STUDIES OF THE EXCRETION OF CHOLESTEROL AND ITS METABOLITES

W.D. Mitchell.

Ph. D. THESIS

1968.

SUMMARY

This thesis describes work on the development of new techniques of faecal neutral steroid and bile acid analysis and the application of these techniques to a variety of clinical circumstances in which the metabolism of cholesterol, and the output of faecal steroids might be altered.

The thesis is in four main parts.

1. A consideration from an historical standpoint of cholesterol metabolism and its regulation; in particular, the reduction of serum cholesterol levels by three groups of compounds;

a) Inhibitors of cholesterol biosynthesis either at an early stage in the biosynthetic pathway or at a stage after the cyclisation of squalene.

b) Compounds which cause increased excretion of faecal bile acids and/or faecal neutral steroids.

c) Compounds which act by impairing cholesterol absorption.

2. A review of the application of thin layer chromatography (TLC) to lipid analysis. In this section the use of silver nitrate impregnated TLC,

reversed phase TLC and derivatives in the separation of structurally similar compounds are discussed together with the application of TLC to quantitative analysis of faecal neutral steroids and bile acids.

3. A detailed consideration of the way in which TLC and other methods have been modified for use in the present study. The extraction of neutral steroids and bile acids from faeces is described together with methods for their qualitative and quantitative analysis. A technique for estimating biliary bile acids is also described.

4. The final section is concerned with the application of quantitative techniques of neutral steroid and bile acid analysis to four problems.

a). The effect of oral taurine on serum cholesterol and biliary bile acid conjugation was studied in three subjects. The results suggest that although the proportion of bile acids conjugated with taurine in human bile can be readily increased by feeding taurine this has no effect on serum cholesterol concentrations.

b). The mechanism by which clofibrate reduces serum cholesterol in patients with hypercholesterolaemia was investigated by three approaches.

1). The effect of clofibrate on the pattern of biliary bile acids in five subjects with hypercholesterolaemia.

2). The effect of clofibrate on faecal neutral steroids and bile acids in twenty one subjects.

3). A comparison of the effect of clofibrate and L-thyroxine on serum and faecal lipids in four hypothyroid patients.

Clofibrate did not appear to alter the conjugation ratio or the pattern of biliary bile acids in the five subjects studied. The results of the comparative study with L-thyroxine suggest that the mechanism of action of clofibrate is quite different to that of L-thyroxine in lowering serum lipids. Studies of faecal bile acids and neutral steroids in the 21 subjects strongly suggest that the reduction of serum cholesterol by clofibrate is not produced by an increased excretion of cholesterol or its metabolites in faeces. Inhibition of hepatic synthesis of cholesterol seems a more likely explanation.

c). The effect of increased oral calcium on faecal neutral steroids and bile acids was studied in six subjects. Although calcium markedly increased faecal bile acid excretion there was no reduction in serum cholesterol concentration.

d). The final problem studied was the effect of dietary cholesterol on serum and faecal lipids in a single subject. The results of this experime suggest that there is a negative feedback mechanism in which the level of dietary cholesterol controls the hepatic synthesis of cholesterol.

STUDIES OF THE EXCRETION OF CHOLESTEROL AND ITS METABOLITES

A thesis presented in part fulfilment of the requirements for the admittance to the degree of Doctor of Philosophy of the University of Glasgow.

by

William Derek Mitchell, A.H.-W.C., A.R.I.C.

1968.

CONTENTS Pg.

CONTENTS (Contd.) Pg.

ACKNOWLEDGEMENT

I would like to express my appreciation to Professor Sir Edward Wayne, Regius Professor of Medicine (Retired), University of Glasgow and the late Dr. B. Bronte-Stewart, Director, Medical Research Council, Atheroma Research Unit, Western Infirmary, Glasgow for the opportunity to carry out this work and for their guidance.

I would also like to thank the staff of the Atheroma Research Unit, especially the medical staff, for their cooperation and assistance throughout this study. I am also grateful to Dr. C.J.W. Brooks and Dr. A.F. Lever, Director, Medical Research Council, Blood Pressure Research Unit (formerly Atheroma Research Unit) for their valuable criticism of this work. I would also like to thank Professor G.M. Wilson who assumed the role of supervisor after Professor Wayne retired. Finally, I would like to thank my wife Elizabeth for her patience and understanding.

SUMMARY

This thesis describes work on the development of new techniques of faecal neutral steroid and bile acid analysis and the application of these techniques to a variety of clinical circumstances in which the metabolism of cholesterol, and the output of faecal steroids, might be altered.

The thesis is in four main parts.

I. A consideration from an historical standpoint, of cholesterol metabolism and its regulation.

II. A review of the application of thin-layer chromatography (TLC) to lipid analysis.

III. A detailed consideration of the way in which TLC and other methods have been modified for use in the present study.

IV. The final section is concerned with the application of quantitative techniques of neutral steroid and bile acid analysis to states of altered cholesterol metabolism.

NOMENCLATURE

The following trivial names have been used.

Cholesterol	: cholest-5-en-3β-ol.
Coprostanol	: 5 β-cholestan-3β-ol.
Coprostanone	: 5 β-cholestan-3-one.
β-sitosterol	: 24 α-ethyl — cholest-5-en-3β-ol.
Campesterol	: 24 α-methyl — cholest-5-en-3β-ol.
Cholestanol	: 5 α-cholestan-3β-ol.
Lathosterol or Δ^7-cholestenol	: 5 α-cholest-7-en-3β-ol.
Δ^4-cholesten-3-one	: cholest-4-en-3-one.
Lanosterol	: 4, 4, 14-trimethyl-cholesta-8:24-dien-3β-ol.
Epicholestanol	: 5 α-cholestan-3α-ol.
Epicoprostanol	: 5 β-cholestan-3α-ol.
Cholic acid	: 3 α, 7α, 12α-trihydroxy-5β-cholanoic acid.
Chenodeoxycholic acid	: 3 α, 7α-dihydroxy-5β-cholanoic acid.
Deoxycholic acid	: 3 α, 12α-dihydroxy-5β-cholanoic acid.
Lithocholic acid	: 3 α-hydroxy-5β-cholanoic acid.

PART I: A.

CHOLESTEROL METABOLISM

CONTENTS Pg.

INTRODUCTION

DISCOVERY:

Cholesterol is the major sterol present in the tissues of mammals. It was first described in the latter half of the 18th century by De Fourcroy, (1789) but it was not until 1815 that Chevreul showed that the substance, obtained by ether extraction of gall stones, could be differentiated from other waxes as it was unsaponifiable. He named the material cholesterine from the Greek "chole", (bile) and "stereos" (solid). Between 1824 and 1838 cholesterol was found in human and animal bile (Chevreul, 1824), in human blood (Lecanu, 1838) and in brain (Couerbe, 1834).

DISTRIBUTION OF CHOLESTEROL:

The amount of cholesterol present varies from tissue to tissue and from species to species. Table I shows the estimated distribution of cholesterol in a 70 Kg. man (Cook, 1958(a).

Table I. The estimated distribution of cholesterol in man.

System	Tissue sterol as approximate % of total body sterol
Brain and nervous system	23
Connective tissue (including adipose) and body fluids.	22
Muscle	21
Skin	9
Blood	8
Bone marrow	5
Liver	4
Heart, lungs, kidneys, spleen, blood vessels	4
Alimentary tract	3
Adrenal glands	1
Other glands	-
Skeleton	-

Cholesterol is also present in considerable concentration in bile (Polonovski and Bourrillon, 1952) and faeces (Eneroth et al, 1964) but only in very small concentrations in urine (Kayser and Balat, 1952).

The presence of cholesterol is not confined to mammalian tissues: it occurs abundantly in egg yolk (Gobley, 1846) but is present only to a minor extent in plants (Heftmann, 1965).

BIOSYNTHESIS:

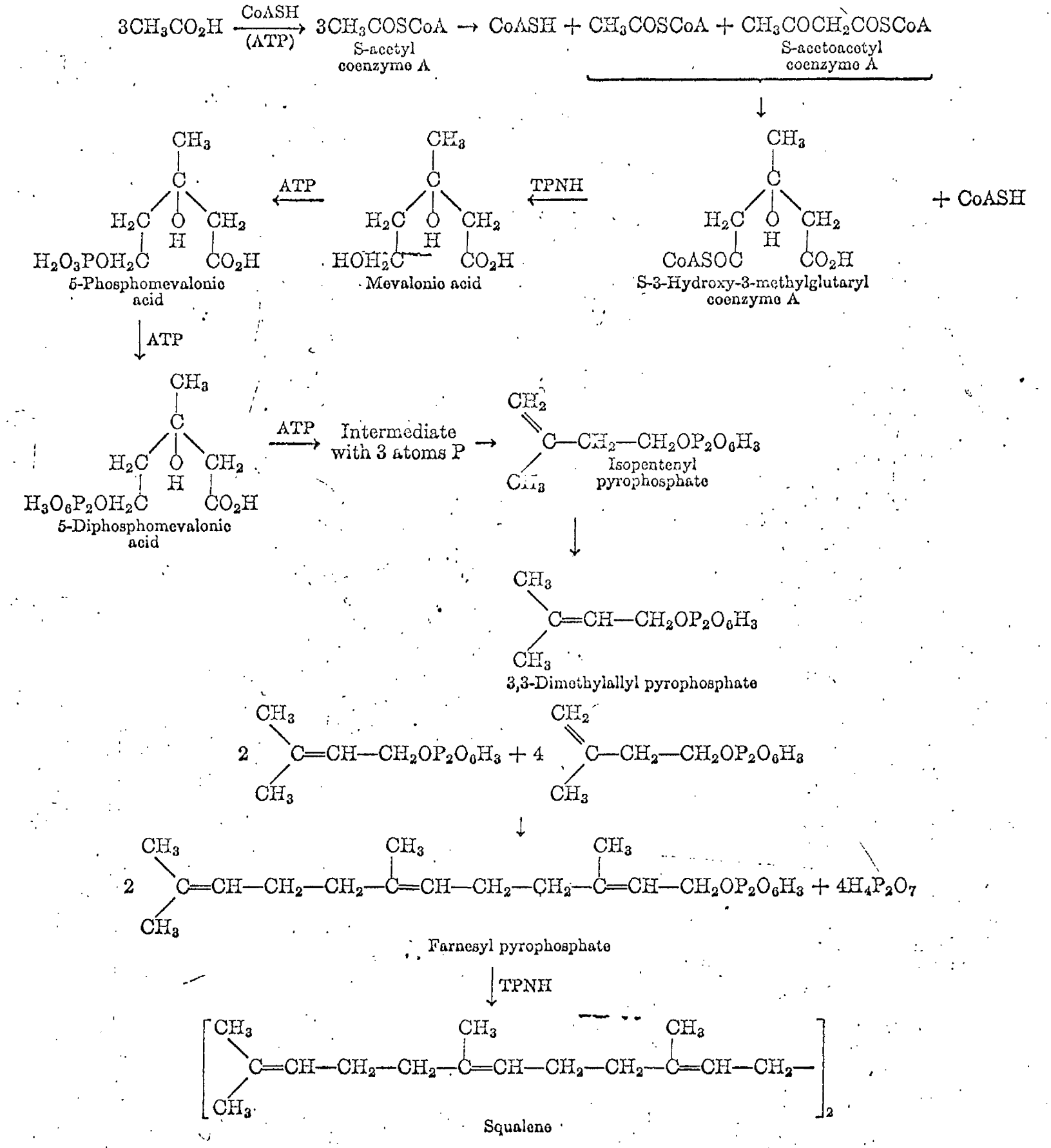
Bloch and Rittenberg (1942a, 1942b) using deutero acetate, CD_3COONa, first showed that an intact animal could convert oral acetate to cholesterol. More exact data on the incorporation of the two carbon atoms of acetate into cholesterol were obtained by Little and Bloch (1950) who found from experiements with double labelled acetate that fiteen carbon atoms of the cholesterol molecule were derived from the methyl carbon of acetic acid and the remaining twelve from the carboxyl group.

The biosynthetic pathway of cholesterol from acetate is outlined in the following scheme. The pathway may be divided into two parts,

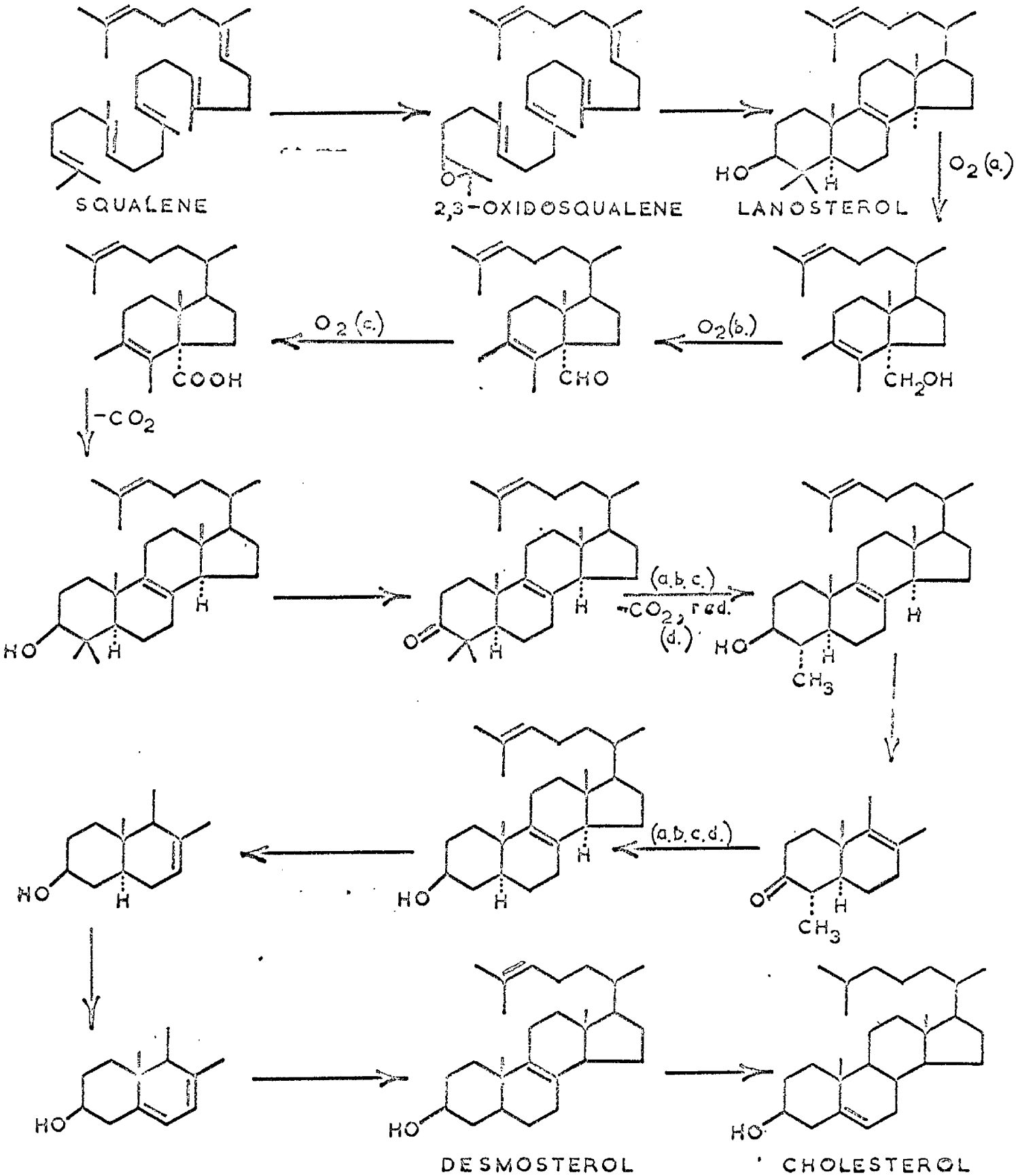
1) The biosynthesis of squalene from acetate is derived from an interpretation by Cornforth (1959).

2) The biosynthesis of cholesterol from squalene. The pathway outlined by Cornforth (1959) for this part of the biosynthetic sequence no longer holds true and scheme 2 is therefore an interpretation derived from works of Clayton (1965), Popják and Cornforth (1966), Corey et al. (1966) and van Tamelen et al. (1966).

THE FORMATION OF CHOLESTEROL - Part I. Biosynthesis of squalene from acetate.



Part II. Biosynthesis of cholesterol from squalene. A scheme based on work of Corey *et al.*, van Tamelen and Clayton.



Sites of Synthesis.

The synthesis of cholesterol has been demonstrated in virtually every tissue of the mammalian body with the exception of the mature nervous system (Srere et al. 1950). As the cells of the adult brain are incapable of regeneration it is possible that there might be a relationship between the ability of cells to regenerate and their ability to synthesise cholesterol. The liver was originally reported to be the sole endogenous source of circulating cholesterol (Hotta and Chaikoff, 1955). This is no longer true as cholesterol is now known to be synthesised by the rat intestinal wall and to enter the circulating cholesterol pool (Lindsey and Wilson, 1965; Dietschy and Siperstein, 1965). Table II summarises various sites and the efficiency which these sites convert acetate - C^{14} to cholesterol (Kritchevsky, 1958(a) in the rat.

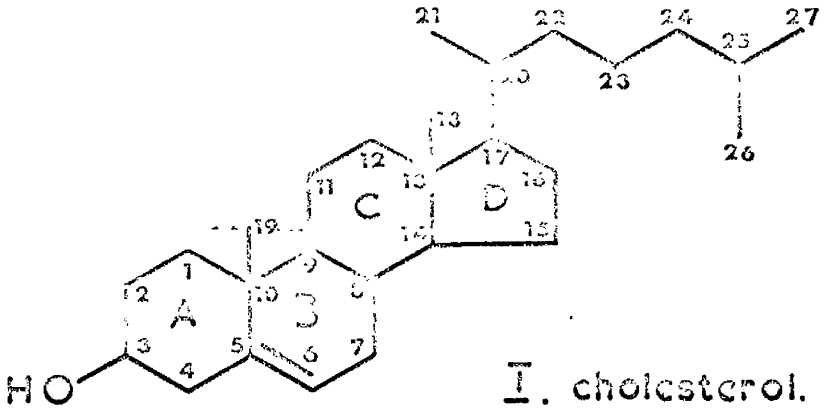
Table II. The efficiency with which various sites convert acetate - C^{14} to cholesterol in the rat.

Tissue	Liver = 1
Liver	1.00
Intestine	0.60
Testes	0.31
Kidney	0.04
Adult skin	0.90
Newborn skin	1.93
Adult brain	0.00
Newborn brain	1.85
Aorta	0.02 (chicken) 0.002 (rabbit)

STRUCTURE:

The elucidation of the structure of cholesterol was achieved mainly by Windaus and his associates between 1919 and 1932, who however failed to arrive at a satisfactory formulation. The structure (I) (figure 2) was finally established by Rosenheim and King in 1932.

Figure 2.



I. cholesterol.

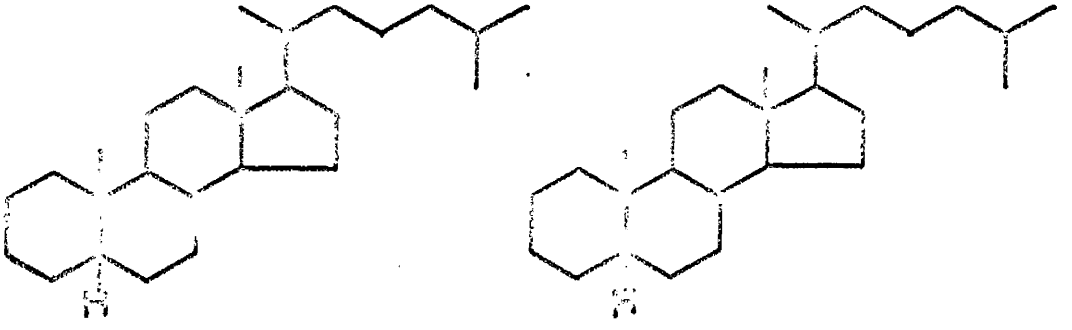
Cholesterol, a C_{27} steroid, has a double bond at the A/B ring junction as shown, and a hydroxyl group in the 3β position. The systematic name for cholesterol is Δ^5-cholesten-3β-ol, or cholest-5-en-3β-ol.

Steroids are numbered as in I. The nucleus being a relatively flat structure is conveniently represented as planar. Groups attached to the nucleus which are above the plane of the ring system (i.e. on the same side as the angular-methyl groups at C-10 and C-13) are called β and bonds joining them to the nucleus are drawn as heavy lines. Groups below the plane of the ring

system are called α and the bonds are drawn as dotted lines.

Rings A and B may be fused either trans- (e.g. 5 α-cholestane II) or cis (e.g. 5 β-cholestane III) but rings B and C are trans in all naturally occurring steroids. The rings C and D are fused trans in nearly all types of steroids. (Figure 3).

Figure 3.



II 5α-cholestane III 5β-cholestane

Metabolism of cholesterol.

Cholesterol may be derived from the diet or be synthesised within the body. The principal

site of synthesis is the liver where cholesterol is also converted to bile acids (Byers and Biggs, 1952).

Most of the cholesterol lost from the body is excreted in the bile. Approximately 80% of the cholesterol so excreted is in the form of bile acids. In man, these are conjugated with glycine or taurine (Wootton and Wiggins, 1953). Most of the bile acids reaching the intestinal lumen are reabsorbed and carried to the liver via the portal vein (Siperstein and Chaikoff, 1952), a process known as the enterohepatic circulation of the bile acids. The remainder is excreted as faecal bile acids.

