaccharide fraction isolated from bran and husk could overcome the effects of feeding a high cholesterol diet at doses of 15 mg/day (Vijayagopolan and Kurup, 1973). The polysaccharide fraction had little effect on cholesterol absorption but increased the hepatic breakdown of cholesterol to bile acids. The mechanism for increasing bile acid excretion is still unknown.

A high intake of dietary fibre will decrease transit time and increase faecal bulk (Burkitt, 1972). It has been previously mentioned that a decreased transit time will shorten the period available for bacterial metabolism and absorption of bile salts: such changes can have a significant effect on the daily excretion of bile acids considering that 20% of the total bile acid pool is in the large intestine (Norman and Sjovall, 1958). The ability of dietary fibre to adsorb and retain water has been recorded (Eastwood, 1973; McConnell et al, 1974) and this is reflected in the ability for pectin and other hydrophilic polysaccharides to lower plasma cholesterol with an increased excretion of bile acids. Pectin will decrease plasma cholesterol in humans after 3 weeks (Keys et al, 1959) and in rats fed high cholesterol diets (Leverille and Sauberlich, 1966). The gel structure formed within the gut by feeding hydrophilic polysaccharides increased bile acid excretion by retaining the acids in the gel phase of the intestine and therefore decreasing their availability for absorption. Wheat bran will retain water (Chapter 5) resulting in an increased weight of faeces: water in the faeces may also be caused by bacterial metabolism of dietary fibre components to volatile fatty acids which will act as cathartic agents. The faeces produced by rats fed diets containing wheat bran had a higher water content than animals fed a cellulose diet and this higher water content may have resulted in an increased retention of bile acids within the large intestinal lumen. Pectin appeared to be more active than wheat bran in lowering the plasma cholesterol of hamsters (Chapter 4, Experiment 3): pectin did not lower plasma cholesterol compared

to cellulose in hamsters fed diets low in cholesterol and wheat bran could only produce a cholesterol-lowering action when diets were supplemented with cholesterol and sodium cholate. The contribution of the bulking properties of bran, either resulting from its water holding capacity or by indirect cathartic action, may not be as great as for pectin.

The mould had a water retention capacity three times greater than wheat bran and ten times higher than cellulose. The moisture content of the faeces from rats fed a diet containing 40% mould was substantially higher than for diets containing bran or cellulose and the hypercholesterolaemic action of the mould may, in part, be similar to that of hydrophilic polysaccharides. Mould would lower plasma and liver cholesterol concentrations in hamsters fed diets low in cholesterol whereas wheat bran had no hypocholesterolaemic activity. Rats fed diets low in cholesterol containing 40% mould did not have their cholesterol levels lowered compared to a cellulose group but there was an increased excretion of neutral sterols and bile acids in their faeces (Chapter 3, Experiment 2). Unlike wheat bran, the mould did not display a high capacity to bind bile acids *in vitro* and the increased excretion of bile acids could not be attributed to its bile acid sequestering properties. It is more probable that the water retention properties of the mould caused bile acids to become integrated within the non-digested matrix and therefore become less available for absorption.

Several studies have been performed on commonly eaten foods in Japan, including seaweeds (Kaneda *et al*, 1963), konjac (Kiriyama *et al*, 1969) and spinach (Iritani, 1972) and each has been reported to overcome the effects of feeding high cholesterol diets to rats. Shiitake, the mushroom *Lentinus edodes*, has also been shown to lower plasma cholesterol concentrations in rats fed diets containing 1% cholesterol. No increase in bile acid excretion was found but there was a large increase in faecal neutral sterols (Kaneda and Tokuda, 1966). The mould, which is a fungal biomass produced as a novel protein source for human consumption produced hypocholesterolaemia

in rats fed diets supplemented with cholesterol and sodium cholate. The mould did not display cholesterol lowering properties in rats fed diets low in cholesterol but hamsters maintained on similar diets did have lowered plasma and liver concentrations of cholesterol. The hypocholesterolaemic response in hamsters did not become evident until the diets had been fed for 45 days: similar delays in the development of the hypocholesterolaemic response of pectin and carragheen were observed for rats in which no effect was observed until 42 days (Tsai et al, 1976).