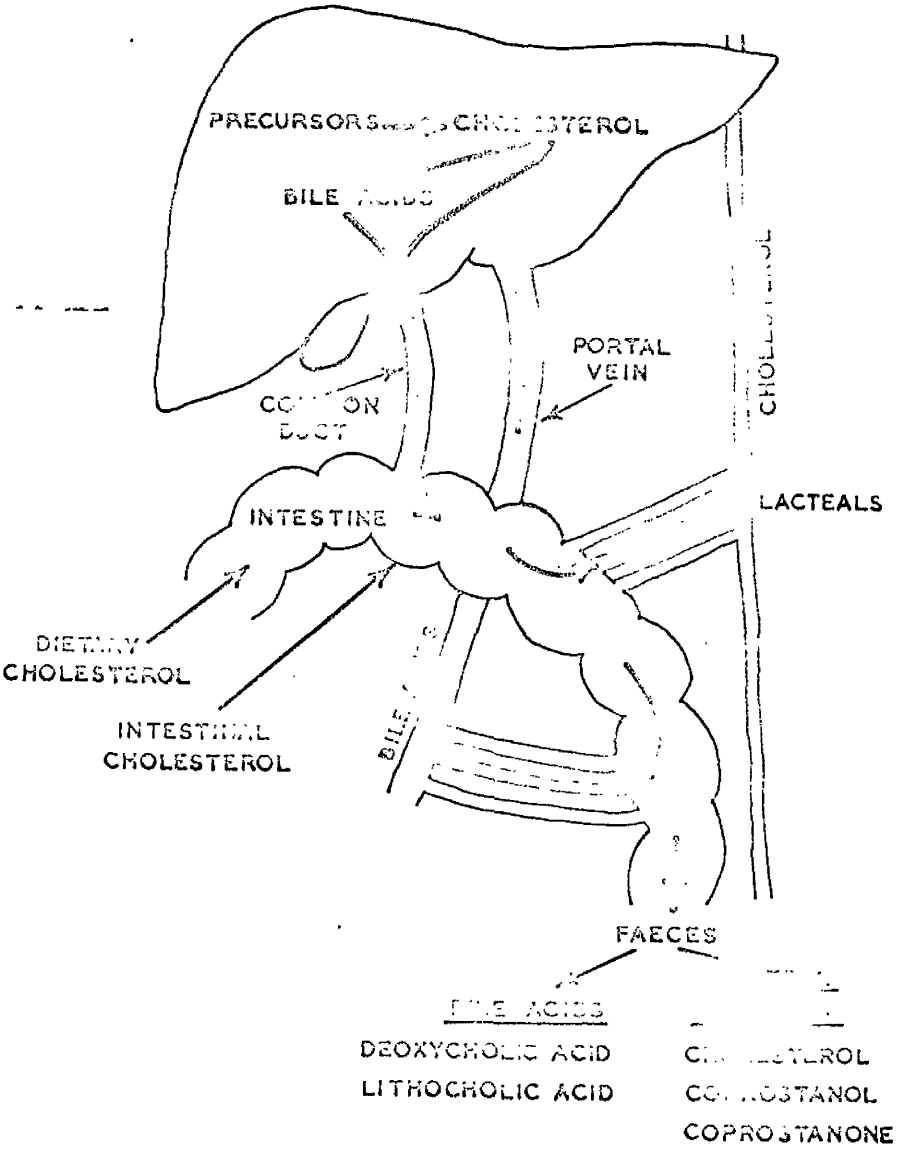
The remaining 20% of biliary cholesterol is in the form of free cholesterol. In the small bowel this mixes with ingested cholesterol and as the enterohepatic circulation of cholesterol is not as active as that of the bile acids a larger proportion reaches the colon. The cholesterol which is absorbed is transported via the lymphatic

system and not by the portal venous system to the systemic circulation (Chaikoff et al, 1952).

Bile salts and cholesterol which are not reabsorbed are finally excreted in the faeces, after further transformation by the intestinal micro-organisms.

These pathways are illustrated in Figure 4, and the processes of absorption, catabolism and excretion are explained in more detail in the following sections.

Fig. 4.



ABSORPTION OF CHOLESTEROL FROM THE INTESTINE:

The earliest demonstration that cholesterol was absorbed from the intestinal tract was made by Jankau in 1892 using dogs. Twenty-three years later Mueller (1915) showed that dietary

cholesterol is absorbed via the lymphatic system and that the ratio of free to esterified cholesterol in the lymph was not altered by feeding free or esterified cholesterol or fatty acids. It was not until 1952 however, that Siperstein et al using cholesterol-4-C^{14}, demonstrated the obligatory function of bile salts in cholesterol absorption. Investigations by Swell et al (1953) and Pihl (1955(a))showed that cholic or taurocholic acids were the most active in promoting cholesterol absorption. The conveyance of intestinally absorbed cholesterol to the systemic circulation via the thoracic duct in preference to the portal venous system has been confirmed by Biggs et al (1951) in the rat and by Hellman et al (1960) in men. This was achieved using tritium labelled and C^{14} labelled cholesterol.

In man the site of cholesterol absorption from the intestine has been shown by Borgström (1960) to be in the proximal part of the jejunum.

Balance experiments on the apparent absorption

of cholesterol in man have shown that cholesterol is poorly absorbed: the degree of absorption is however greatly influenced by the form in which it is fed. When fed as crystalline cholesterol only 16% is absorbed, whereas fed as cholesterol in the form of eggs 60% is absorbed (Cook et al, 1956). Cook (1958(b)) suggested that on a body-weight basis the smaller experimental animals have a more marked absorption than do the larger species. Table III.

Table III. Absorption of cholesterol in different animals.

Specimen	Typical Weight	Amount fed/day g.	%	Absorption gm./kg. body-weight/day
Rat	300 g.	0.3	40	0.4
Guinea pig	400 g.	0.25	50	0.3
Rabbit	1.5 kg.	0.5	75	0.3
Dog	10-12 kg.	1.6	50-80	0.1
Man	70 kg.	Crystalline 10.0	15	0.01
		Egg 6.9	60	0.02

However if the amount of cholesterol fed

to the animal is calculated per unit weight of animal then it is evident that the smaller animals were presented with a much greater load of cholesterol than the larger species Table IV.

Table IV.

Specimen	Typical Weight	Amount of cholesterol fed/day g.	Amount of cholesterol fed/kg. of animal g.
Rat	300 g.	0.3 g.	1.0 g.
Guinea pig	400 g.	0.25 g.	0.7 g.
Rabbit	1.5 kg.	0.5 g.	0.33 g.
Dog	10-12 kg.	1.6 g.	0.16 g.
Man	70 kg.	Crystalline 10.0 g.	0.14 g.
		Egg 6.9 g.	0.10 g.

It is therefore unjustified to conclude from the evidence presented in Table III that there is a species difference in cholesterol absorption.

MECHANISM OF ABSORPTION OF CHOLESTEROL:

Several studies on the mechanism of

absorption of cholesterol in rats have established that the sterol can enter the mucosal cells only as unesterified cholesterol (Treadwell *et al*, 1959, Treadwell *et al*, 1962). The fact that cholesterol esters are not absorbed to any greater extent than free cholesterol and that conditions in the intestinal lumen favour hydrolysis (Vahouny and Treadwell, 1958) lend support to this theory. Swell *et al*. (1958) suggested that the cholesterol esters formed in the mucosa do not pass into the lymph alone, but are transferred along with triglycerides, phospholipids, free cholesterol and proteins, in the form of chylomicrons. The exact site of formation of such a complex is unknown. Swell and his colleagues further suggest that cholesterol absorption is closely interrelated with the absorption and transport of other lipids and is not an independent mechanism. Recently, David *et al*. (1966) have suggested that cholesterol esters in the diet are hydrolysed by cholesterol

ester hydrolase of either pancreas or mucosal brush border and that the free cholesterol is transferred to the mucosal cell. Here, they believe, it is re-esterified for transport in the lymphatic system. The finding that the rate of re-esterification in the mucosal cell is parallel to the rate of removal of the ester from the cell and that the brush border rapidly takes up dietary cholesterol without effecting any quantitative change in its own cholesterol content, suggests absorption by a displacement mechanism. This had been postulated earlier by Glover and Green (1955) and by Murthy *et al.* (1963).

EXCRETION OF CHOLESTEROL:

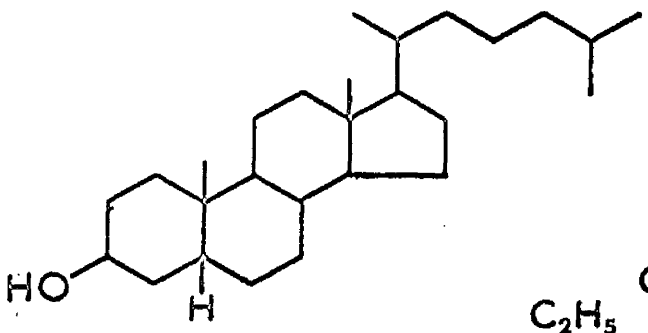
Cholesterol is eliminated from the body in two main ways: 1) direct excretion as cholesterol into the gastrointestinal tract from the liver in bile and from the intestinal mucosa; and 2) hepatic oxidation to cholanoic acids which are then excreted into the bile as their taurine

or glycine conjugates.

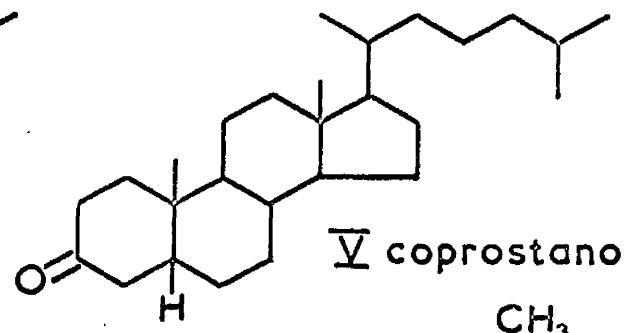
1) Excretion of cholesterol as neutral steroids:

Although cholesterol is catabolised mainly to bile acids it is also excreted in the faeces as neutral steroids. Faecal neutral steroids consist of cholesterol and its metabolites (mainly coprostanol (IV) and coprostanone (V)) together with phytosterols of dietary origin, such as β-sitosterol (VI) and campesterol (VII) which are also accompanied by their metabolites (Eneroth et al. 1964). Small amounts of cholestanol (VIII) and lathosterol (IX) (Kritchevsky, 1958(b), Cook, 1958(c), have also been detected. (figure 5).

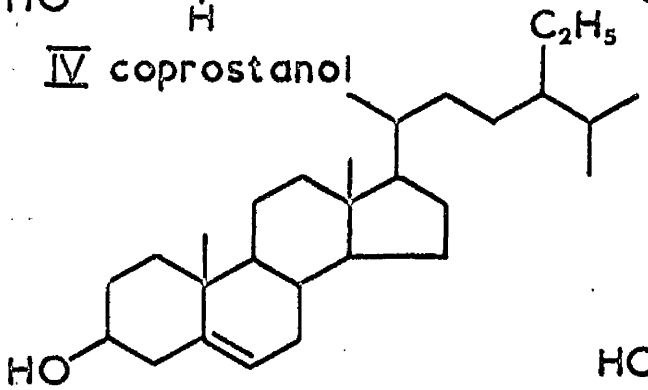
Figure 5.



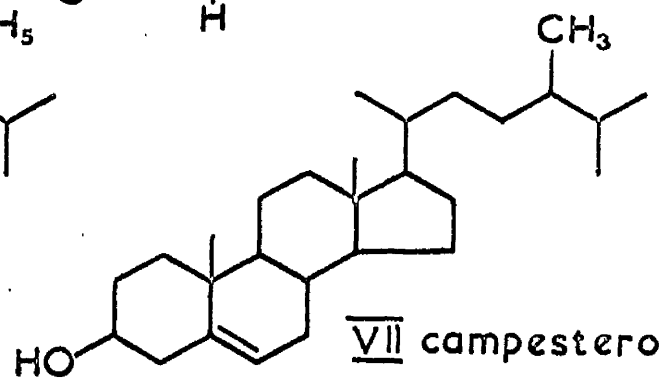
IV coprostanol



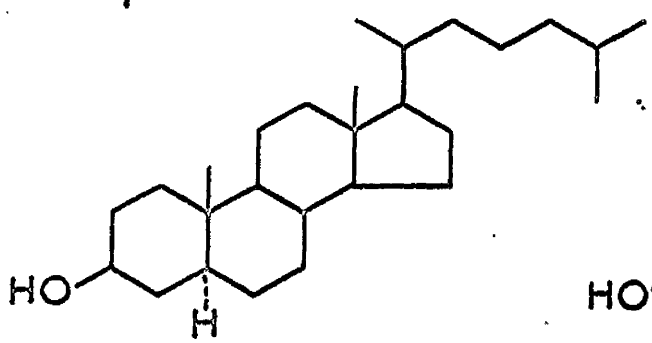
V coprostanone



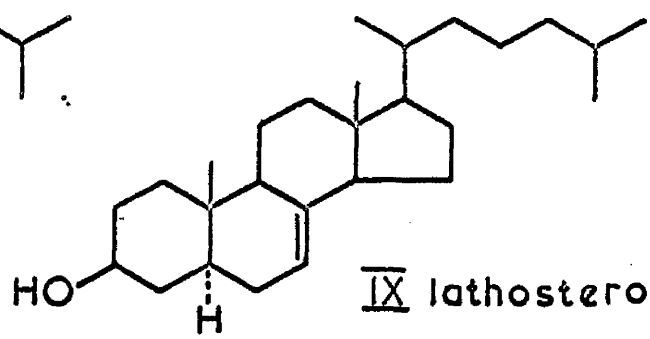
VI β-sitosterol



VII campesterol



VIII cholestanol



IX lathosterol

COPROSTANOL EXCRETION:

The most abundant faecal steroid is normally coprostanol. It is formed by microbiological reduction of cholesterol in the lower intestinal tract, especially in the colon (Rosenfeld et al.

1954, Wilson, 1961). In addition the conversion of cholesterol to coprostanol by bacterial cultures isolated from faeces has also been demonstrated conclusively (Rosenfeld et al. 1954). No coprostanol is excreted by germ free animals (Danielsson and Gustafsson, 1959) and coprostanol formation can also be abolished by the administration of a number of antibacterial agents (Rosenheim and Webster, 1943(b), Wainfan et al. 1952, Coleman and Baumann, 1957). In addition to antibacterial agents various dietary constituents have been found to influence coprostanol formation (Wilson, 1961).

Two explanations have been advanced to explain the conversion of cholesterol (I) to coprostanol (IV).

a) a direct stereospecific reduction of the double bond at C5-6 (Schoenheimer et al. 1930),

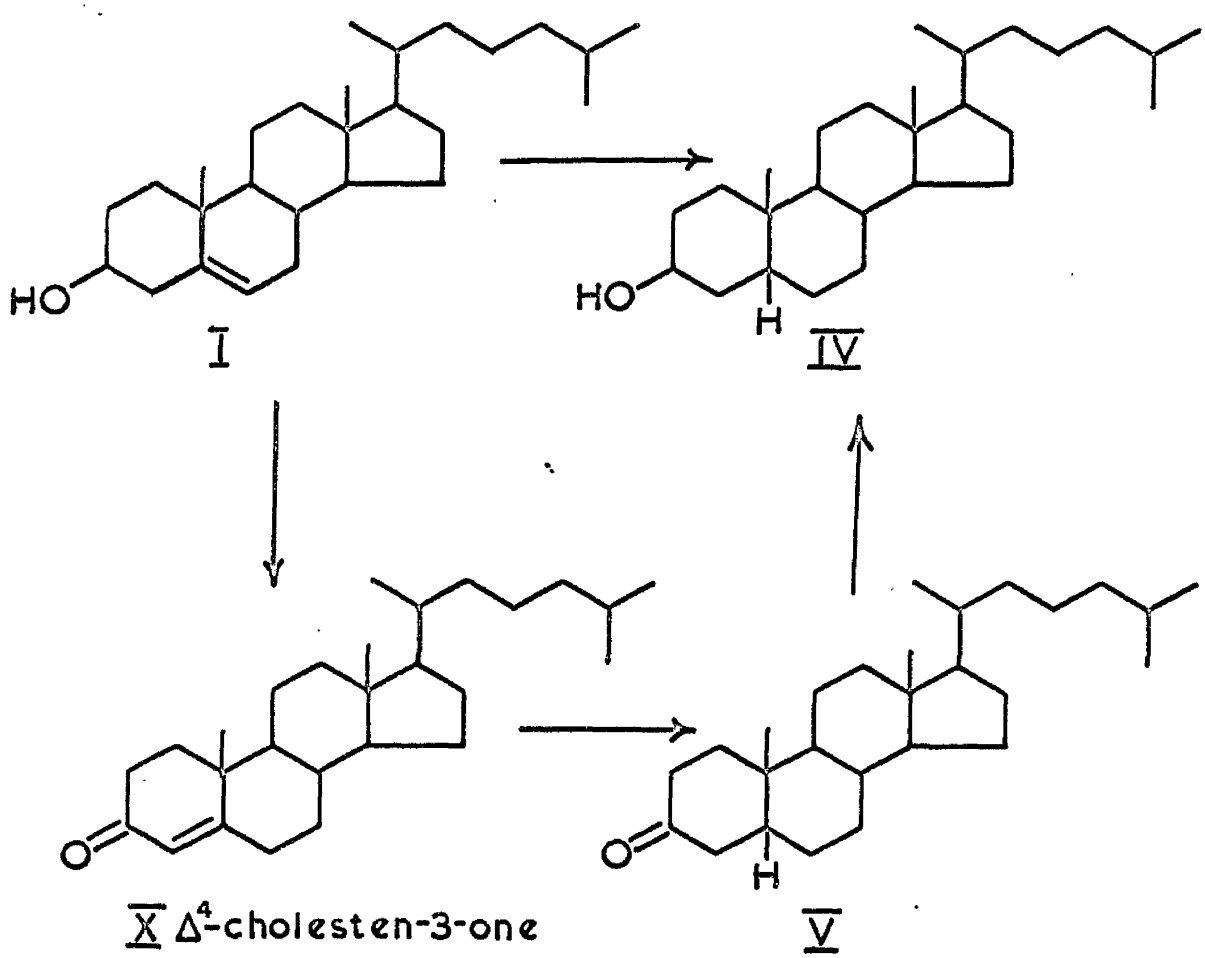
and

b) a three stage conversion from cholesterol (I)

involving the intermediates ^{4}-cholesten-3-one (X) and coprostan-3-one (V) Rosenheim and Webster, 1943).

These routes are illustrated in figure 6.

Figure 6.



However, Rosenfeld et al. (1954) showed

using radio-active cholesterol that coprostanol is produced principally by the direct saturation of the C5-6 double bond of cholesterol.

2. Excretion as bile acids:

A. Formation of bile acids in the liver.

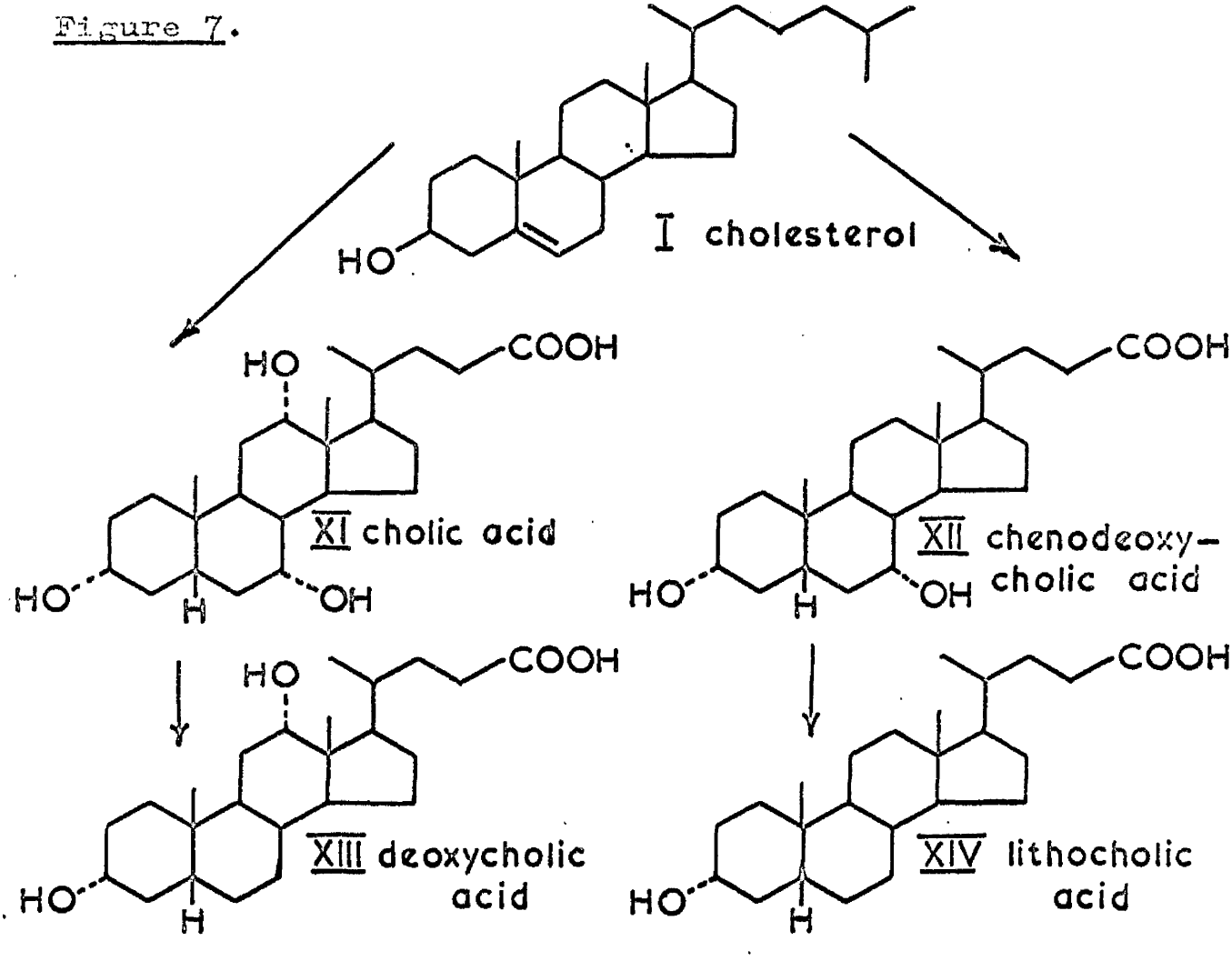
Bloch et al. (1943) using deuterium labelled cholesterol demonstrated the direct transformation of cholesterol into bile acids in the liver. This was confirmed in 1952 by Byers and Biggs using tritiated cholesterol.

Investigations into the exact nature of the biliary end products of cholesterol have shown that in the rat the major biliary products are cholic (XI) and chenodeoxycholic acids (XII), both present as the taurine conjugates (Siperstein et al. 1954), and in man, cholic acid and chenodeoxy-cholic acid plus a small proportion of deoxycholic acid (XIII) conjugated with glycine or taurine (Wootton and Wiggins, 1953).

Although most of these conjugated bile acids are re-absorbed from the intestinal lumen a small

proportion of them pass on to the colon where they are converted by bacterial action to free bile acids as a result of removal of the glycine or taurine fragment. The 7α-hydroxyl groups is also largely removed by bacterial reduction. In this way, cholic acid is broken down to deoxycholic acid, whereas chenodeoxycholic acid is converted to lithocholic acid (XIV) (Carey and Williams, 1962, Danielsson et al. 1963) figure 7.

Figure 7.



The "primary" bile acids are cholic acid and chenodeoxycholic acid. A comparison of the formulae of these acids with that of cholesterol would suggest that the following reactions take place:-

1) hydroxylation at C-7 and C-12

2) inversion of the C-3 hydroxyl group

3) saturation of the double bond

and

4) degradation of the side chain

Bergström et al. (1954) and Fredrickson and Ono (1956) suggested that in the conversion of cholesterol to bile acids, alteration of the cholesterol nucleus occurs before degradation of the side chain. However, the observation that 26-OH cholesterol is metabolised to chenodeoxycholic acid (Danielsson, 1961(a), and Berséus and Danielsson, 1963) suggests that this is not invariably true and that Bergström's conclusions require modification i.e. the nucleus may be elaborated after terminal oxidation of the side-

chain.

The first reaction taking place in the nucleus seems to be the hydroxylation at C-7 (Lindstedt, 1957(a) Danielsson, 1961(b)). However, it may be considered that both hydroxylations at C-7 and C-12 occur simultaneously. Once the nucleus has been hydroxylated at C-7 and C-12, the hydroxyl group at C-3 is inverted from the β- to the α- orientation (Danielsson, 1961(b), Hutton and Boyd, 1963). Hutton and Boyd (1963) also showed that the inversion appeared to precede the saturation of the double bond.

The side-chain is then oxidised very rapidly to give a C-24 acid (Briggs and Staple, 1960) but the initial step is the oxidation of one of the terminal methyl groups to a hydroxymethyl group (Danielsson, 1960).

If the view that the nucleus may be elaborated after terminal oxidation of the side-chain is accepted, then it may be concluded that that part of the cholesterol which is oxidised to 26-OH

cholesterol (Danielsson, 1961, Bergström and Danielsson, 1963) would give rise to the chenodeoxycholic acid present in the organism while the cholic acid pool is derived from the cholesterol which undergoes modification of the nucleus before any oxidation in the side-chain.

In summary, the degradation of cholesterol may be as follows:-

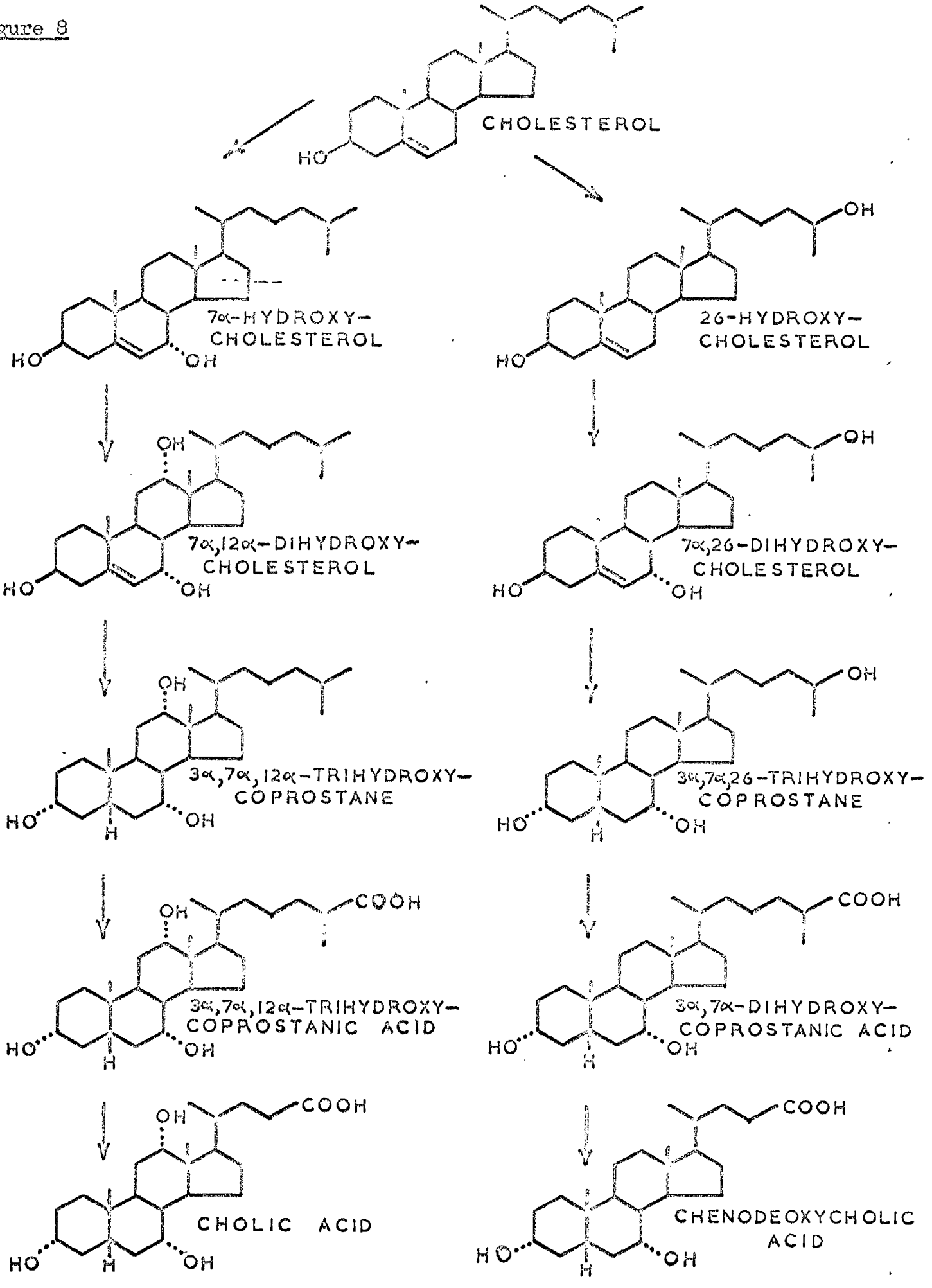
1) hydroxylation at C-7 and C-12,
2) inversion of the C-3 hydroxyl group,
3) saturation of the C5-C6 double bond,
4) oxidation of a terminal CH_3 group to OH,
5) oxidation of this OH group to a C-27 acid,
6) oxidation at C-24,

and

7) cleavage of the terminal 3-carbon unit leaving a C-24 acid.

This is illustrated in figure 8. (Van Belle 1965).

Figure 8


CHOLESTEROL
7α-HYDROXY-CHOLESTEROL
26-HYDROXY-CHOLESTEROL
7α,12α-DIHYDROXY-CHOLESTEROL
7α,26-DIHYDROXY-CHOLESTEROL
3α,7α,12α-TRIHYDROXY-COPROSTANE
3α,7α,26-TRIHYDROXY-COPROSTANE
3α,7α,12α-TRIHYDROXY-COPROSTANIC ACID
3α,7α-DIHYDROXY-COPROSTANIC ACID
CHOLIC ACID
CHENODEOXYCHOLIC ACID

B. Extrahepatic metabolism of the bile acids.

The bile acids in human bile are conjugated with glycine or taurine, the normal ratio of glycoconjugates to tauroconjugates being 3.2:1 (Sjövall, 1960). This ratio has been found to be the same for cholic, chenodeoxycholic and deoxycholic acids in human bile. In the large intestine most of the bile acids are present as free bile acids (Norman and Sjövall, 1958). Norman and Sjövall (1958) concluded from their experiments that the action of micro-organisms does not start until the bile acids have entered the caecum and that the first reaction appears to be the splitting of the peptide bond. This splitting was positively demonstrated to occur in the caecum by Portman (1958). The administration of chemotherapeutic agents to rats resulted in only 1-15% of faecal bile acids as free acids (Norman, 1955). This suggested that the peptide bonds are split by the intestinal micro-organisms and not by digestive enzymes.

The findings that labelled cholic acid and chenodeoxycholic acid were extensively metabolised (Danielsson et al. 1963) and that only traces of these "primary" acids have been detected in human faeces (Carey and Williams, 1962) suggested marked changes in the bile acid molecules during their passage through the intestine. The non-transformation of lithocholic acid in the human liver (Carey and Williams, 1963) also indicates intestinal transformation of the bile acids. These changes, according to Portman (1958) occur in the caecum and colon.

The transformation of the bile acids by the micro-organisms results in the excretion of a complex mixture of free bile acids in faeces (Eneroth et al. 1966a, 1966b). The principal bile acids in human faeces are deoxycholic acid and lithocholic acid (Rosenfeld and Hellman, 1962, Eneroth et al, 1966a). These two acids are formed by the dehydroxylation at C-7 of cholic acid and chenodeoxycholic acid (Carey and Williams,

1962, Danielsson _et al._ 1963) respectively.

A considerable amount of bile acids is reabsorbed from the intestine and reaches the liver via the portal blood, in contrast to cholesterol which is absorbed via the lymph (Siperstein and Chaikoff, 1952). In the rat and other species e.g. dog, rabbit and chicken, the absorptive activity of the terminal ileum for conjugated cholate was found to be four times as great as that of the duodenum (Baker and Searle, 1960, Lack and Weiner, 1961, 1963). This restriction of absorption of bile salts to the distal small intestine allows those substances to exert their digestive function in the proximal region where fat absorption is maximal. Norman and Sjövall (1958) showed by administering cholic acid orally or by an intracaecal catheter that over 80% was reabsorbed from the small intestine. They suggested that the incomplete absorption from the caecum is

probably due to the conversion of cholic acid into non-absorbable metabolites. This would account for the large variety of metabolites present in the small intestine and the few free bile acids found in bile.

Lindstedt (1956, 1957(b)) using isotope labelled bile acids, calculated the bile acid pool in man to be approximately 3.58 g. of which cholic acid accounted for 1.35 g. chenodeoxycholic acid 1.45 g. and deoxycholic acid 0.78 g. The half-life of cholic acid varied from 1.2 to 4.2 days with a mean value of 2.8 days. Ivy *et al.* 1957 calculated that the average daily synthesis of cholesterol in man on sterol-free diet was 2.1 g.; of this, 1680 mg. was catabolised, 1460 mg. of which were converted to bile acids. This is much higher than the value proposed by Bergström (1959) who suggested that only 0.7 g. of cholesterol is degraded to bile acids per day and based his value on half-life times and pool sizes of cholic and chenodeoxycholic acids.

The values reported for the daily excretion of bile acids in man show a marked difference from laboratory to laboratory. This is reviewed by Grundy *et al.* (1965).

In summary cholesterol is derived from two sources, dietary cholesterol which is absorbed as such, from the intestine, and synthesised cholesterol. Cholesterol is lost from the body by two main pathways; a) direct excretion into the gastrointestinal tract. In the colon bacterial action then produces a mixture of neutral steroids which are excreted in the faeces, and b) hepatic oxidation of cholesterol to bile acids. These are excreted in bile into the gastrointestinal tract where they also undergo bacterial transformation to produce a complex mixture of bile acids which are excreted in the faeces. However a proportion of bile acids and cholesterol in the gastrointestinal tract undergo enterohepatic circulation to reappear in bile.

PART I: B.

FACTORS AFFECTING THE LEVEL OF CRIMINALITY IN LAGOS.

CONTENTS

INTRODUCTION

A multiplicity of factors are associated with the development of ischaemic heart disease. One of these factors which correlates well with the incidence of coronary heart disease is the level of serum cholesterol (Dawber et al. 1962, Keys et al. 1963). It would therefore seem important to study factors which affect serum cholesterol values in the hope that the results obtained might lead to a better understanding of the aetiology and control of atheroma. Thus dietary and other methods of altering serum cholesterol levels may affect the incidence of atherosclerosis.

As part of the work reported in this thesis is concerned with therapeutic and dietary alteration of serum cholesterol and the associated effect on the excretion of cholesterol and its metabolites a brief review on dietary conditions and therapeutic agents is given here.

THE INFLUENCE OF DIETARY CHOLESTEROL ON SERUM CHOLESTEROL LEVEL.

A. A feedback effect on cholesterol synthesis.

The early balance experiments of Schoenheimer and Breusch (1933) with mice suggested that the concentration of cholesterol in animals was regulated by a homeostatic mechanism; evidence for which was first provided by Gould (1951) who found that hepatic cholesterol synthesis in dogs and rabbits was reduced by feeding cholesterol. Other investigators have since demonstrated a similar effect in rats (Tomkins et al. 1953(a); Langdon and Bloch, 1953; Frantz et al. 1954) and chicken (Sakakida et al. 1963). This depression of cholesterol synthesis may well be an example of "negative feedback" control in that the rate at which material is formed is inversely related to the amount provided from an exogenous source.

The mechanism by which dietary cholesterol inhibits endogenous cholesterol synthesis has been the subject of extensive studies: Gould and

Popják (1957) showed, in rats, that inhibition occurred before formation of mevalonic acid in the biosynthetic pathway. Siperstein and Guest (1960) obtained evidence that the inhibition was localised more specifically at the conversion of β-hydroxy-β-methylglutaric acid (HMG) to mevalonate. These conclusions were based on the fact that cholesterol feeding had no effect on the conversion of mevalonate-C^{14} to cholesterol. However a more direct effect of cholesterol on mevalonate synthesis was not demonstrated.

Recently, Siperstein et al. (1966) were able to separate mevalonate and β-hydroxy-β-methylglutarate by gas-liquid chromatography. With this technique Siperstein and Fagan (1966) showed that feedback inhibition by dietary cholesterol occurs through inhibition of mevalonate synthesis. This is in agreement with the conclusion that the major site of the cholesterol feedback system is located at the reaction responsible for the conversion of

β-hydroxy-β-methylglutarate to mevalonate; namely β-hydroxy-β-methylglutaryl reductase. The nature of the inhibitor, possibly cholesterol itself or one of its metabolites is yet to be determined.

B. Limitation of cholesterol absorption.

Earlier Karvinen et al. (1957) suggested that the human intestine has a limited capacity for the absorption of dietary cholesterol and this limiting effect is considered by Taylor et al. (1965) to be man's principal protection from hypercholesterolaemia of dietary origin, rather than a negative feedback mechanism regulating cholesterol synthesis. This is in agreement with the views of Kaplan et al. (1963).

It has been shown that decreasing the cholesterol content of the diet alone has little effect on serum concentrations (Keys et al. 1965) although the ingestion of a high cholesterol diet especially in the form of egg yolk and in the presence of fat produces a marked elevation

of serum cholesterol (Steiner and Domanski, 1941; Messinger et al. 1950; Connor et al, 1961; Wells and Bronte-Stewart, 1963). Crystalline cholesterol even in the presence of fat has usually failed to increase serum cholesterol levels (Messinger et al. 1950; Connor et al. 1961). Wells and Bronte-Stewart (1963) however found no difference between the serum cholesterol responses with egg yolk and crystalline cholesterol as long as the fat fed with the crystalline cholesterol had the same iodine value as the egg saponifiable fraction. This would suggest that the absorption of cholesterol in man is dependent in part on the dietary cholesterol and also on dietary fat.

INFLUENCE OF DIETARY FAT ON SERUM CHOLESTEROL.

Although dietary fat is known to stimulate cholesterol absorption in rats (Bollman and Flock, 1951; Kim and Ivy, 1952; Pihl, 1955(b)) it is not an essential factor. Dietary fat can be broadly classified as saturated fat

(typically of animal origin or derived by hydrogenation of unsaturated fat) and unsaturated fat (typically of vegetable or fish origin). The degree of unsaturation is given by the iodine value. Many dietary studies involving these two types of fat have been carried out in man and rats and although the majority of workers agree that unsaturated fat does cause a decrease in serum cholesterol levels in man (Ahrens et al. 1957; Keys et al. 1957a; Malmros and Wigard, 1957; Keys et al. 1957b; Joliffe, 1961; Kinsell, 1963) and the rat (Bloomfield, 1964a, Bloomfield, 1964b) others claim that it is the cholesterol rather than the unsaturated fat content of the diet which is more important (Connor et al. 1964; Erickson et al. 1964).

The mechanism by which the unsaturated acids exert their influence is not clear. Bloomfield (1964a; 1964b) suggested that the unsaturated fats cause increased absorption of

cholesterol with markedly increased cholesterol accumulation in the liver of the rat. This is assumed to alter the equilibrium between serum cholesterol and liver cholesterol, causing a decrease in the concentration of serum cholesterol. More recently however, Wood et al. (1966) using synthetic triglycerides, have suggested that the decrease in serum cholesterol in man, noted after ingestion of unsaturated fats, is due to increased faecal excretion of bile acids and neutral steroids resulting from diminished absorption.

Earlier workers had also found this increase in faecal neutral steroids and bile acid excretion with unsaturated fat diets but many of them had used corn oil as their unsaturated fat (Hellman et al. 1957; Moore et al. 1962; Antonis and Bersohn, 1962) and were thus introducing plant sterols such as β-sitosterol, and campesterol as well as unsaturated fatty acids. The cholesterol-reducing effect of corn oil and other vegetable

oils has not yet been fully explained. Some of the reasons given are: reduced solubility of cholesterol in the oil, reduced absorption of cholesterol from the intestinal tract, increased degradation, increased excretion of bile acids and more rapid transport of lipids (Nutrition Reviews 1964). The unsaponifiable fraction of corn oil has been shown to be an effective agent for reducing serum cholesterol in experimental animals (Peterson, 1951) and man (Beveridge, 1958). The most active compound in this fraction is β-sitosterol.

THE INFLUENCE OF PHYTOSTEROLS ON SERUM CHOLESTEROL.

In 1953 Pollak showed that although β-sitosterol caused complete inhibition of hypercholesterolaemia in rabbits on a cholesterol diet, the effect was dependent on the ratio of the two sterols. Rabbits required six times as much β-sitosterol as cholesterol and rats only a ratio of 3:1 of β-sitosterol to cholesterol. Two years later, Glover and Green (1957) carried

out absorption experiments with guinea pigs and suggested that β-sitosterol may compete with cholesterol at the level of the "acceptor sites" of the lipoprotein in particular the lipoproteins present in the mucosal cells, which control the absorption through the mucosa itself. It was originally thought (Hernandez et al. 1953; Swell et al. 1954) that β-sitosterol might compete with cholesterol for esterification within the gut lumen. This is no longer accepted as it has been shown that cholesterol is absorbed into the mucosal cell in the free state (Friedman et al. 1956; Treadwell, 1962).

While earlier studies have suggested that β-sitosterol acts predominantly in the gut and affects absorption, there is the possibility that the small proportion of β-sitosterol absorbed could function in some other manner perhaps as a competitive inhibitor and so bring about a decrease in serum cholesterol

level. Swell *et al* in 1959 showed that phytosterols obtained from tobacco leaves were absorbed into the rat mucosal cell to quite an appreciable extent with virtually all of it present as free sterol. However, after 24 hours there was only 2.1% of the fed dose in the lymph, 50% of which was esterified, compared to 20 to 40% of cholesterol found in the lymph when a comparable amount of cholesterol-4-C^{14} had been administered. They suggested that the principal block in phytosterol absorption occurs within the mucosa, at a step in the transfer mechanism from mucosa to the lymph; this may be the esterification or chylomicron formation. Swell *et al*. (1959) also found that administration of phytosterols produced an increased faecal excretion of cholesterol and related sterols and a lower lymph cholesterol concentration than was found in a control group of rats.