The difference in response between the rat and hamster suggested that the hamster may have been a more suitable experimental model for evaluating the cholesterol-lowering effects of dietary components. Further experiments (Chapter 4, Experiment 3) indicated that wheat bran could only produce hypocholesterolaemia in hamsters fed diets supplemented with cholesterol and sodium cholate, and there were therefore no substantial differences in response of the hamster and rat to wheat bran. The poor response of hamsters to wheat bran was confirmed by Truswell and Kay (1975).

Since hypocholesterolaemic mechanisms of the mould seem to be different to that of wheat bran these mechanisms were further evaluated in hamsters. Kaneda and Tokuda (1966) prepared six fractions from the mushroom: a water soluble and a 30% (v/v) ethanol/water fraction were found to possess cholesterol-lowering properties. A similar method of fractionation was employed for the mould but only a diethyl ether soluble fraction would lower the plasma and liver cholesterol concentrations of hamsters. This fraction will contain sterols such as ergosterol which can be present in yeasts and fungi in concentrations as high as 2% of the dry weight. Plant sterols will lower plasma cholesterol by inhibiting cholesterol absorption, although this effect is only seen when concentrations of up to 5% of the diet are used (Best and Duncan, 1957). The hamsters received 1.1 g extract/kg body weight/day whereas 4 g β-sitosterol/kg body weight/day were required to overcome the

effects of feeding 1% cholesterol in rat diets. No cholesterol was added to the hamster diets and the amount of phytosterol required to lower plasma cholesterol would therefore be correspondingly less than for a high cholesterol diet.

The mould did not cause hypocholesterolaemia in rats receiving diets low in cholesterol and this difference compared to that of hamsters may be due to the rat having a continuous secretion of dilute bile whereas the bile of the hamster will be more concentrated and released from the gall bladder when food is transferred from the stomach to the small intestine. A further explanation for the differences in response between rats and hamsters to the mould may involve the contribution of the small intestine to the exchangeable body cholesterol pool. The small intestine has been shown to be a major extrahepatic source of newly synthesised cholesterol (Wilson, 1968) and in the rat it can constitute 18-20% of the total cholesterol synthesis. No data is available for hamsters but if the contribution is substantially greater than in the rat an alternative mechanism for lowering body cholesterol would be the inhibition of cholesterol synthesis in the small intestine. The amount of cholesterol absorbed from the intestine is thought to be responsible for regulating the synthesis within the small intestine (McIntyre and Isselbacher, 1973) and there may be a component of the mould, present in a diethyl ether soluble fraction, which will inhibit intestinal cholesterol synthesis.

The hypocholesterolaemic action of the mould can be partly attributed to its lipid component which may inhibit cholesterol absorption: the mould might also produce a lowering of plasma and liver cholesterol in the hamster by inhibiting endogenous cholesterol absorption. In the rat hypocholesterolaemia was observed only when diets were supplemented with cholesterol and sodium cholate and the mould was probably decreasing the absorption of exogenous cholesterol under these conditions. The decrease in cholesterol absorption was further supported by analyses of rat faeces for neutral sterols (cholesterol and coprostanol): rats

fed a diet containing the mould had a substantially greater excretion of neutral sterols compared to diets containing cellulose or bran. No lowering of cholesterol was observed in hamsters fed the residue remaining from sequential extracting of the mould (Chapter 4) but the fibrous components of the mould may still be responsible for some of the hypocholesterolaemic activity. The composition and particle size of konjac mannan was found to be a critical factor in lowering cholesterol in rats (Kiriyama et al, 1972) and it is quite possible that there were changes in the structural integrity and removal of indigestible components from the mould during the preparation of the residual fraction. The ability of the mould to absorb and retain water resulted in a high moisture content in the faeces. The increase in bile acid excretion could be explained by the 'gel-effect' which is observed in pectin and hydrophilic colloids.