More recently, Haust and Beveridge (1963)

observed that β-sitosterol caused a decrease in serum cholesterol levels in humans. This decrease was accompanied by a significant increase in the faecal excretion of neutral sterols even after correcting for the excretion products of the fed β-sitosterol. This led them to suggest that the hypocholesterolaemic effect of β-sitosterol may be due to increased excretion of endogenous sterols in the faeces. Enoroth et al. (1964) also reported an increase in faecal neutral steroid excretion in human subjects given corn oil but suggested that this increase was due mainly to the increased intake of plant sterols. Unlike Haust and Doveridge (1963) and Enoroth et al. (1964), Spritz et al. (1965) did not find an increase in faecal neutral steroids but found that plant sterols caused an increase in faecal bile acid excretion. They suggested that the changes in human plasma cholesterol levels were due to the redistribution of cholesterol to other tissues. This

increase in faecal bile acid excretion may also be explained by decreased absorption of bile acids which will in turn cause an increased hepatic degradation of cholesterol to bile acids. Gerson et al. (1963; 1965) found that although intraperitoneal injections of β-sitosterol into rats produced a lowering of cholesterol and lipid concentrations in the tissues, there was no change in the faecal excretion of neutral steroids although more $^{14}CO_2$ with higher ^{14}C content was expired in rats given β-sitosterol. Together these observations suggest that the effect of β-sitosterol on serum cholesterol may be produced both in the intestinal mucosa and in the liver.

Although β-sitosterol does lower the cholesterol blood levels in man the effect is achieved only by continuing administration at a dose of at least 5-10g./day (Preziosi, 1964): once the treatment is suspended the cholesterol blood levels tend to return rapidly to the

initial values. β-sitosterol has the disadvantage that many subjects cannot tolerate it with the result that they quickly discontinue treatment and consequently there are no hypocholesterolaemic effects.

MISCELLANEOUS STEROIDS.

Various other sterols have also been tried but they normally produced undesirable side-effects. Dihydrocholesterol (5α-cholestan-3β-ol (VII)) was found to prevent hypercholesterolaemia in cockerels (Siperstein et al. 1953(a)) and rabbits (Nichols et al. 1953) maintained on a cholesterol diet, but it was absorbed itself to quite an extent and was shown to be atherogenic in rabbits when added to the diet (Curran and Costello, 1956).

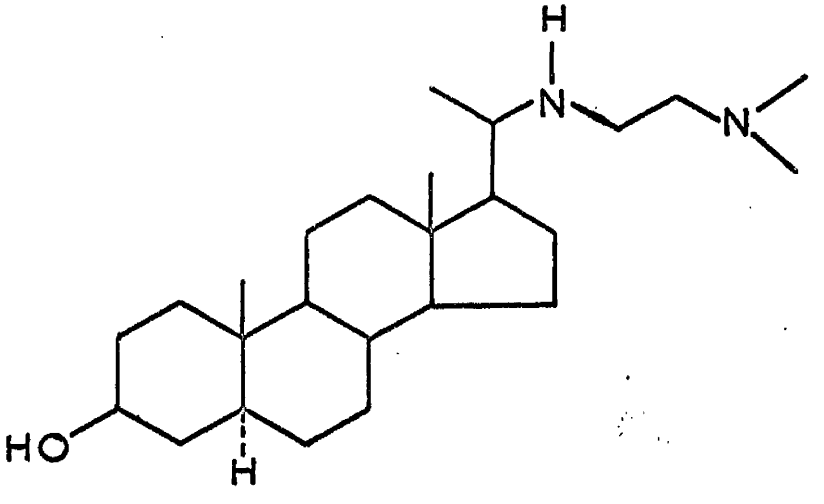
Δ^{4}-Cholesten-3-one (IX) was shown to be an inhibitor of cholesterol biosynthesis in the rat (Tomkins et al. 1953(b)) but is converted in vivo to dihydrocholesterol as one of its major metabolic products (Anker and Bloch,

1949; Stokes et al. 1955). This renders Δ^4-cholesten-3-one unsuitable as dihydrocholesterol is itself atherogenic (Curran and Costello, 1956).

AZASTEROLS.

Oral administration of diaza-derivatives of either cholestanol or cholesterol has been reported to lower serum cholesterol levels in normal and hypercholesterolaemic rats, in normal dogs and in man (Counsell et al. 1962a; Counsell et al. 1962b). The effects of 22, 25-diazacholestanol have been studied in great detail.

Figure 9.



22,25-diazacholestanol

Rats treated orally for 10 days showed a fall of 24% and 17% in plasma and liver cholesterol respectively (Ranney and Counsell, 1962) and Sachs and Wolfman (1962) reported a similar finding in human subjects. From their results Sachs and Wolfman (1962) suggested that although the primary action of this compound appeared to be the inhibition of cholesterol synthesis at the level of hydroxymethylglutaryl CoA reductase, a desmosterol-like compound was also detected in the serum of treated patients suggesting an additional site of inhibition occurring late in the cholesterol biosynthetic cycle. More recently, Sachs and Wolfman (1965) showed that 20, 25-diazacholestenol dihydrochloride moderately reduced plasma total sterols while effectively lowering the plasma cholesterol concentration in thirteen hypercholesterolaemic subjects; but this was accompanied by the progressive accumulation of a precursor sterol which they identified by thin layer chromatography as desmosterol.

[illegible].

The oral administration of this antibiotic neomycin to human subjects resulted in a significant decrease in serum cholesterol levels (Samuel and Steiner, 1959) which was accompanied by an increased faecal excretion of neutral sterols (Powell _et al_. 1962) and bile acids (Goldsmith _et al_. 1960; Goldsmith, 1962; Powell _et al_. 1962). Neamine, kanamycin and chlortetracycline resulted in a less consistent and less marked decrease (Samuel and Waithe, 1961; Steiner _et al_. 1961). Intramuscular neomycin as well as oral streptomycin, dihydrostreptomycin, tetracycline, oxytetracycline, chloramphenicol, polymycin B, bacitracin and novobiocin were ineffective (Samuel and Waithe, 1961; Steiner _et al_. 1961).

Since neomycin is poorly absorbed from the gut, the effect of the drug must be dependent on its effect on the gastrointestinal tract. Three possible mechanisms of action have been

proposed.

a) alternation of intestinal bacterial flora due to its antibiotic properties (Goldsmith, 1962; Powell et al. 1962).

b) intestinal malabsorption of lipids due to diarrhoea consequent upon alteration of the bacterial flora (Jacobson et al. 1960) and

c) the formation of insoluble salts of the antibiotic with bile acids (Dyssen et al. 1966).

The last hypothesis is the most recent and the work by Dyssen et al. (1966) with chicks does suggest that, in the case of neomycin, it is the basic and not the antibiotic property which causes the decrease in serum cholesterol. Using a neutral N-acetylated neomycin which had no antibiotic properties and a condensation product of two molecules of streptomycin with diaminoguandine which had seven basic groups and had about 5% of the antibiotic activity of

streptomycin they showed that N-acetylated neomycin was inactive but the condensation compound was very effective in lowering serum cholesterol levels. More compelling observations were made by Van den Bosch and Claes (1967) who showed that N-methylated neomycin, a compound without antibacterial activity but with several basic groups lowered serum cholesterol levels in human subjects and increased the faecal excretion of neutral steroids and bile acids.

AGENTS FORMING INSOLUBLE BILE SALTS

The formation of insoluble bile salts as a mechanism for lowering serum cholesterol levels was also studied by Siperstein et al. in 1953(b). They found that the addition of ferric chloride, which precipitates the bile acids, to a diet rich in bile concentrate and cholesterol impeded the increase in cholesterolaemia in cockerels. However, Boher et al. (1957) did not find any change in the intestinal

absorption of cholic acid or the rate of hepatic cholesterol mobilization by mice following the oral administration of ferric chloride. Similar differences were encountered with the feeding of aluminium hydroxide gel. Rodbard et al. (1950) found that aluminium hydroxide gel lowered blood cholesterol and inhibited atherogenesis in cholesterol fed cockerels but in rats, aluminium phosphate gel was ineffective in increasing bile acid excretion and in fact increased absorption of cholesterol (Setty and Ivy, 1960). Dietary calcium has also been shown to be hypocholesterolaemic (Yacowitz et al. 1965). Its mode of action in rats is thought to be due to increased excretion of faecal bile acids and faecal 3β-hydroxysterols (Fleischman et al. 1967). In the latter part of this thesis the effect of increased dietary calcium on cholesterol metabolism in six human subjects is presented.

An important development in the lowering of serum cholesterol levels by the removal of

bile acids as insoluble compounds by the application of bile acid binding anion-exchange resins. Borjon et al. (1959) and Tennent et al. (1960, 1961) found a cholestyramine resin (MK-135) capable of binding the biliary acids selectively in the intestinal tract and thus favouring their excretion. The resin is insoluble, has a polystyrene skeleton, is neither degraded nor absorbed in the intestinal tract and seems to have no toxic effects. It increases faecal bile acid excretion by a factor of 8 in doses of 10 g./day without causing steatorrhea. Tennent et al. (1960) found that it lowered the serum cholesterol levels in cockerels on a cholesterol enriched diet and also in normocholesterolaemic cockerels and dogs. The clinical use of cholestyramine has been extended to the treatment of pruritis in primary biliary cirrhosis and obstructive jaundice (Carey, 1960; Carey and Williams, 1961).

THYROID HORMONES AND SERUM CHOLESTEROL.

It is by now well established that serum cholesterol levels are markedly influenced by thyroid hormones (Gildea et al. 1939; Strisower et al. 1954). In 1960, Loyd and Oliver studied the effect of thyroxine and some of its analogues on serum cholesterol levels. They concluded that of the analogues studied, the compounds iodinated at the 3,5,3' - positions were the most active in all respects and that the laevo-isomers were more active than the dextro-isomers in those compounds which were optical active. However, the dextro-isomers were found to be less likely to cause angina than the laevo-isomers and were still able to lower serum cholesterol levels. Khitchevsky (1960) suggested that the thyroid hormone, despite the fact that it causes increased synthesis of cholesterol, lowers serum cholesterol levels by increasing the degradation and excretion of cholesterol.

p-AMINOSALICYLIC ACID./

p-AMINOSALICYLIC ACID.

Tygstrup et al. in 1959 discovered that patients with pulmonary tuberculosis had a marked decrease in serum cholesterol levels after treatment with p-aminosalicylic acid (P.A.S.). Later Tygstrup and co-workers (1960) observed that P.A.S. administered orally (12 g./day) could reduce serum cholesterol levels by as much as 30% but that intravenous administration of the drug was considerably less effective. From their results they suggested that P.A.S. acts by impairing cholesterol absorption. Unfortunately, approximately 50% of the subjects treated with P.A.S. complained of nausea, vomiting and/or diarrhoea.

SALICYLIC ACID.

This compound was found by Reid (1960) to lower serum cholesterol levels but due to side effects such as tinnitus, deafness and gastro-intestinal irritation, caused by the high dosage required, it is unsuitable for practice.

[illegible]

The following three compounds are known to depress cholesterol synthesis; α-phenylbutyric acid (Steinberg and Avigan, 1960(a)) triparanol (MER-29) (Bloom and MacKenzie, 1959, Avigan et al. 1960a, and Avigan et al. 1960b) and nicotinic acid (Parsons, 1961).

It is thought that α-phenylbutyric acid inhibits the first step in cholesterol biosynthesis i.e. formation of acetyl Coenzyme A, whereas triparanol was shown to inhibit cholesterol biosynthesis mainly at the desmosterol stage (Steinberg and Avigan, 1960(b), Steinberg et al. 1960 and Frantz et al. 1960). Triparanol has also been found to inhibit other stages of cholesterol biosynthesis (Holmes and Di Pullio, 1962). This drug however has serious side effects, giving rise to cataracts, ichthyosis and alopecia (Goodman and Gilman, 1965) together with accumulation of desmosterol in serum, and it is no longer used. In 1955 Altschul et al.

showed that large doses of nicotinic acid lowered plasma cholesterol levels. Parsons (1961) showed that nicotinic acid caused an inhibition of the incorporation of acetate into serum cholesterol. Similar results have been obtained by Nunn et al. (1961) and Goldsmith (1962). The experiments of Miller et al. (1962) suggest that nicotinic acid inhibits the synthesis of cholesterol prior to the squalene cyclisation step. This is based on their finding only cholesterol in the serum of a patient treated with nicotinic acid. However, the side effects of nicotinic acid, flushing and pruritus, make it unsuitable for long term administration as a cholesterol lowering agent (Altschul et al. 1955; Goodman and Gilman, 1965).

ARYLOXYISOBUTYRIC ACIDS.

In 1962, Thorp and Waring found that a series of aryloxyisobutyric acids and related compounds reduced total lipid and cholesterol

concentration in the liver and blood of rats. The most effective of these was ethyl 2-(p-chlorophenoxy)-2-methylpropionate (CPIB) or its free acid. In the same year Oliver (1962) reported that a mixture of CPIB plus androsterone, "Atromid", lowered serum cholesterol and serum triglyceride levels in patients with ischaemic heart disease. It was shown in 1963 that CPIB alone (known as Clofibrate or Atromid-S) was as effective as "Atromid" in reducing serum cholesterol and serum triglyceride levels in man. The mechanism of action of this compound is still unknown although it has been suggested that it inhibits either the hepatic synthesis of cholesterol (Azarnoff and Tucker, 1964, and Nestel et al. 1965) and triglycerides (Thorp, 1962) or the low density lipoproteins which transport triglycerides in the serum (Strisower et al. 1965). Others have found no change in triglyceride production and have suggested that Clofibrate acts by increasing the removal of

circulating lipid (Ryan and Schwartz, 1964 and Spritz, 1965). Minor side effects such as nausea and dyspepsia (Goodman and Gilman, 1965) have been observed in about 10% of patients treated with this drug. Studies of faecal neutral steroid and bile acid excretion in patients treated with this drug are reported in this thesis.

In summary, the compounds reviewed which reduce serum cholesterol levels in hypercholesterolaemic human subjects and experimentally induced hypercholesterolaemia in animals may be divided into three groups according to their supposed mechanism of action:

a) Inhibitors of cholesterol biosynthesis either at an early step in the biosynthetic pathway or at a stage after the cyclisation of squalene. These agents include azasterols, α-phenylbutyric acid, triparanol, nicotinic acid and clofibrate.

b) Compounds which cause increased excretion of faecal bile acids and/or faecal neutral

steroids. Examples include unsaturated fats, phytosterols, soy fats, calcium salts, anion exchange resins and thyroid hormones. Ferric chloride and aluminium hydroxide gel have been shown to increase faecal bile acid excretion in experimental animals.

c) The final group contains p-aminosalicylic acid, which is thought to act by impairing cholesterol absorption, β-sitosterol may also act in this way, and salicylic acid. The mechanism of the action of salicylic acid on lipids is not completely understood.

Two practical issues are raised by this consideration of cholesterol metabolism:

1) That a wide variety of approaches have been tried in order to control serum cholesterol levels.

2) That relatively little work had been carried out on the excretion of cholesterol and its

metabolites.

The work in this thesis arose from this situation. As the techniques used were modifications of classical thin-layer chromatography (TLC), the following sections will be devoted to a consideration of the use of TLC in lipid analysis (Part II) and to specific modifications and to techniques (Part III) used in assessment of quantitation of excretion of cholesterol and its metabolites.

PART II.

A REVIEW OF TLC AND ITS APPLICATIONS TO LIPID ANALYSIS

CONTENTS Pg.

SEPARATION OF LIPIDS BY CHROMATOGRAPHY

THIN-LAYER CHROMATOGRAPHY (TLC)

During the last 15 years, the more traditional techniques of lipid analysis, such as fractional crystallization, have been almost completely replaced by chromatographic methods. Initially column chromatography was used exclusively but later the introduction of cellulose and glass-fibre papers impregnated with silicic acid enabled small quantities of lipid to be separated with greater resolution and speed. More recently paper-chromatographic methods have been largely replaced by thin-layer chromatography (TLC) which has the advantages of giving more discrete spots with consequently better resolution and, in adsorption systems, of allowing more corrosive and sensitive detection agents to be employed than are possible with impregnated cellulose papers.

Thin-layer chromatography was first introduced in a convenient form as a procedure for

analytical adsorption chromatography by Stahl in 1956 who used glass plates coated with a layer of adsorbent material.

By ascending one-dimensional thin-layer chromatography, the developing solvent rises and transports the individual substances at rates which depend on their physical characteristics. Separation also depends on the solvent and on the chromatographic matrix. The processes involved in separation may be adsorption, partition or ion-exchange or a combination of those.

Various adsorbents for TLC are manufactured commercially and are almost exclusively oxides, hydrated oxides or salts e.g. alumina, silicic acid and magnesium carbonate. The most generally useful adsorbent is silica gel, available with or without calcium sulphate as a binder to afford a firmer layer. It may also be impregnated with phosphors which cause fluorescence under ultraviolet light. Various ionic materials may be incorporated which form complexes with

the compounds to be separated and hence increase their separation from each other. Impregnation of the adsorbent on the plate with hydrocarbons such as undecane or tetradecane allows reversed-phase partition-TLC to be carried out. Coating the plate with cellulose ion-exchange resins or with dextran gel ("Sephadex") widens the scope of the method considerably although these modifications have not yet been fully exploited.

Thin layer plates may be "poured", "sprayed" or "dipped" but to obtain a uniform layer it is much better to use "spread" plates.

LOCATION OF COMPOUNDS ON TLC PLATES

Detection of compounds on TLC plates may be achieved destructively or non-destructively. A convenient destructive procedure entails spraying the plate with a charring reagent such as 50% sulphuric acid and heating in an oven at a high temperature (approx. 180°C.). This produces dark spots on a relatively white background. Milder treatments with acidic reagents

or with other reagents such as phosphomolybdic acid in ethanol, frequently afford characteristic coloured spots on heating. For non-destructive detection, the plate may be sprayed with water (when hydrophobic compounds may appear as glistening spots) or with a fluorescent material allowing detection by quenching of fluorescence under ultraviolet light. Scanning equipment can be used to detect radioactive material. Non-destructive techniques must be used if the compounds are to be recovered from the thin-layer plate for further analysis.

MEASUREMENT OF R_F VALUES

As in paper chromatography, the rate of advance of each compound in a given solvent on a given adsorbent is expressed as an R_F value:

$$R_F \text{ value} = \frac{\text{distance of the substance from the origin}}{\text{distance of the solvent front from the origin}}$$

However to overcome the effect of variations

in conditions and in layer thickness it is advisable to run a known comparison compound, for instance a dye or a known standard compound, together with the unknown mixture on the same chromatogram so that effective comparison can be made between plates. In this way a relative R_F value is obtained.

$$\text{Relative } R_F \text{ value} = \frac{\text{distance of substance from origin}}{\text{distance of known substance from origin}}$$

APPLICATIONS OF TLC

Adsorption TLC has been used extensively in the analysis of naturally occurring fats, oils (Malins and Wekell, 1963; McKillican and Sims, 1963) and waxes (Haahti et al. 1963). Human skin fat and sebum have also been analysed by adsorption-TLC (Haahti and Nikkari, 1963) and total lipid extracts of human and animal blood serum, as well as various organs have been fractionated by a similar technique (Chalvardjian et al. 1962, 1964; Hofmann and Bergström, 1962;

Kaunitz et al. 1962; Krell and Hashim, 1963; Stein and Stein, 1962). Fractionation of steroids (Avigan et al. 1963) and free and conjugated bile acids (Eneroth, 1963; Hofmann, 1962) has also been carried out using adsorption TLC. Convenient separations of phospholipids (Hofmann, 1963), sulpholipids (Martensson, 1963), glycolipids (Sweeley and Klinosky, 1963) and carotenoids (Davies et al. 1963) have also been made possible.

Until fairly recently, adsorption TLC was used almost exclusively but reversed-phase partition TLC, and TLC on layers containing complexing agents have permitted separations that previously could not be accomplished by any simple method. By impregnating TLC plates with silver nitrate, various classes of compounds can be resolved even further according to their degree of saturation. This has been applied to vegetable oils and animal fats (Barrett et al. 1962; Barrett et al. 1963), human skin

lipids (Haahti et al. 1963) to sterol esters isolated from human blood serum (Haahti et al. 1963; Morris, 1963) and to the separation of higher fatty acid isomers and vinylogues (Morris, 1962).

Reversed-phase partition chromatography, although it has not been used as widely as adsorbent or silver nitrate impregnated TLC, has been found to be useful in the resolution of triglycerides and cholesterol esters (Michalec et al. 1962).

THE SEPARATION OF STRUCTURALLY SIMILAR STEROLS BY TLC.

The analysis of certain groups of sterols in biological material into their constituent compounds has proved extremely difficult. For example cholestan-3β-ol (dihydrocholesterol) and Δ^7-cholestenol (lathosterol) which accompany cholesterol in serum, faeces and several tissues (Kritchevsky, 1958(b); Cook, 1958(c); Mosbach et al. 1963) cannot be separated from cholesterol by ordinary TLC (Cargill, 1962; Avigan et al. 1963)

or glass paper chromatography (Swartwout et al. 1960; Hamilton and Muldrey, 1961). With gas-liquid chromatography, resolution of mixtures of cholesterol and cholestanol is also difficult. Partial separation of these sterols has been achieved using a selective stationary phase (Horning et al. 1963) but it is only when derivatives are used that complete separation occurs (Horning et al. 1963).

Cargill (1962) and Michalec (1963) separated cholestanol from cholesterol on thin-layer plates after bromination, which converted cholesterol to the faster-moving dibromide but left the saturated cholestanol unaltered. Unfortunately, more polar oxidation products of cholesterol were formed at the same time (Cargill, 1962) which made it difficult to estimate the cholesterol after developing the chromatogram. Avigan et al. (1963) were able to separate Δ^7-cholestenol from cholesterol with benzene-ethyl acetate (20:1) on Silica Gel G, but the plates had to be 40 cm.

long and the development took 24-36 hours. De Vries (1962) introduced the use of silica gel impregnated with silver nitrate for the separation of *cis* and *trans* isomers of fatty acids by column chromatography. This principle has been extended to the TLC of lipids of several classes differing only by one double bond (Avigan *et al*. 1963; Morris, 1962; 1963; Barrett *et al*. 1963; Gréen and Samuelsson, 1964).

The efficiency of this technique is amplified if sterol derivatives rather than free sterols are used. Copius-Peereboom and Beekes (1965) obtained better separation of cholestanol and Δ^7-cholestenol acetates than of the free sterols. Claude (1965) also found that the acetates of cholesterol and cholestanol could be separated from each other and also from desmosterol acetate, but better separations were obtained of their propionates. Svoboda and Thompson (1967) reported the separation of

the isomers 24- and 25- dehydrocholesterol acetates on silver nitrate impregnated TLC.

Although silver nitrate impregnated TLC has been successful in resolving mixtures of closely related sterol derivatives it fails to separate cholesterol from the closely related phytosterols such as β-sitosterol and campesterol either in acetate or free form.

However Copius-Peereboom and Beekes (1965) used reversed-phase TLC to successfully separate the acetates of cholesterol and phytosterols. The incorporation of bromine into the solvent system for the reversed-phase TLC improved the separation of cholesterol, β-sitosterol, campesterol and stigmasterol acetates from each other. These authors also found that cholesterol was not separated from Δ^7-cholestenol by reversed-phase TLC. It is also important to note that the use of bromine results in the decomposition of sterols with a system of conjugated double bonds and sterols devoid of the normal $\Delta^{5(6)}$ double

bond (e.g. Δ^7-sterols and lanosterol).

QUANTITATIVE ANALYSIS OF FAECAL NEUTRAL STEROIDS AND BILE ACIDS

ESTIMATION OF NEUTRAL STEROIDS

The quantitative determination of individual faecal neutral steroids has proved to be very difficult because of the similarities in their molecular structures and physical properties. Over the past few years there have been several methods published, many of which rely on precipitation of the sterol with digitonin followed by regeneration of the sterol and photometric analysis frequently by the Liebermann Burchard reaction. (Antonis and Bersohn, 1962; Haust and Beveridge, 1963). However these techniques do not measure all the steroids present in faeces. For example, coprostanol, normally the predominant faecal neutral steroid, is incompletely precipitated by digitonin (Wells and Moore, 1961) and coprostanone, which may account for one third of the total faecal neutral steroid (Miettinen et al. 1965), is not measured by the

Liebermann Burchard reaction. Isotopic balance techniques (Hellman et al. 1957) have the disadvantage that radioactive sterols must be administered to the patients.

Of the earlier methods the most accurate would appear to be that of Goldsmith et al. (1960). This technique separated the neutral steroids by glass paper chromatography into three main groups, the principal compound of each group being cholesterol, coprostanol and coprostanone respectively. The optical density (OD) of each spot was measured after charring with sulphuric acid, the relationship between the OD and the amount of steroid charred being linear over a range of 0.6 μg. to 1.2 μg. It had several disadvantages; one being that it could not differentiate between the plant sterols of dietary origin and the endogenous sterols; another that the charred spots had to be cut from the glass paper chromatogram before the OD could be measured. . The more

recent methods of Eneroth et al. (1964) and Miettinen et al. (1965) incorporate column chromatography, TLC and GLC and are able to measure all faecal neutral steroids, whether of plant or animal origin. The method of Miettinen et al. (1965) is especially well controlled, using an internal standard to compensate for any losses through the procedure. The sensitivity of this method is such that 1 g. of faecal homogenate containing as little as 25 μg. of mixed neutral sterols can be analysed accurately. It has a disadvantage that there are several steps between the extraction of the faeces and the measurement of the steroid concentration.

ESTIMATION OF FAECAL BILE ACIDS

The complexity of the mixture of faecal bile acids has rendered separation and quantification extremely difficult. Most methods currently available have relied upon the titration of total bile acids with alkali which results in spuriously high values due to incomplete removal

of acidic contaminants. These methods have recently been reviewed by Grundy et al.(1965). In the method devised by Grundy et al. (1965) the bile acids are isolated by column chromatography, thin-layer chromatography and then estimated as the trimethylsilyl ethers of their methyl esters by gas-liquid chromatography. This technique has the advantage that not only total bile acid measurements can be made but changes in individual bile acids can also be observed. The method appears to be very reproducible with recoveries of the order of 90% and extremely sensitive. Faecal samples containing 50 μg. of mixed bile acids can be analysed accurately (Grundy et al. 1965). More recently Eneroth et al. (1966(a),(b)) have identified the bile acids in faeces with gas-liquid chromatography combined with mass spectrometry following a similar purification procedure to that of Grundy et al. (1965) and concluded that deoxycholic acid and lithocholic acid were the

main faecal bile acids. Semenuk and Beher (1966) have devised a method of direct densitometry of bile acids on thin-layer chromatograms using phosphomolybdic acid to locate and measure the compounds. The colour density response was linear over a range of 0.25 to 4.0 μg.

The remarkable resolving power of thin-layer chromatography and the fact that compounds can be estimated in situ on the thin layer plate indicated that it would be an ideal technique for the qualitative and quantitative analysis of faecal neutral steroids and bile acids. The next section contains a description of the methods devised and the results of these investigations.

PART III.

METHODS

CONTENTS Pg.

CONTENTS (Contd.) Pg.

MATERIALS

1. Silica gel. MN-Kieselgel G-HR (Camlab Ltd., England).

   Dowex 50W-X8 cation exchange resin (H^+ form) (V.A. Howe Ltd., London).

   Dowex 1-X2 anion exchange resin (Cl^- form) (V.A. Howe Ltd., London). (This resin was converted to the hydroxyl form by suspension in 1 M. sodium hydroxide).

2. Solvents

2,2,4-Trimethyl pentane	Analar (BDH)
Sulphuric Acid	Analar (BDH)

   All other solvents were redistilled before use.

3. Reagents

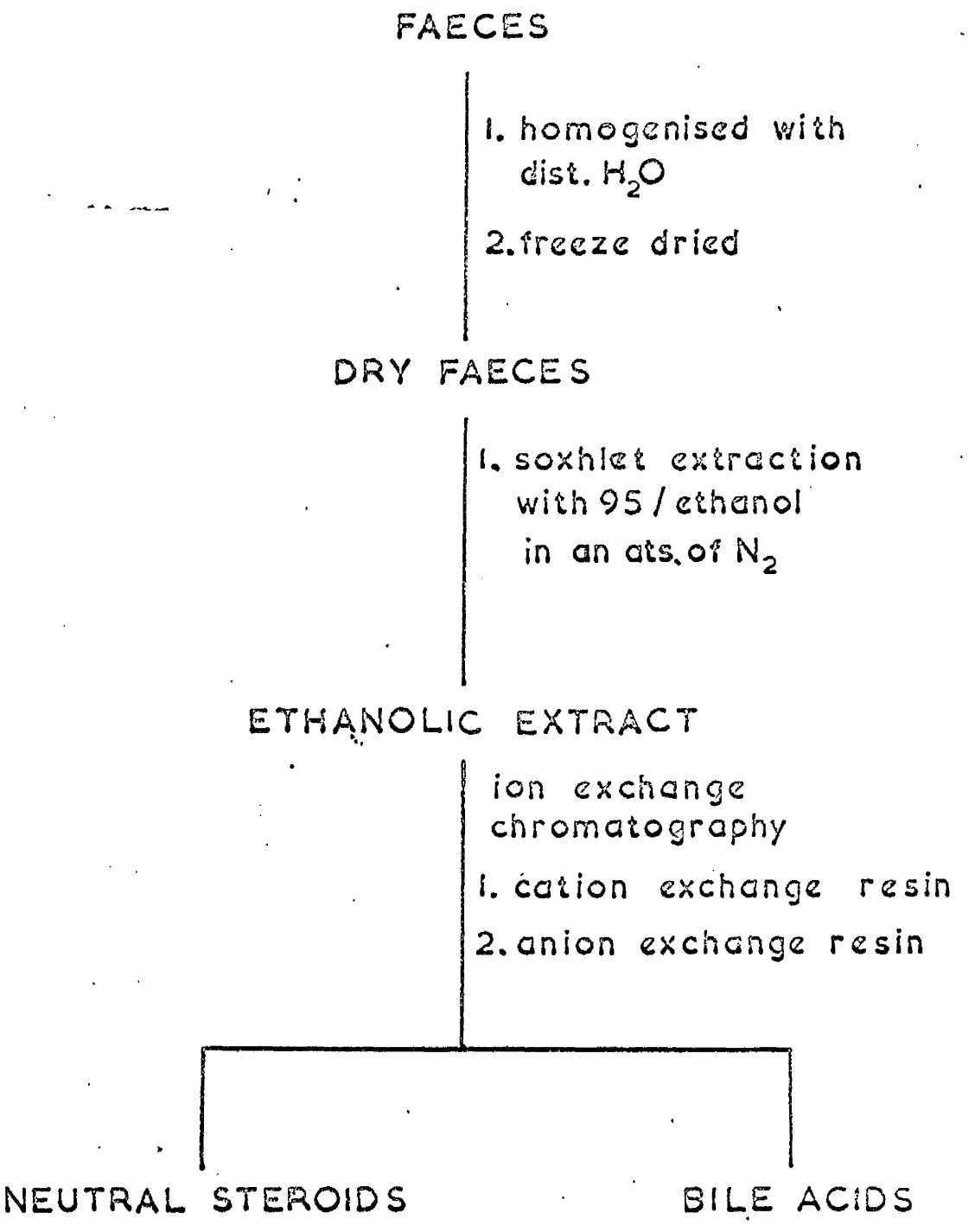
Silver nitrate	Analar (BDH)
Phosphomolybdic acid	" (")
Potassium iodate	" (")
Sodium hydroxide (pellets)	" (")
Ammonium carbonate	" (")
Sodium chloride	" (")

Hexamethyldisilazane.	Koch-Light Laboratories Ltd.	
Trimethylchlorosilane.		(B.D.H.)
Diazomethane.		

Standards

Coprostanol	Southeastern Biochemicals	(U.S.A.)
Coprostanone	" "	(")
Cholestanol	" "	(")
Δ^7-cholestenol	" "	(")
cholesterol	recrystallised	(B.D.H.)
Epicholestanol	Steraloids Ltd.	(Croydon)
Epicoprostanol	" "	(")
Cholic acid	B.D.H.	
deoxycholic acid	Sigma	(London)
lithocholic acid	Calbiochem	(U.S.A.)
glycocholic acid	Calbiochem.	(U.S.A.)
taurocholic acid	Southeastern Biochemicals	(U.S.A.)
12-Hydroxystearic acid	Calbiochem.	(")

Figure 10(a).



Procedure for the separation of faecal neutral steroids from bile acids.

PREPARATION OF FAECAL NEUTRAL STEROID AND BILE ACID EXTRACTS.

(1) Collecting and Drying of Faeces

Faeces were collected in weighed plastic containers during a period of 3-7 days and stored in a deep freeze until required. The specimens were then weighed and homogenised with distilled water in a weighed homogeniser. An aliquot of the homogenate representing a known weight of faeces was transferred to a tared 250 ml. round bottom flask, freeze-dried for approximately 24 hours, and the flasks re-weighed. The dried faeces were removed and transferred to small screw-capped jars which were then stored in a desiccator until required.

(2) Extraction of Faeces

A known weight (between 1.-2.0 g.) of dried faeces was extracted with 95% ethanol in a Soxhlet extraction apparatus under nitrogen atmosphere for 6 hours. The extract was then separated into acidic and neutral fractions by passing it through Dowex ion-exchange resins, as

described below.

(3) Ion-Exchange Column Chromatography.

Column I.

A glass column of inside diameter 1.3 cm. and length 39 cm. was fitted with a stopcock, a coarse sintered disc and a reservoir to hold approximately 50 ml. Dowex 50W-X8 cation exchange resin (hydrogen form) (approx. 15.0 g.), suspended in 80% ethanol, was poured into the column and allowed to settle to a depth of 16 cm. above the sintered disc. The resin was then washed with a further 100 ml. of 80% ethanol.

Column II.

Dowex 1-X2 anion exchange resin (approx. 14.0 g.) was converted from the chloride form to the hydroxyl form by suspension in aq. 1 M. sodium hydroxide. The suspension was poured into a glass column similar to column I and allowed to settle to a depth of 13 cm. above the sintered disc before washing with 100 ml. aq. 1 M. sodium hydroxide. The column was

then brought to neutrality with distilled water before being washed with 100 ml. of 80% ethanol.

The columns were then arranged so that the eluate from column I drained directly into the reservoir of column II.

Neutral Steroid Fraction

The faecal extract was poured into column I and allowed to percolate at a flow rate of 4-5 ml./min. into column II. When approximately 5.0 ml. of the extract was left in column I, 50 ml. of 80% ethanol was added in 15 ml. aliquots and the eluate was allowed to run through into column II. Column I was allowed to run dry and the eluate from column II containing neutral steroids was collected. This fraction contains cholesterol, coprostanol and coprostanone together with their phytosterol analogues.

Bile Acid Fraction

The faecal bile acids retained on column II were then eluted with 250 ml. 0.1 M. ammonium carbonate in 50% ethanol after washing the column

in the following manner according to Kuron and Tennent (1961):-

a) 25 ml. 50% ethanol
b) 50 ml. distilled water
c) 25 ml. CO_2 free water
d) 100 ml. aq. 1M sodium chloride
e) 100 ml. aq. 0.5 M ammonium carbonate
f) water to neutrality
g) 25 ml. CO_2 free water
h) 25 ml. 50% ethanol

The bile acid fraction consists of a mixture of trihydroxy bile acids, when present, and the dihydroxy and monohydroxy bile acids which are predominant. The main constituents of these three groups of bile acids are cholic acid, deoxycholic acid and lithocholic acid respectively.

The faecal neutral steroid fraction and the bile acid fraction were then separately evaporated to dryness on a rotary evaporator and the residues dissolved in acetone, transferred with washings to 15 ml. vials and then evaporated to dryness with a stream of nitrogen. The residues were dissolved in 3 ml. of

re-distilled acetone and stored in tightly-capped vials in a deep-freeze until analysed by TLC.

THIN-LAYER CHROMATOGRAPHY

PREPARATION OF TLC PLATES

The following procedure was used. A smooth suspension of silica gel in distilled water (30 g. in 60 ml. water) was obtained by shaking the mixture in a stoppered 250 ml. conical flask for 90 secs. The plates were then spread with a Camag Spreader, left overnight at room temperature to dry and then stored in a desiccator until required. This method produced TLC plates with a uniformly thick (0.25 mm.) layer. (The Desaga and Shandon spreaders were both tried but the layers obtained were not uniformly thick.) Solutions of the compounds to be separated were applied with a 10 µl. Hamilton syringe about 1.5 cm. from the lower edge of the plate and the plate placed in a chromatography tank filled to a depth of

approximately 0.5 cm. with a suitable developing solvent. The tank was lined with filter paper to ensure saturation of the atmosphere in the tank with solvent vapour.

PREPARATION OF SILVER-NITRATE IMPREGNATED TLC PLATES

When required, a silica gel plate was sprayed with 25% aqueous silver nitrate until evenly moistened and dried in an oven at 110° for 30 min. It was allowed to cool to room temperature before applying the sample. The silver nitrate layer started to darken after standing 12-24 hrs., even in the dark, so that impregnated plates were used on the day of preparation.

PREPARATION OF REVERSED-PHASE TLC PLATES

A silica gel plate, 20 cm. long, was impregnated with stationary phase by allowing 15% n-undecane in petroleum ether (B.pt. 40-60°) to rise nearly to the top of the plate in a TLC tank. The samples were then applied and the plate left in a fume cupboard for 1 hr.

to allow the petroleum ether to evaporate.

THIN-LAYER CHROMATOGRAPHY OF NEUTRAL STEROID FRACTION

First separation: Silver nitrate - impregnated thin-layer Chromatography

An aliquot from the faecal neutral steroid extract was subjected to silver nitrate impregnated TLC in the solvent system chloroform-acetone (98:2). The solvent front was allowed to rise 15 cm. above the origin. To make the sterols suitable for light photography the plate was sprayed with 10% ethanolic phosphomolybdic acid and then heated at 140° for 15-20 min. to produce brown spots on a yellow background (see Results section, Figure 10).

The effect of impregnating silica gel with silver nitrate on the R_F values of cholesterol, Δ^7-cholestenol and the C_{27} stanols is shown in Tables V and VI (Results section, p. 109-110).

Extraction of sterols from silver nitrate impregnated plate

The sterols were detected by lightly spraying the dried plate with distilled water. The plate was viewed against a dark background

when the sterols appeared as white spots as the thin layer started to dry. The smallest amounts that could be detected with certainty in this way were 15 μg. of cholesterol and 5 μg. of Δ^7-cholestenol. The spots were ringed with a fine point, allowed to dry, scraped off the plate into conical centrifuge tubes and extracted. Extraction with methanol, chloroform and ethanolic ammonia were compared, the latter being found most suitable (Results section, Table VII, p. 113).

Extraction with ethanolic ammonia

The silica gel in the conical centrifuge tube was extracted with 50% ethanolic ammonia (3 x 4 ml.). The combined extracts were diluted with an equal volume of distilled water and re-extracted with 3 x 10 ml. redistilled benzene. The benzene extracts were washed with 20 ml. of distilled water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness at 40-50° under reduced pressure. The residue

was either subjected to further chromatography (see below) or a Liebermann-Burchard reaction was carried out at this stage (p. 100).

Second separation: Reversed-phase chromatography at low temperature

The residue from the single spot containing cholestanol and Δ^7-cholestenol was taken up in a small volume of chloroform. The mixture of Δ^7-cholestenol and cholestanol was resolved conveniently by reversed-phase TLC. The tank containing mobile phase (acetic acid:acetonitrile (1:1), 70% saturated with undecane (Kaufmann and Makus, 1960))was placed in a deep-freeze (-15 to -20°) for 15 mins. (for temperature equilibration) before development of the plate. The prepared plate was developed until the solvent reached a height of 15 cm. (time 3-3½ hours). Undecane was removed from the plate by heating for 1 hour at 150° and the sterols were located with ethanolic phosphomolybdic acid (Results section, Figure 11). If the plate was sprayed immediately

after being taken out of the oven, it was not necessary to heat further to induce colour development.

Adsorption Thin-Layer Chromatography: Quantification of Faecal Neutral Steroid Fraction

The TLC plate was prepared as previously described (p. 91), cleaned by initial development in benzene-ethyl acetate (8:1) and allowed to dry in a fume-cupboard at room temperature. The faecal neutral steroid extracts for quantitative analysis and standards were then applied and the plate was developed at room temperature in benzene-ethyl acetate (8:1) to a height of 15 cm. Subsequently the plate was dried in a fume cupboard at room temperature. The compounds were subjected to densitometry in situ (p. 98).

ADSORPTION THIN-LAYER CHROMATOGRAPHY: QUANTIFICATION OF FAECAL BILE ACID FRACTION.

The bile acid fraction and standards were applied as previously described and the plate developed in hexane-diethyl ether-methanol-acetic acid (90:20:3:2, Brown and Johnston, 1962). This solvent system caused the fatty acids to move approximately 10 cm. in a 15 cm. development and left the bile acids at the origin. The plate was then air dried in a fume cupboard and placed in an oven at 100° for 20 min. to remove traces of solvent. After cooling, the plate was developed in 2, 2, 4-trimethyl pentane-ethyl acetate-acetic acid (5:25:0.2; Eneroth, 1963) to a height of 10 cm. This gave a very good separation of the bile acids and was also effective in separating hydroxy-fatty acids from the bile acids. The bile acids were then subjected to densitometry in situ (see below).

Measurement of Steroid Concentration by Densitometry

The plate was sprayed with 1% potassium

iodate in 10% sulphuric acid and heated at 100° for 20 min. to produce dark brown spots on a white background which proved extremely suitable for scanning (see Results section, Figures 12 and 13).