The influence of gut flora on the response of rats to diets containing cellulose, bran or the mould is difficult to assess. Gustafsson and Norman (1969) found that the gut flora was of little importance in the hypocholesterolaemic activity of a commercial diet compared to a semi-synthetic diet. The major bacterial degradation product of cholesterol, coprostanol, was greatest in the mould group but these animals also had a higher concentration of cholesterol in the faeces. The ratio of cholesterol:coprostanol in the mould group was 0.93 and a similar ratio (0.94) was found in a cellulose group. The bran group had substantially more coprostanol present than cholesterol (0.62) indicating that a higher proportion of cholesterol was metabolised by the intestinal bacteria. The bulk of the powdered cellulose diet and the soft gel-like matrix of the undigested mould may have partially protected the neutral sterols from bacterial metabolism whereas the relatively non-protective residue of a bran diet may have resulted in a more extensive metabolism by the gut flora. The relevance of bacterial metabolism of sterols is still uncertain but there are indications that the increased faecal bulk and decreased transit times associated with high fibre diets may protect against possible carcinogens produced by bacterial metabolism

of bile acids and maybe decrease the incidence of colon cancer (Hill and Aries, 1972).

Summary

Wheat bran and the mould will lower the concentration of plasma and liver cholesterol when rats are fed diets supplemented with cholesterol and sodium cholate. Neither bran or the mould will alter the cholesterol concentrations of rats fed on diets low in cholesterol, although bran increased the faecal excretion of bile acids and the mould will increase bile acid and neutral sterol excretion. Wheat bran and the mould increased the rate of turnover of cholesterol in hypercholesterolaemic rats compared to cellulose.

The mould is probably acting in two ways: firstly, a diethyl ether soluble portion may inhibit cholesterol absorption and secondly, the mould will absorb water and decrease the absorption of bile acids, which will also tend to hinder the absorption of cholesterol. Wheat bran binds bile salts *in vitro* and may decrease the reabsorption of bile acids by binding them within the intestine. The site of action of wheat bran is probably the large intestine and caecum where there will also be a further degradation of wheat bran to produce some volatile fatty acids. These volatile fatty acids will cause an increased water content of the intestinal contents and expand the volume of the solid phase: this will further help to decrease the availability of bile acids for reabsorption.

The majority of experiments performed in humans have been unable to demonstrate hypocholesterolaemic activity in wheat bran. There is a minimal period before experimental animals develop lowered cholesterol concentrations and perhaps further studies in humans, using hypercholesterolaemic patients fed for longer periods than in reports published to date, will confirm the hypocholesterolaemic action of wheat bran in rats.

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APPENDIX

The term 'mould' applies to a microfungal protein, Fusarium graminearum produced by Rank-Hovis-McDougall Ltd. The fungal biomass is produced by fermentation on carbohydrate substrate and is being developed as a high protein human food.

The mould, code A3/5, was received from the Lord Rank Research Centre, High Wycombe, Bucks, as a pale yellow powder. The composition of the mould on a moisture free basis is given below:

		%
Protein (total α amino-N x 6.25)		36
Amino acids (free)		4
Nucleic acids (as R.N.A.)		8
Carbohydrate :		37
made up of chitin	9	
1:3 and 1:6 glycans	16	
Glycogen	6	
Mannitol	5	
Trehalose	1	
Lipid		10
Ash		5
		100

Acid detergent fibre yield = 16.5% (of dry matter)

The composition of wheat bran, based on the values quoted by Fisher (1974) is shown in Table 1.6. The yields of three preparations of chemical fibre, expressed as mean ± SEM for quadruplicate analyses are:-

	%
Crude fibre	10.3 ± 0.9
Normal acid fibre	12.5 ± 0.9
Acid detergent fibre	13.4 ± 0.6