The optical density (O.D.) of the charred spots was measured with the Joyce Loebl Chromoscan with Thin Layer Attachment. The attachment is suitable for both reflectance and transmittance operation and uses the recording and integrating facilities of the Chromoscan. Best results were obtained using the narrow slit width and transmission on the Thin Layer Attachment combined with the largest cam in the Chromoscan. The TLC plate was placed on the sample table and was scanned in the direction in which the plate was spread and chromatographically developed which helped to eliminate the effect of any irregularities in the layer arising during spreading. The O.D. of each spot was shown graphically in the shape of a peak on a

piece of paper attached to the recording drum and the area under the curve was recorded on an integrator. At the end of each run, the sample table carrying the TLC plate was stopped automatically. The table and plate were then moved back manually so that the slit of light was again at the origin and the plate was moved horizontally to the next lane to be scanned. A linear relationship between the amount of steroid or bile acid applied and the optical density (O.D.) of the charred spot as recorded by the integrator on the Chromoscan was obtained (see Results section, Figures 14 and 15). The standards used were cholesterol and coprostanone (10 to 25 μg.), coprostanol (20 to 50 μg.) and deoxycholic acid and lithocholic acid (10 to 25 μg. in each case).

Measurement of sterol concentration by Liebermann-Burchard Reagent

Sterol concentrations were also estimated according to the procedure of Abell et al (1952). 3 ml. of Liebermann-Burchard reagent (acetic

anhydride, cooled to less than 10° in a glass stoppered bottle:conc. sulphuric acid:glacial acetic acid - 20:1:10) was added to the sterol samples which had been placed in a 25° water bath. The optical density (O.D.) was measured at 620 mμ. after 2 min. (optimum time for Δ^7-cholestenol) and after 35 min. (optimum time for cholesterol). Sterol concentration of the samples was obtained from a standard graph of O.D. plotted against concentration.

Measurement of sterol concentration by the method of Zak et al. (1954).

Tubes containing varying concentrations of cholesterol were evaporated to dryness under nitrogen and the residue dissolved in 0.5 ml. of isopropyl alcohol. The contents were well mixed and 2.5 ml. of Colour Reagent added. This reagent contained 0.075 g. $Fe(NO_3)_3.9\ H_2O$ dissolved in 95 ml. glacial acetic acid + 5 ml. distilled water and 43 ml. concentrated sulphuric acid. The cholesterol extract and colour reagent were remixed and the tubes placed in a 95° water bath for 8 mins. The tubes were

then removed, cooled and the optical density (O.D.) of the colour read at 520 mμ. in a spectrophotometer. Sterol concentrations of the test samples were obtained from a standard graph of O.D. plotted against concentration.

Measurement of sterol concentrations by gas-liquid chromatography (GLC)

Gas-liquid chromatography was carried out to compare the results obtained by the quantitative TLC densitometry technique with an established technique. The GLC quantitative procedure was based on that devised by Brooks (1964) for the measurement of serum cholesterol.

A Pye Argon gas chromatograph was used. Operating conditions were; 1% SE-30 Gas Chrom. Q (80-100 mesh) packed in a glass column (5 ft. long, internal diameter = 5.0mm), temperature 220°, argon flow 35 ml./min., chart speed 150 mm./hour, detector voltage 1,250 volts and sensitivity X 10. Trimethylsilyl ethers were prepared by evaporating the extract to dryness and redissolving the steroids in a mixture of

dimethylformamide (for neutral steroids) or dry pyridine (for bile acids), hexamethyldisilazane and trimethylchlorosilane in the proportions 4:4:1. The tubes were stoppered, well shaken and left at room temperature for at least 1 hour. The samples were then evaporated to dryness in a stream of nitrogen, extracted with chloroform, centrifuged and the supernatant re-evaporated before being taken up in a known volume of chloroform. An aliquot (1.0 µl.) was injected with 0.5 µl. cholestane (0.5 µg.) and the ratios of the emergent peak heights of the sample to that of cholestane were determined. By injecting mixtures of standards, linear relationships were obtained between the ratio of peak heights and the amount of neutral steroid or bile acid injected.

The bile acids were methylated with diazomethane prior to the formation of their trimethylsilylethers.

TECHNIQUE FOR ANALYSIS OF BILE

Collection of Bile

Under X-ray control a nasal tube was passed into the duodenum and the gall bladder stimulated to emptying by an intravenous injection of pancreozymin (Boots). Bile was then collected and frozen until required for analysis. Before analysis the bile was diluted with ethanol and the precipitated protein removed by centrifugation. The supernatant was subjected to further analysis (see below).

The separation of conjugated biliary bile acids and the determination of their conjugation ratio by TLC.

Glycine and taurine conjugates of the bile acids were separated by thin-layer chromatography of the supernatant on silica gel prewashed with water. Triplicate samples of bile and duplicate standards of glycocholic acid and taurocholic acid were run on each plate in the solvent system butanol:acetic acid:water (10:1:1, Gänshirt *et al.* 1960). Compounds were located

by spraying lightly with water. The plate was allowed to dry. Glycocholic and taurocholic acid spots and appropriate blank areas were scraped off into conical centrifuge tubes. They were reacted with 4 ml. of 65% sulphuric acid at 60° in a water bath. After 30 min. the tubes were removed, the contents remixed and the tubes replaced in the water bath for a further 30 min. The tubes were cooled to room temperature under a stream of cold water, centrifuged and the optical densities were read at 387 mμ. (Gänshirt et al. 1960). The linear relationship between the O.D. at 387 mμ. of the supernatant of the extracted spot and the amount of bile acid applied to the plate as described by (Gänshirt et al. 1960) was confirmed (see Results section, Figure 17). The ratio of the values obtained from the extracts corresponding to the glycocholic and taurocholic acid spots gave the conjugation ratio (G/T) of bile acids.

The extraction and separation of biliary bile acids

To determine the pattern of the bile acids conjugated with glycine and taurine it was necessary to hydrolyse the conjugates. One ml. samples of bile were autoclaved with 2 ml. of 2 N. sodium hydroxide in sealed glass ampoules at 120° for 4 hours. After cooling, the contents were neutralised with concentrated hydrochloric acid using Congo Red as indicator. The free bile acids were extracted (3 x 4 ml.) with ether. The ether extracts were washed free of hydrochloric acid, evaporated to dryness and dissolved in acetone. Thin-layer chromatography on silica gel was performed in the solvent system iso-octane:isopropyl ether:acetic acid (2:1:1, Hamilton, 1963). Control and treatment samples from one patient were run on the same plate. After drying, the plate was sprayed with ethanolic phosphomolybdic acid and the bile acid spots which developed after gentle heating were then photographed. (See Part IV, Figure 18).

STATISTICAL METHODS.

Student "t" test.

The significance of changes in serum and faecal lipids from their mean pretreatment levels was determined by the Student "t" test for paired values.

$$t = \frac{\bar{x}}{\sqrt{\frac{\Sigma(x^2) - \frac{(\Sigma(x))^2}{n}}{n(n-1)}}}$$

$\bar{x}$ = mean change.

x = change from mean pretreatment level of each subject.

n = number of values.

Correlation Coefficient.

The association between serum and faecal lipids or the changes from their mean pretreatment levels was determined by the correlation coefficient (r).

$$r = \sqrt{\frac{\left[\Sigma(x.y) - \frac{\Sigma x.\Sigma y}{n}\right]^2}{\left[\Sigma(x^2) - \frac{(\Sigma(x))^2}{n}\right]\left[\Sigma(y^2) - \frac{(\Sigma y)^2}{n}\right]}}$$

RESULTS

THE APPLICATION OF SILVER IMPREGNATED TLC AND REVERSED-PHASE TLC TO THE SEPARATION OF FAECAL NEUTRAL STEROIDS

Separation of cholesterol from Δ^7-cholestenol and cholestanol by silver nitrate impregnated TLC

During a study of human faecal neutral steroids it was found that cholestanol and Δ^7-cholestenol could be completely separated from cholesterol on 20 cm. plates coated with silica gel impregnated with silver nitrate. Evidently cholesterol forms the expected silver complex at its double bond and is retarded while Δ^7-cholestenol moves ahead with cholestanol. The faster spot containing cholestanol and Δ^7-cholestenol could be resolved by a second reversed-phase TLC at low temperature.

The best separation of cholestanol plus Δ^7-cholestenol from other sterols was obtained with the solvent system chloroform:acetone (98:2). The R_F values for these two sterols and cholesterol in other solvent systems are given in Table V.

Table V.

A comparison of the R_F values of cholesterol Δ^7-cholestenol and cholestanol on silver nitrate impregnated TLC plates developed in several solvent systems.

Solvent system	R_F Values		
	cholesterol	Δ^7-cholestenol	cholestanol
Benzene: hexane (9:1)	0.11	0.15	0.15
Benzene: ethyl acetate (20:1)	0.19	0.19	0.19
Benzene: ethyl acetate (5:1)	0.45	0.46	0.46
Chloroform: acetone (92:8)	0.70	0.73	0.73
Chloroform: acetone (96:4)	0.62	0.68	0.68
Chloroform: acetone (98:2)	0.39	0.50	0.50

It is apparent from the results in Table VI that not only did silver nitrate impregnated TLC separate

Δ^7-cholestenol and cholestanol from cholesterol but also that the R_F value of each sterol was increased. Cholesterol migrated 1.2 times further while Δ^7-cholestenol and the C_{27} stanols all moved 1.4 to 1.5 times further. Consequently cholesterol was separated from its companions. Table VI.

Table VI.

Effect of silver nitrate impregnation on R_F values* of cholestenols and C_{27} stanols on TLC plates.

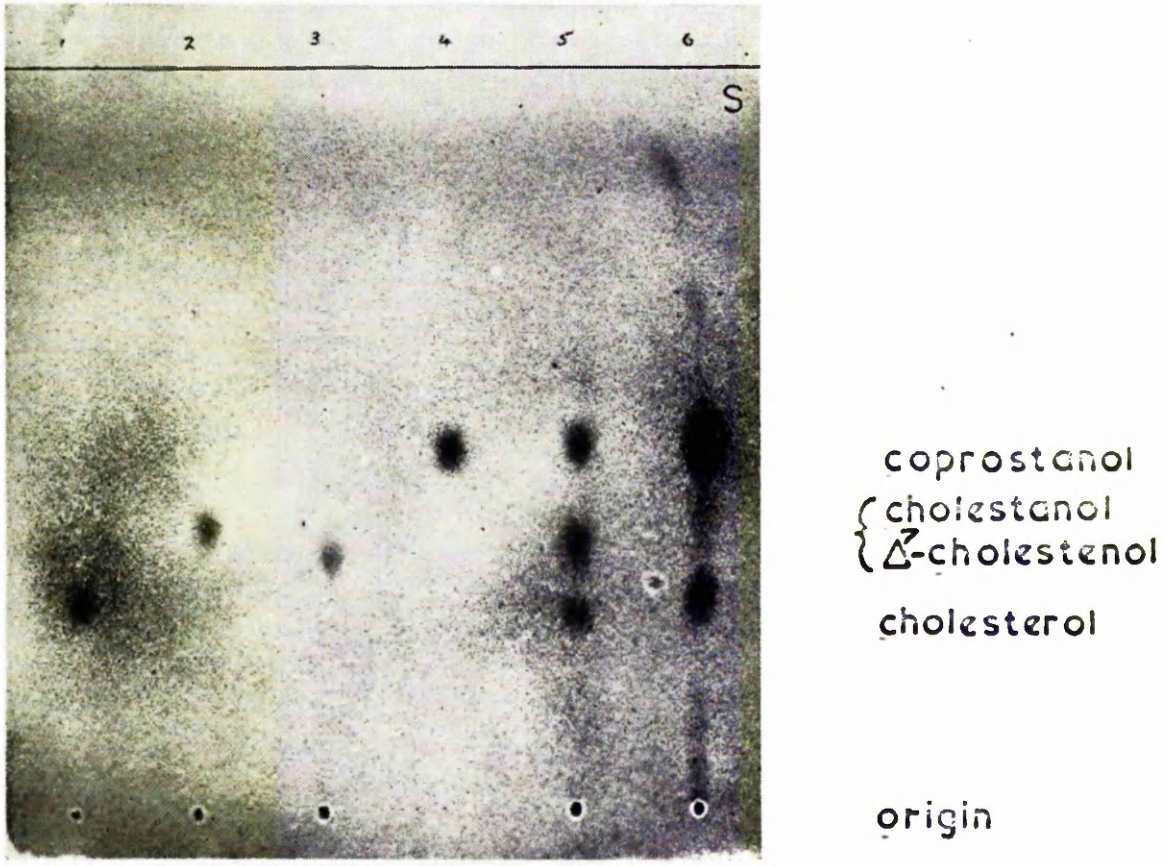
	On Silica Alone	On Silica Plus Silver Nitrate	Ratio R_F on $\frac{\text{Silica} + AgNO_3}{\text{RF on Silica alone}}$
Cholesterol	0.40	0.48	1.22
Δ^7-Cholestenol	0.39	0.57	1.46
Cholestan-3β-ol	0.39	0.59	1.51
Coprostan-3β-ol	0.51	0.72	1.40
Coprostan-3α-ol	0.51	0.74	1.46
Cholestan-3α-ol	0.58	0.80	1.40

Solvent:chloroform-acetone 98:2, run 15 cm.

*Means of four replicates on each of two pairs of plates run in the same tank simultaneously.

Although cholestanol and Δ^7-cholestenol moved further than cholesterol they ran well behind coprostanol, both in a mixture of pure sterols and in a naturally occurring mixture (Figure 10).

Figure 10.



Photograph of thin-layer chromatogram on Silica Gel G impregnated with silver nitrate. Sprayed with phosphomolybdic acid. 1, 10 µg cholesterol; 2, 10 µg cholestanol; 3, 10 µg Δ^7-cholestenol; 4, 10 µg coprostan-3β-ol; 5, mixture; and 6, neutral lipid extract of human faeces. S, solvent front. Origins at base of the photograph were enlarged after chromatography for easier visualization. The fast spots in the faecal extract are steroid 3-ketones and esters.

In experiments in which this separation method was applied for neutral lipid extracts from human faeces, spots of up to 70 µg. of cholesterol and 200 µg. of coprostanol have been very well separated from cholestanol and Δ^7-cholestenol.

Comparison of the three extraction methods used to elute the sterols from the silver nitrate impregnated plate

To separate cholestanol from Δ^7-cholestenol the spot containing these two sterols was extracted from the silver nitrate plate and then subjected to reversed-phase thin-layer chromatography. Three methods of extraction were examined; methanol, chloroform and 50% ethanolic ammonia. The extracted sterols were measured by the Liebermann-Burchard reaction (see Method section, p. 100). The results are shown in Table VII.

Table VII.

A comparison of the three extraction procedures.

Extraction method	Sterol Added (μg.)	% Recovery Cholesterol	% Recovery Δ^7-Cholestenol	Number of Estimations
Methanol	50 μg.	-	10%	3
Chloroform	50 μg.	-	40%	5
50% Ethanolic Ammonia	50 μg.	89%	81%	5

It was thought that the Ag^+ might have inhibited the Liebermann-Burchard reaction and this was investigated.

The effect of Ag^+ on the Liebermann-Burchard reaction.

The results are shown in Table VIII.

Table VIII.

The effect of Ag^+ on the Liebermann-Burchard reaction.

Sample	Ag^+ concentration	O.D. after 35 mins.
50 µg. cholesterol	0	0.070 0.070
"	1.58 µg.	0.065 0.072
"	7.90 µg.	0.042 0.054
"	158.0 µg.	0.010 0.000
"	790.0 µg.	0.000 0.000

The optical density of the colour resulting from the Liebermann-Burchard reaction of 50 µg. of cholesterol was reduced by 50% in the presence of 7.9 µg. Ag^+.

The effect of Ag^+ on the method of Zak et al. (1954).

The method of Zak et al. 1954 (see Method section, p. 101) was tried as an alternative to the Liebermann-Burchard reaction. The Zak

procedure, when modified slightly by replacing ferric chloride with ferric nitrate to avoid cloudiness produced by silver chloride, was not inhibited by Ag^+. Table IX.

Table IX.

The effect of Ag^+ on the method of Zak et al. (1954).

Sample	Ag^+ concentration	O.D. at 520 mμ.	cholesterol concentration
50 μg. cholesterol	0	0.280	50 μg.
"	7.9 μg.	0.261 0.279	48 μg.
"	15.8 μg.	0.239 0.288	47 μg.
"	31.6 μg.	0.268 0.248	46 μg.
"	158.0 μg.	0.252 0.268	46 μg.
"	790.0 μg.	0.290 0.232	46 μg.

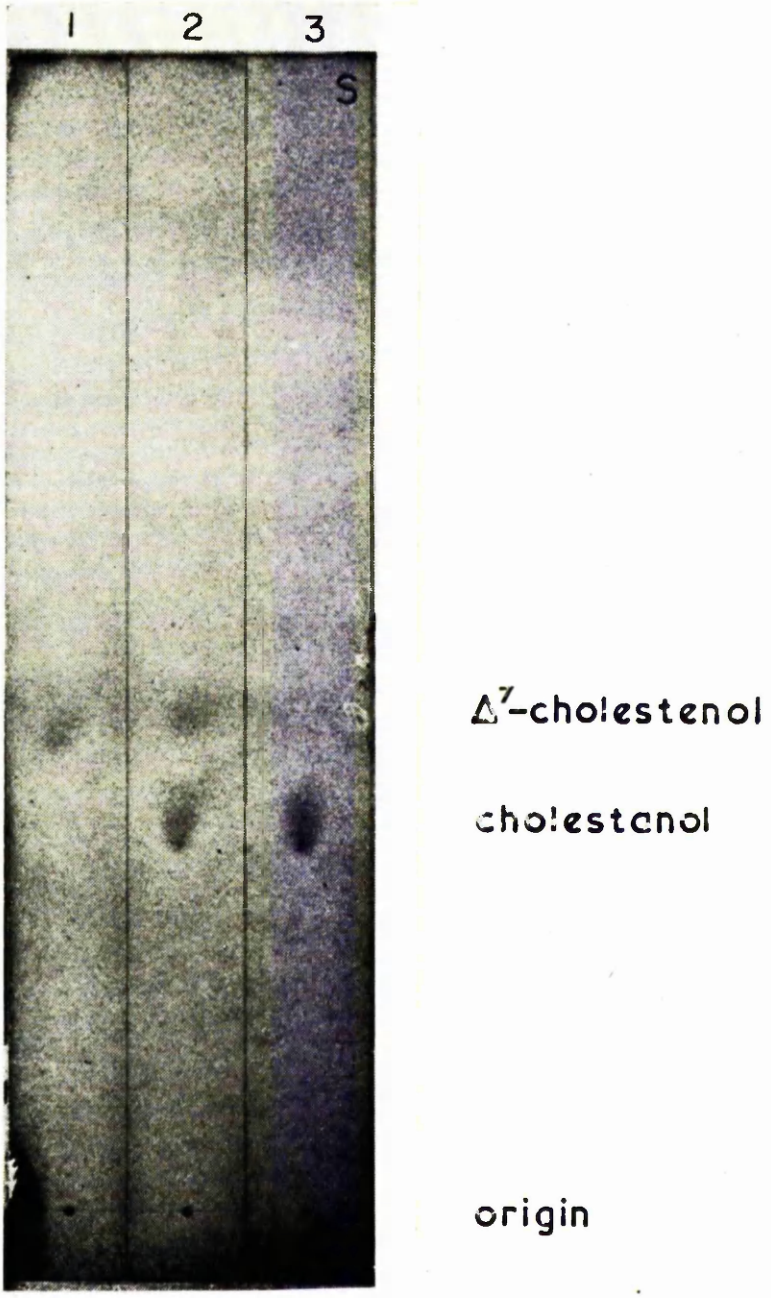
However this method gave less consistent recoveries for Δ^7-cholestenol than the Liebermann-Burchard reaction. The mean percentage recovery from 5 samples of 50 μg. of Δ^7-cholestenol was

46% with a range of 24-78%.

The separation of Δ^7-cholestenol from cholestanol by reversed-phase TLC.

After extraction from the silver nitrate impregnated plate the mixture of cholestanol and Δ^7-cholestenol was separated by reversed-phase TLC. (Figure 11).

Figure 11.



Photograph of low temperature reversed-phase thin-layer chromatogram of 1, Δ^7-cholestenol (1 μg.); 2, mixture; 3, cholestanol (1 μg.); S, solvent front. Origin at base of the photograph.

With this technique the unsaturated Δ^7-cholestenol migrated faster than cholestanol, even at room temperature. By lowering the temperature to between -15 and -20° the separation factor was increased from 1.175 to 1.39. This improved separation was necessary because the edges of the spots were not as sharp as on layers of silica gel alone. The loads of sterols which could be completely separated in this system were rather small since with more than 5 µg. of each sterol some blurring of the spots occurred. Cholesterol migrated with cholestanol in this second system, hence the need for a preliminary step to resolve a mixture of cholesterol, Δ^7-cholestenol, and cholestanol by TLC.

QUANTITATIVE TLC DENSITOMETRY

Comparison of colour development reagents.

For precise densitometry it is necessary to produce the maximum possible contrast between the spot and background. Several spray reagents were tried e.g.:-

a) 10% phosphomolybdic acid in methanol: this gave blue spots on a yellow background which was not uniform in optical density.

b) 1% ceric ammonium sulphate in 10% sulphuric acid: this gave coloured spots on a faintly yellow background.

c) Potassium dichromate in 80% sulphuric acid: this gave an uneven greyish background with black specks.

d) 35% phosphoric acid: this gave a background similar to c).

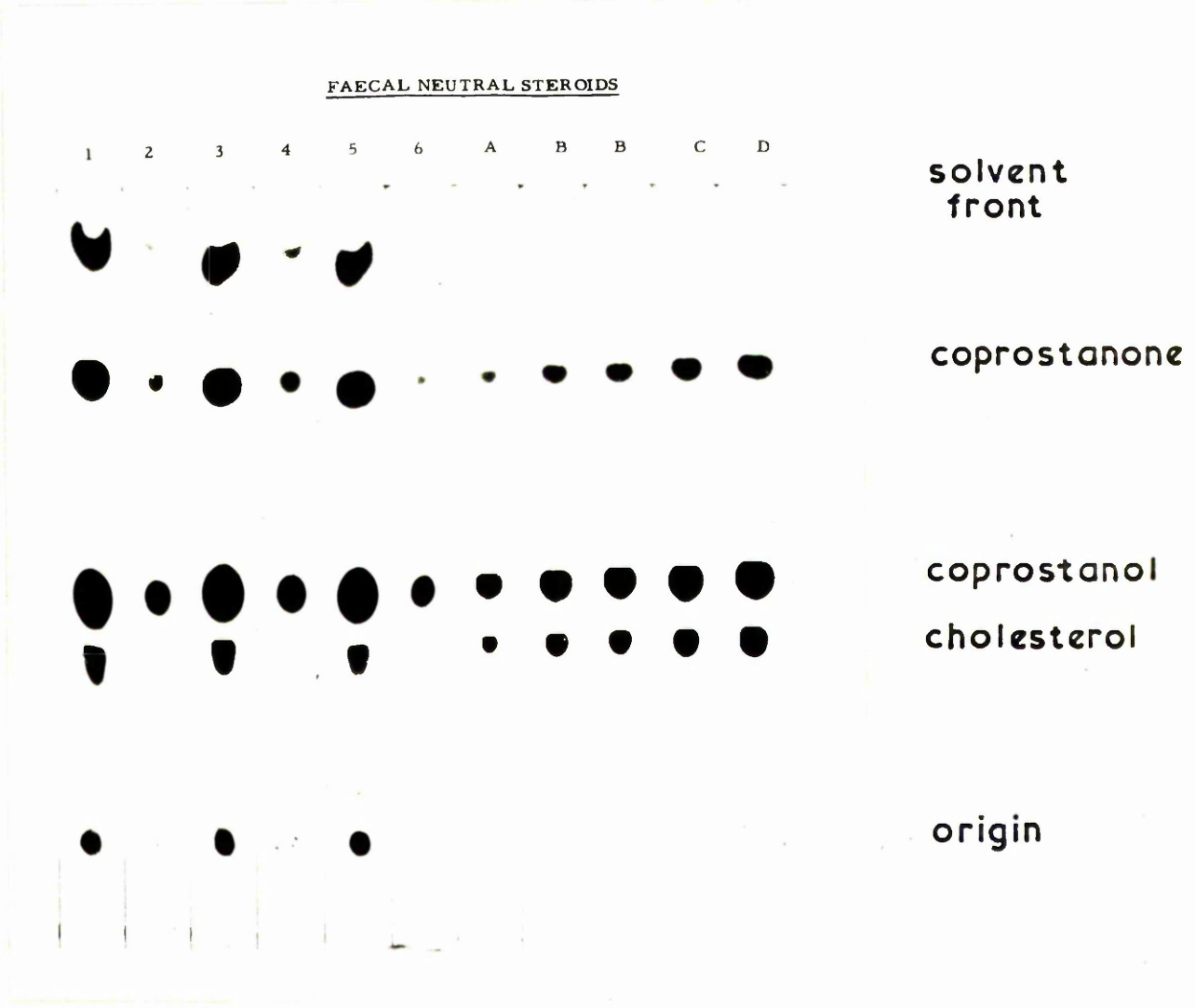
e) 50% sulphuric acid: this also gave a background similar to c).

The only suitable spray reagent was found

to be 1% potassium iodate in 10% sulphuric acid (see Method section, p . 98). This gave dark well defined spots under the conditions employed. (Figures 12, 13). Using this spray reagent linear relationships were obtained between the optical density (O.D.) of the neutral steroids and the amount of steroid applied to the TLC plate. Similarly for the faecal bile acids. (Figures 14, 15).

The separation of the free fatty acids, especially the 12- hydroxy stearic acid from the faecal bile acids is shown in Figure 16.

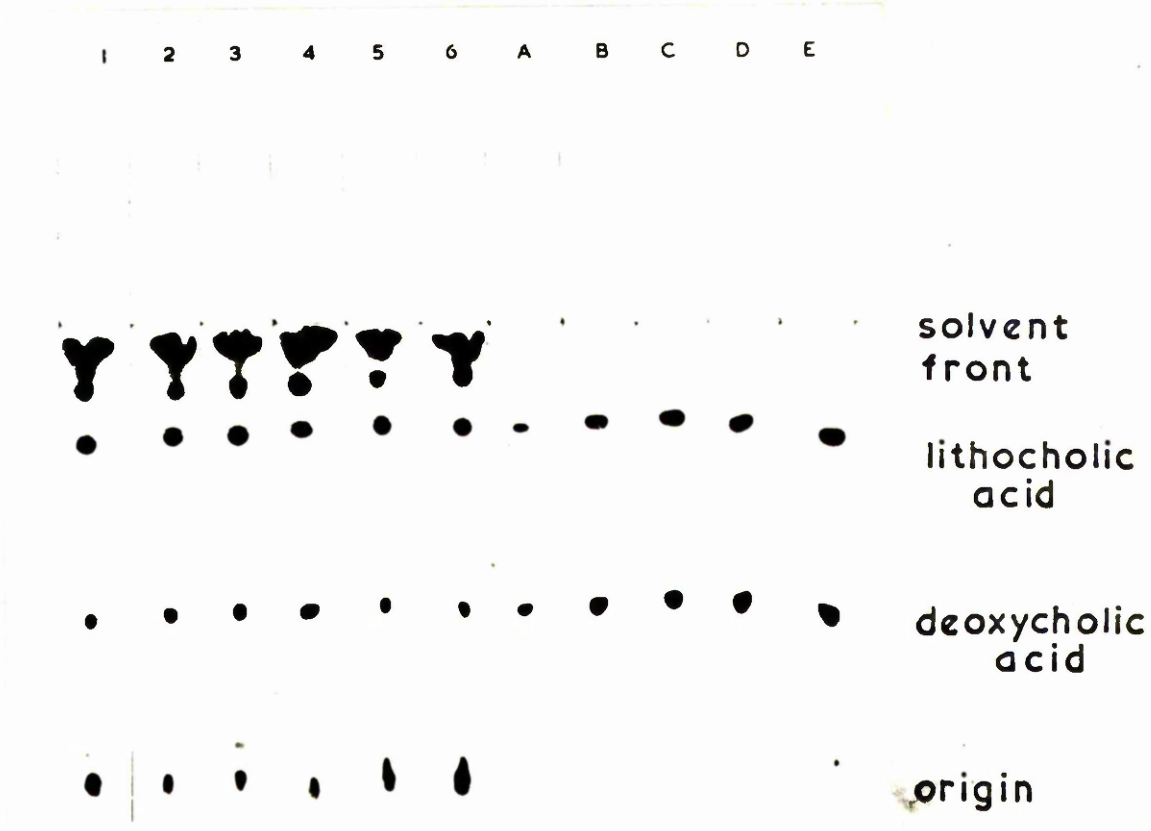
Figure 12.



A photograph of a typical thin-layer chromatogram of faecal neutral steroids and standards. Origin at bottom. Lanes 1-6 are six faecal neutral steroid extracts and lanes A B C and D contain cholesterol standards (spots nearest origin) 10, 15, 20 and 25 µg., coprostanol standards (2nd spot from origin) 20, 30, 40 and 50 µg. and coprostanone standards (3rd spot from origin) 10, 15, 20 and 25 µg.*. The spot nearest the solvent front in the extracts is a mixture of sterol esters, triglycerides and hydrocarbons.

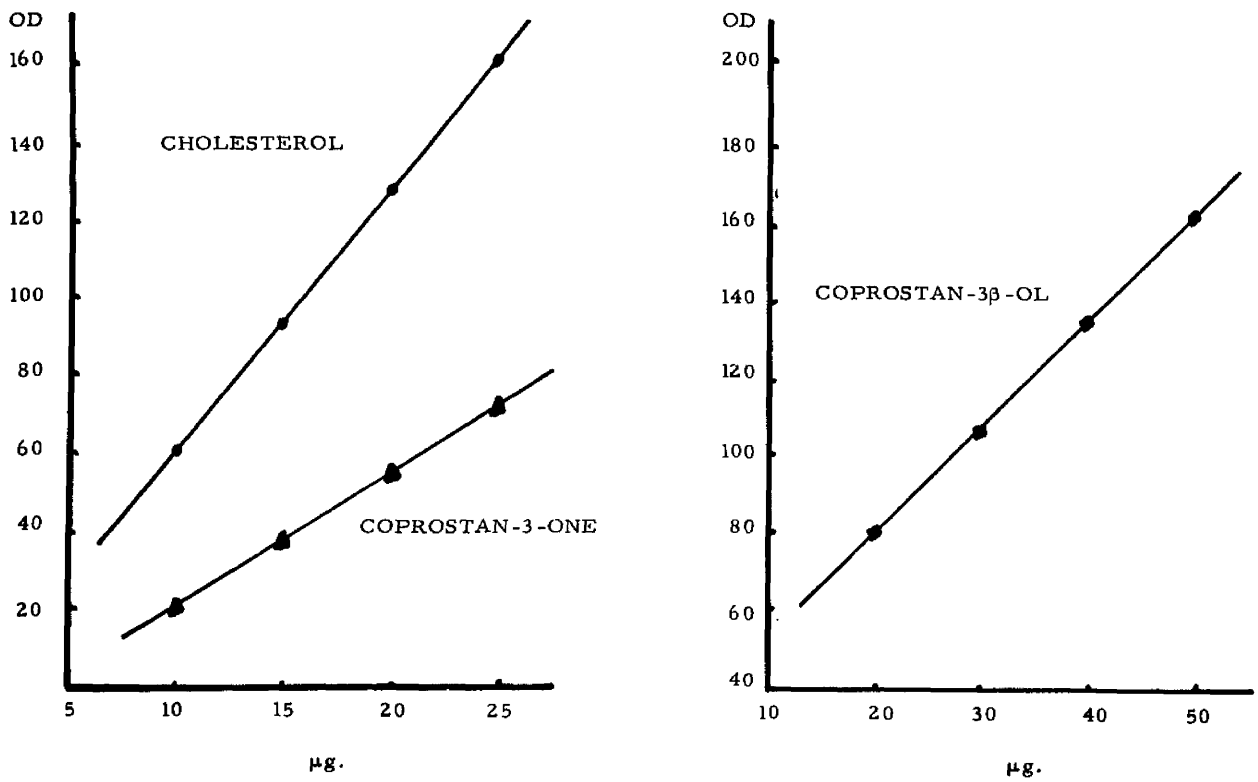
*Standard B is duplicated.

Figure 13.



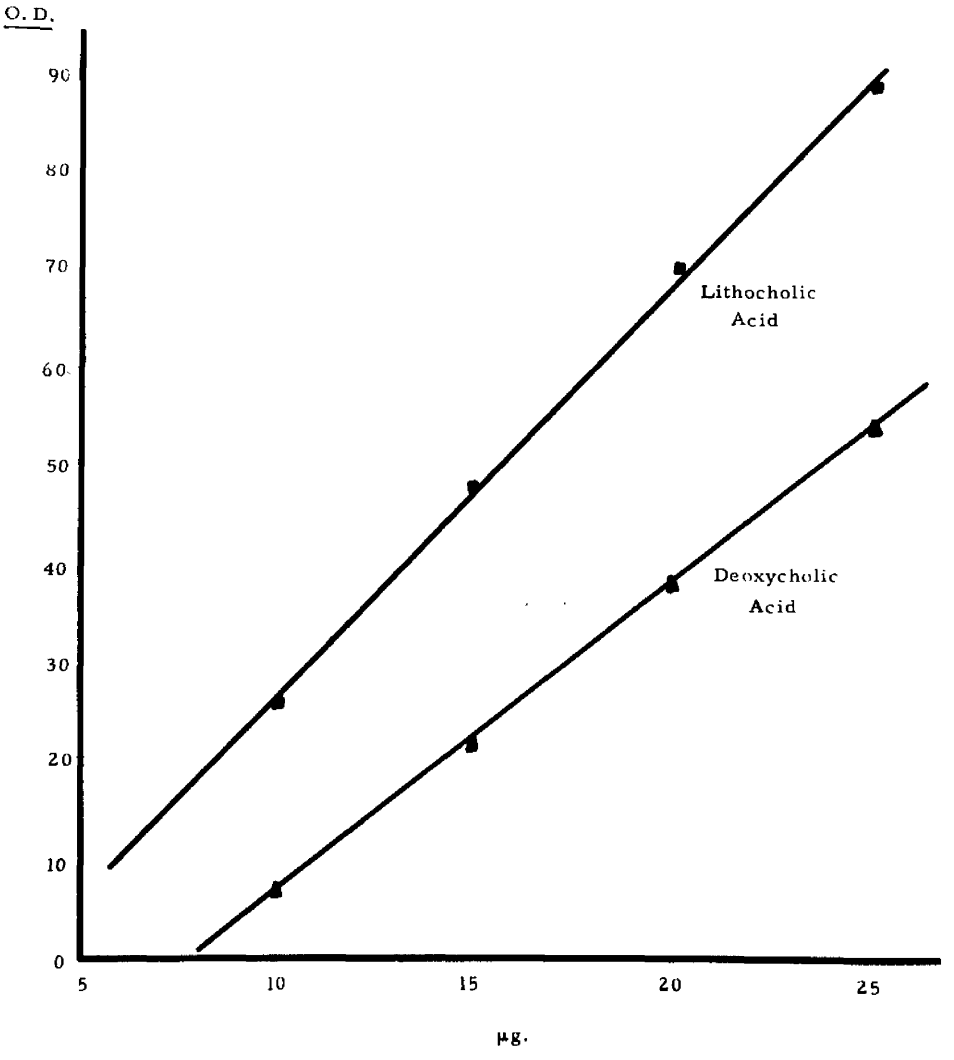
Thin-layer chromatogram of faecal bile acids and standards. Origin at bottom. From left to right lanes 1-6 are six faecal bile acid extracts and lanes A - E contain deoxycholic acid standards (spot nearest origin) 15, 20, 25 and 30 µg. and lithocholic acid standards 10, 15, 20 and 25 µg. *. The spot nearest the solvent front in the faecal extracts is a mixture of fatty acids and pigments. The spot just ahead of lithocholic acid is a hydroxy fatty acid thought to be 12 hydroxystearic acid.
Deoxycholic acid 20 µg. standard is duplicated in B and C.
Lithocholic acid 20 µg. standard is duplicated in C and D.

Figure 14.



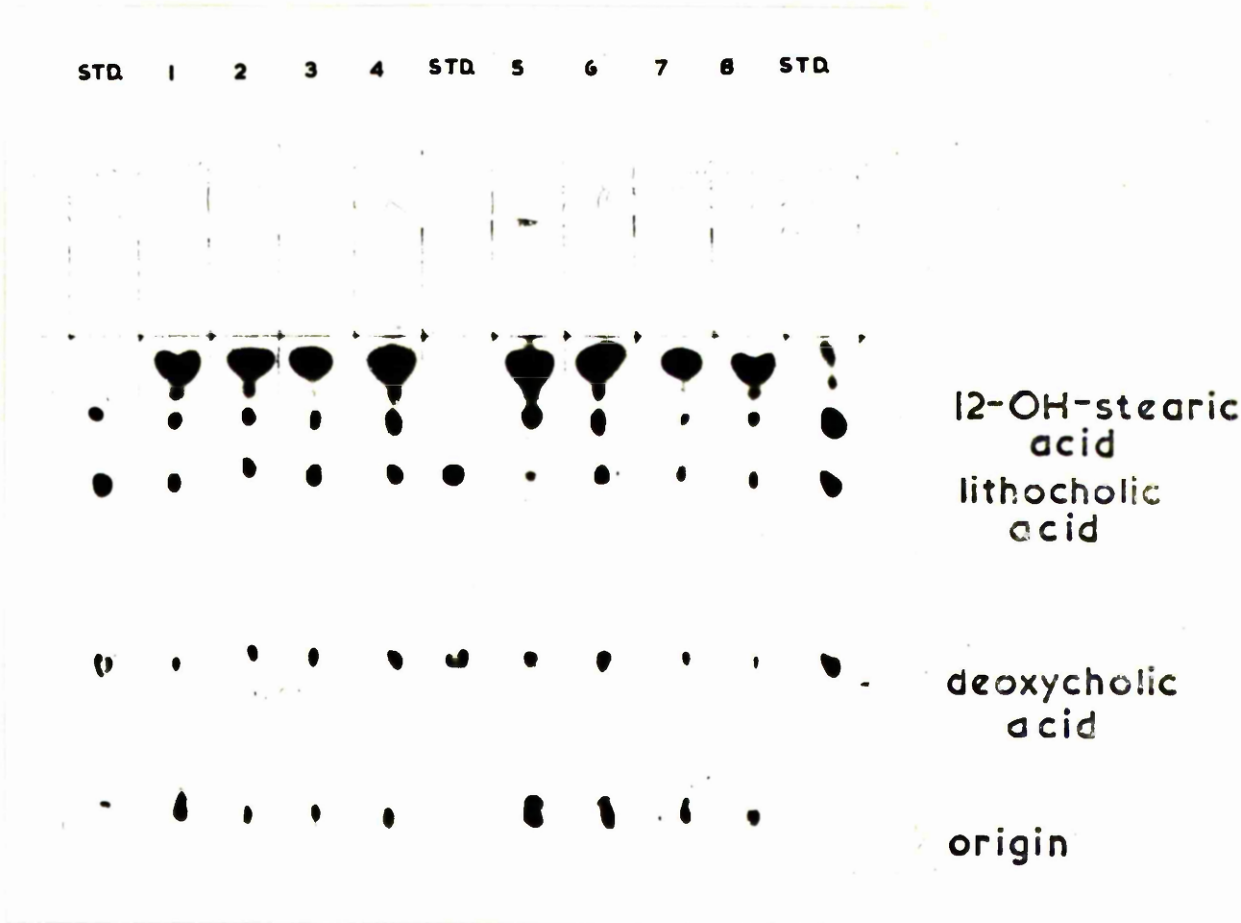
Standard graphs for cholesterol, coprostanol and coprostanone showing the straight line relationship between O.D. of the charred spot and quantity of steroid present.

Figure 15.



Standard graphs for deoxycholic acid and lithocholic acid showing the straight line relationship between O.D. of the charred spot and quantity of bile acid present.

Figure 16.



Thin-layer chromatogram of faecal bile acid extracts 1 to 8 and mixtures of deoxycholic acid, lithocholic acid and 12-hydroxystearic acid standards.

RECOVERIES AND REPLICATE VARIATION

RECOVERY FROM ETHANOLIC EXTRACTION PROCEDURE

The procedure for extracting sterols and bile acids from faeces was checked by recovery experiments. Cholesterol and deoxycholic acid standards were added to extraction thimbles and the extraction procedure was carried out. After 6 hours extraction with 95% ethanol under nitrogen, the extracts were evaporated to dryness with a rotary evaporator and the residue dissolved in 3 ml. of re-distilled acetone. Aliquots were then applied to a thin layer plate for quantification. The results are shown in Table X.

Table X.

Recovery of cholesterol and deoxycholic acid by the extraction procedure.

	Cholesterol		Deoxycholic Acid	
Sample	Added µg.	Recovered µg.	Added µg.	Recovered µg.
1	22.7	22.3	11.5	10.9
2	18.3	18.7	10.4	9.8
Mean	20.50	20.50	10.95	10.35

Recovery from Ion-Exchange Columns

The presence of neutral steroids and bile acids in their respective eluates was checked by passing cholesterol and deoxycholic acid standards through the ion-exchange columns and running samples from the various eluted fractions on TLC. Cholesterol was detected only in the 80% ethanolic fraction and deoxycholic acid in the fraction eluted by 0.1M ammonium carbonate in 50% ethanol.

Recovery of Neutral Steroids and Bile Acids Added to TLC Plate

Recovery of Neutral Steroids

Duplicate TLC plates each containing five standard mixtures of cholesterol, coprostanol and coprostanone and six equal aliquots of a faecal extract, three of which had known amounts of a mixture of cholesterol, coprostanol and coprostanone added to them were developed. The spots were charred and the amount of cholesterol, coprostanol and coprostanone in the faecal extracts calculated (see Methods

section, p. 98). The results are shown in Table XI.

Table XI.

Recovery of neutral steroids from faecal extracts by TLC.

	Cholesterol		Coprostanol		Coprostanone	
	Added µg.	Recovered µg.	Added µg.	Recovered µg.	Added µg.	Recovered µg.
	15	14.8	20	21.3	10	9.9
Plate 1	"	16.3	"	20.3	"	9.9
	"	16.3	"	18.1	"	8.4
	"	15.8	"	19.6	"	10.5
Plate 2	"	15.8	"	20.5	"	9.0
	"	15.5	"	22.6	"	8.0
Mean		15.75		20.40		9.28
S.D.		±0.560		±1.226		±0.975
%.		105%		102%		93%

Bile Acids

A similar recovery experiment was carried out using deoxycholic acid and lithocholic acid.

The results are shown in Table XII.

Table XII.

Recovery of bile acids from faecal extracts by TLC.

	Deoxycholic Acid		Lithocholic Acid	
	Added µg.	Recovered µg.	Added µg.	Recovered µg.
	15	14.0	15	15.8
Plate 1	"	14.0	"	13.0
	"	13.6	"	16.3
	"	13.6	"	16.5
Plate 2	"	13.8	"	16.3
	"	13.8	"	16.3
Mean		13.8		15.7
S.D.		±0.179		±1.341
%.		92%		105%

RECOVERY OF NEUTRAL STEROID AND BILE ACIDS AFTER COMPLETE ANALYTICAL PROCEDURE

Faecal specimens, with and without added neutral steroid and bile acid standards were subjected to the complete analytical procedure i.e. solvent

extraction under nitrogen, ion exchange chromatography and thin layer chromatography as described in Method section (p.87-92; 97-100). The results are shown in Table XIII.

Table XIII.

Recovery of neutral steroids and bile acids after complete analytical procedure.

	Sample 1.		Sample 2.	
Compound	Added µg.	Recovered µg.	Added µg.	Recovered µg.
cholesterol	10.3	10.0	10.3	10.4
coprostanol	10.5	10.3	10.5	10.7
coprostanone	13.5	13.0	13.5	13.8
deoxycholic acid	9.5	8.8	9.5	8.9
lithocholic acid	13.4	13.3	13.4	12.3

Variation of Replicate Estimations

To check the reproducibility of the technique, aliquots from a single faecal specimen were analysed by the complete procedure. The results are analysed in Table XIV.

Table XIV. Variation of replicate estimations.

	Neutral Steroids (mg./100 g. dry faeces)			Bile Acids (mg./100 g. dry faeces)	
	Cholesterol	Coprostanol	Coprostanone	Deoxycholic acid	Litho-cholic acid
	190.0	1320.0	1170.0	171.0	94.3
	233.0	1150.0	1000.0	179.0	95.9
	206.0	1150.0	1020.0	150.0	96.6
	190.0	1320.0	1040.0	180.0	97.4
	180.0	1340.0	980.0	186.0	100.6
	-	1420.0	1010.0	151.0	91.2
Mean	199.8	1283.3	1036.7	169.5	96.0
S.D.	±20.8	±109.6	±68.3	±15.5	±3.1
S.D.%.	10.4	8.5	6.5	9.1	3.1

Control Data: Faecal Neutral Steroid and Bile Acid Excretion in Normal Human Subjects.

The faecal excretion of neutral steroids and bile acids in three young (26, 27 and 29 yrs.) healthy subjects was studied for three weeks. Faeces were collected for five days each week. The subjects were staff members and were not on a restricted diet. The results and standard deviations are shown in Tables XV. and XVI.

Table XV. Neutral Steroids

Subject	Cholesterol (mg./24 hr.)	Coprostanol (mg./24 hr.)	Coprostanone (mg./24 hr.)	Total (mg./24 hr.)	N
1	129.8±31.5	1133.4±361.9	8.8± 9.8	1272.0±377.4	3
2	45.1±12.9	703.9±212.4	16.7±15.3	765.6±194.8	3
3	644.5±155.9	638.5± 40.1	18.3±31.6	1301.3±191.6	3

Table XVI. Bile Acids

Subject	Deoxycholic Acid (mg./24 hr.)	Lithocholic Acid (mg./24 hr.)	Total (mg./24 hr.)	N
1	69.9±29.8	91.1±17.7	161.0± 47.4	3
2	137.2±32.2	86.1±12.4	223.3± 37.8	3
3	247.9±63.8	147.3±63.4	395.2±120.9	3

Comparison of the daily excretion of faecal neutral steroids and bile acids measured by TLC densitometry and GLC.

The daily faecal excretion of neutral steroids and bile acids in six faecal specimens was measured by TLC densitometry (Method section, p. 98) and by GLC (Method section, p. 102). The results are shown in Table XVII.

Table XVII. Comparison of the daily excretion of faecal neutral steroids and bile acids measured by TLC densitometry and GLC.

Fraction	Sample	TLC	GLC
Total Neutral Steroids (mg./24 hr.)	1	196.2	210.2
	2	1321.1	1628.1
	3	289.2	254.4
	4	571.0	655.9
	5	410.0	368.5
	6	736.9	715.7
	Mean	587.4	638.8
Total Bile Acids (mg./24 hr.)	1	27.0	30.8
	2	42.3	37.1
	3	73.9	74.5
	4	79.0	67.8
	5	67.0	67.7
	6	77.0	72.9
	Mean	61.0	58.5

The total sterol content of the diet was also analysed and the values ranged from 184 to 375 mg./day (mean = 194 mg./day) (five daily diets were analysed).

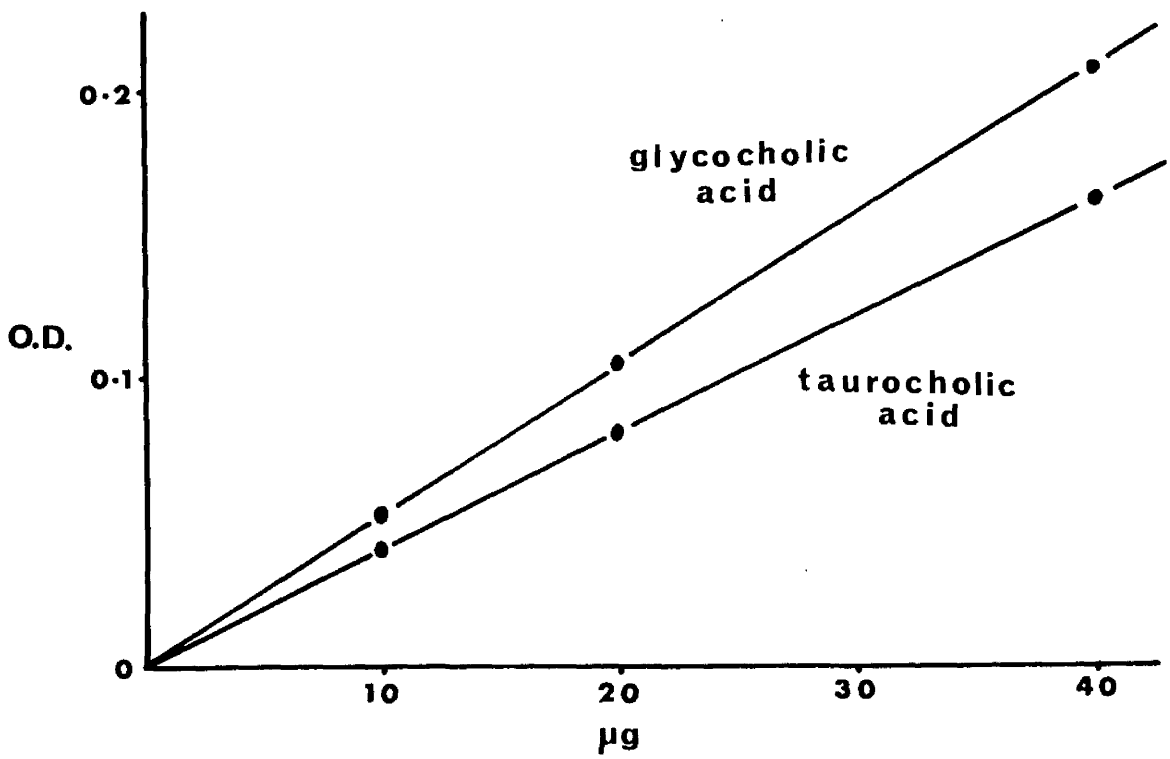
To determine whether there was much variation in the excretion of plant sterols in subjects during an ordinary ward diet, the faecal neutral steroid extracts of eight subjects were examined by GLC (Method section, p. 102). The mean percentage of steroids excreted as plant sterol metabolites three to six days after admission was 9.7% ($\pm$4.6%) and ten to fifteen days later was 9.1% ($\pm$2.7%).

BILIARY BILE ACIDS

The method of Gänshirt *et al.* (1960) (see Method section, p.104) was used in the measurement of glycocholic acid and taurocholic acid as it was a relatively simple and accurate method. The adsorbent used, Macherry Nagel Kieselgel MN-GHR, was found to be the most suitable as the others gave high and variable blank values.

To check the reproducibility of the method two bile samples were analysed three times. In one sample the glycine:taurine ratios (G/T) were 4.0, 4.4 and 4.6 and in the other the ratios were 3.5, 4.2 and 4.2. The linear relationship between the optical density (O.D.) at 387 mμ. of the supernatant of the extracted spot and the amount of bile acid applied to the plate is shown in figure 17.

Figure 17.



Standard graphs for taurocholic acid and glycocholic acid showing the straight line relationship between O.D. of the extracted spot and quantity of bile acid present.

DISCUSSION

SEPARATION OF CHOLESTEROL, CHOLESTANOL AND Δ^7-CHOLESTENOL BY SILVER NITRATE IMPREGNATED TLC AND REVERSED-PHASE TLC.

a) Silver nitrate impregnated TLC

The separation of these compounds from each other has proved extremely difficult (Part II). It has not been possible to accomplish separation of cholesterol from Δ^7-cholestenol by ordinary TLC as reported by Bennett and Heftman (1962) but the separation of these sterols was achieved on plates 40 cm. long (Avigan et al. 1963). The introduction of silver nitrate impregnated TLC to lipid analysis (see Part II) suggested a means of separating cholesterol, cholestanol and Δ^7-cholestenol.

Surprisingly enough, silver nitrate TLC systems accomplished the separation of Δ^7-cholestenol from cholesterol even although they both possess a single double bond. This difference in R_F may be explained by suggesting that the double bond at position 7 is less

accessible than that at position 5 and that this reduces the tenacity with which it is adsorbed by the silica gel surface. The ease of separation of cholesterol and cholestanol by these TLC systems is more easy to explain since they differ in structure by a single double bond. The general increase in sterol R_F values on silver nitrate impregnated thin-layer chromatograms compared with those in the more standard adsorption TLC systems is probably due to a reduction in surface area available for adsorption or alternatively, to increased hydration brought about by less thorough drying of the layer.

The increased resolving power of the technique was somewhat offset by a number of associated disadvantages. Standard extracting solvents, such as methanol and chloroform gave low recoveries of Δ^7-cholestenol from the impregnated plates as measured by the Liebermann-Burchard reaction (see sections, Method ,

Results Table VII), an effect later shown to be due to inhibition of the reaction by the large concentration of silver ions present (results section, Table VIII). This difficulty was overcome by eluting with 50% ethanolic ammonia which cleaves the silver-sterol complex and facilitates assay of the free compound. An attempt to further enhance the reliability of the quantitative assay of sterols from silver nitrate impregnated TLC plates was made using the reagent of Zak et al. (1954) (Method section, p. 101), the ferric chloride in the reagent being replaced by ferric nitrate to avoid cloudiness due to silver chloride precipitation. However, little success was obtained by this method, the recoveries being more variable and generally lower than those given by the Liebermann-Burchard reaction (results section, p. 115), possibly due to high blank values relative to the colour-response of Δ^7-cholestenol.

b) Reversed-phase TLC.

While it proved impossible to separate cholestanol from Δ^7-cholestenol using silver-nitrate impregnated TLC, even although many solvent systems were tried, this separation was achieved with relative simplicity using reversed-phase TLC at low temperature (results section, Figure 11). With this procedure however, only small amounts of these sterols (1 µg.) could be well separated. This together with the fact that charring resulted in a dark grey background limited the detecting spray reagent to phosphomolybdic acid. The improved separation at low temperature compared with that at room temperature is probably due to the stationary phase and the mobile phase becoming less miscible at the low temperature, resulting in an increase in the development time which together enhance the partitioning of the compounds.

From these results (Part II; Results section, p.108-118) it appears that thin layer

chromatographic separation of cholesterol, Δ^7-cholestenol and cholestanol from each other, either as free sterols or as derivatives is best achieved by the combination of silver nitrate impregnated TLC and reversed-phase TLC.

QUANTITATIVE ESTIMATION OF FAECAL NEUTRAL STEROIDS AND BILE ACIDS BY TLC DENSITOMETRY

Quantification of steroids and bile acids by densitometry of the spot produced by charring on a TLC plate is dependent on several factors, a few of which are listed below.

a) A uniformly thick layer of silica gel.

b) A good separation between well defined spots and

c) A suitable spray reagent.

a) The procedure and apparatus for producing a uniformly thick layer has been described (Methods section, p. 91). This is especially important when using an automatic integrator as any irregularities in thickness of the layer might result in inaccuracies in the recorded peak areas for the compounds scanned.

b) Effective separation is necessary to allow the discrete scanning of each spot ensuring that the area recorded under each peak is due entirely to a single spot. The procedures described

for neutral steroids and bile acids (see Method section, p. 97) resulted in the compounds being well separated from each other (see Results section, Figs. 12, 13 and 16).

c) To obtain spots suitable for precise densitometry *in situ* it is necessary to produce the maximum possible contrast between spot and background. The most suitable reagent for this purpose was 1% potassium iodate in 10% sulphuric acid as it produced dark brown spots on a white background (see Results section, Figs. 12 and 13). The other spray reagents tried all produced non uniform backgrounds (see Results section, p. 119).

Although these three criteria were adhered to it was necessary to include a series of standards with each plate as the degree of charring varied from plate to plate even when the temperature of the oven and the period of heating were kept constant. This was probably due to the amount of spray reagent varying from

plate to plate. The number of extracts which could be run on each plate was six (Results section, Figs. 12 and 13).

Comparison with other techniques

The recoveries through the method were adequate and its reproducibility compared favourably with that of the GLC technique (Results section, Table XVII) while having the considerable advantage of increased simplicity and speed. For example, there are only two stages between the extraction and the quantification by densitometry which not only reduces the time required for assay but also tends to improve recoveries. In addition the mass measurement *in situ* on the thin layer plate is quicker and losses are less likely to occur than if extraction from the plate is carried out before for example measurement by colorimetry (Haust and Beveridge, 1964) or by GLC (Miettinen *et al*, 1965). The method of Goldsmith *et al* (1960) although one of densitometry is not carried out

in situ (see Part II, p. 77).

A disadvantage in the densitometry method described in this thesis is that plant sterols are not measured separately from endogenous sterols. Complete faecal sterol analysis has been achieved by a combination of TLC and GLC (Eneroth *et al.* 1964; Miettinen *et al.* 1965). However from patients on an ordinary mixed diet, the contribution of plant sterols to total faecal steroids is small and relatively constant (see Results section, p. 136) and so the densitometry technique is not seriously affected. The plant sterols are in fact measured with their corresponding homologue of animal origin viz. cholesterol, coprostanol and coprostanone and so an accurate measurement of the faecal excretion of total neutral steroids is obtained by the densitometry technique.

Faecal bile acids

In the measurement of faecal bile acids by densitometry, the initial development in the

solvent system of Brown and Johnston (1962) is essential as this ensures removal of fatty acids and hydroxy fatty acids (see Results section, Figure 16) and acidic pigments from the bile acids. The presence of these compounds causes over estimation of bile acid excretion when titration techniques are used (see Grundy et al. 1965). The TLC technique of Semenuk and Beher (1966) is similar to the method described in this thesis but the spray reagent used by these authors was found to be unsuitable (see Results section, p. 119). However, their method in their hands had considerable sensitivity.

The densitometry method reported here measures the bile acids as three groups i.e. the monohydroxycholanoic acids, the dihydroxycholanoic acids and the trihydroxycholanoic acids. The principal components of these three groups are lithocholic acid, deoxycholic acid and cholic acid respectively. Although it measures

the total bile acids present, it does not assay individually the isomers present in each group. This is relatively unimportant as the principal bile acids in faeces are deoxycholic acid and lithocholic acid. The important point is that the excretion of total faecal neutral steroids and total bile acids can be measured accurately by the densitometry technique.

This concludes Part III on Methods. The following and final section Part IV describes the application of these techniques and discusses the results obtained.

PART IV.

THE APPLICATION OF QUANTITATIVE TECHNIQUES OF NEUTRAL STEROID AND BILE ACID ANALYSIS TO STATES OF ALTERED CHOLESTEROL METABOLISM

CONTENTS Pg.

INTRODUCTION

The relationship between ischaemic heart disease and elevated serum cholesterol levels has resulted in a wide variety of compounds being tested in an attempt to control serum cholesterol concentrations. A brief review of the effects of some of these compounds has been presented in Part IB of this thesis.

There are two main routes by which cholesterol is lost from the body.

a) direct excretion into the gastrointestinal tract from the liver and from the intestinal mucosa and

b) the conversion in the liver to bile acids which are then excreted as their taurine or glycine conjugates (see Part IA).

Compounds which affect either of these two pathways would therefore be expected to exert some influence on serum cholesterol levels. In the following sections a more detailed review of a small number of these compounds

has been given followed by a description of experiments designed to determine their effects on the excretion of cholesterol and its catabolites.

THE EFFECT OF TAURINE

It has been suggested that the proportion of taurine to glycine conjugated biliary bile acids may influence the serum cholesterol concentration (Kritchevsky, 1958(c),1961; Howe et al. 1960; Hellström and Sjövall, 1961; Failey et al. 1962). In general, conjugation with taurine is associated with low serum cholesterol; glycine conjugation with high serum cholesterol. On the one hand the dog and the rat are resistant to experimental hypercholesterolaemia induced by adding cholesterol to the diet (Steiner and Kendall, 1946; Gould, 1951) and their bile acids are conjugated entirely with taurine (Bremer, 1956). On the other hand, the rabbit is susceptible to cholesterol feeding (Wang et al. 1954) and has its bile acids conjugated with glycine (Hellström and Sjövall, 1962).

If Cebus monkeys, rats or mice are fed diets deficient in sulphur containing amino acids,

but rich in cholesterol they develop hypercholesterolaemia (Mann et al. 1953). Addition of taurine to these diets decreases the levels of serum cholesterol (Portman and Mann, 1955; Portman and Stare, 1959; Seidel et al. 1960). Similarly the raised serum cholesterol of rats given cholic acid and a high-cholesterol diet can be reduced by taurine (Herrmann, 1959). In mice 0.1% of taurocholic acid in the diet induces less hypercholesterolaemia than either glycocholic acid or free cholic acid (Howe et al. 1960).

In man the ratio of glycine-to taurine-conjugated bile acids (G/T ratio) averages 3:1 (Wiggins and Wootton, 1958; Sjövall, 1960). The hypercholesterolaemia of myxoedema is associated with an increased G/T ratio of approximately 7 (Hellström and Sjövall, 1961), an abnormality which is corrected by thyroid hormones. In thyrotoxicosis on the other hand the G/T ratio is about 2 (Hellström and Lindstedt, 1964).

Doses of nicotinic acid sufficient to reduce serum cholesterol also reduce the proportion of bile acids conjugated with glycine (Failey et al. 1959). Nicotinic acid is excreted in the urine as its glycine conjugate nicotinuric acid (Miller et al. 1960) and this might deplete the body of glycine (Kritchevsky, 1961). Diets rich in unsaturated fat appear to produce more cholic acid in bile than those rich in saturated fats (Lindstedt et al. 1965; Hellström and Lindstedt, 1966). The type of dietary fat does not however have a consistent effect on the G/T ratio (Hellström and Lindstedt, 1966).

Sjövall (1959) was able to increase the proportion of taurine conjugated bile acids in human bile by feeding as little as 1.5 g./day of taurine whereas large amounts of glycine (21.0g./day) did not affect the G/T ratio. Unfortunately Sjövall did not record the serum cholesterol level of the normal men in this study.

Since taurine is derived from cysteine

it is likely that diet can influence the conjugation of bile acids and if serum cholesterol levels can be changed by altering the taurine conjugation of biliary bile acids a relatively simple way of controlling serum cholesterol levels in man might be developed.

These considerations prompted this study of the effect of feeding taurine on the serum cholesterol concentration and bile acid conjugation pattern of men with normal lipid metabolism.

THE EFFECT OF CLOFIBRATE

Although clofibrate has been widely used in clinical trials, not only in patients with disorders of lipid metabolism, but also in apparently healthy adults, the mechanism by which it reduces the serum lipids has not yet been established. In 1963, Thorp suggested that it displaces thyroxine from the plasma albumin into the liver, thereby inhibiting hepatic synthesis of lipids. If this were so

then the effects of clofibrate on lipid metabolism would probably be similar to those produced by the administration of thyroxine. It is well established that serum cholesterol levels are markedly influenced by thyroid hormones (Gildea et al. 1932; Strisower et al. 1954) and that hypothyroidism leads to elevated serum cholesterol levels in a number of animal species including man (Pitt-Rivers and Tata, 1959). The question of whether or not the plasma concentration of cholesterol is abnormally low in thyrotoxicosis has been much debated (Peters and Man, 1950) but is not generally agreed.

From the work of Karp and Stetten (1949), Byers et al. (1952), Friedman et al. (1952) and Eriksson (1957) it appears that thyroxine stimulates both the synthesis and excretion of cholesterol and that the changes observed in the plasma cholesterol level when thyroid activity is altered are the net results of these

two opposing effects. As has been described (page 156) the ratio of glycine to taurine conjugated bile acids (G/T) is also affected by thyroid hormones. The hypercholesterolaemia which accompanies myxoedema is associated with an increased G/T ratio whereas in thyrotoxicosis the G/T ratio is low.

As a result of their cholesterol lowering ability thyroxine and its analogues, have been used in the treatment of hypercholesterolaemia in euthyroid subjects (Oliver and Boyd, 1956; Goolden, 1956; Beierwaltes and Ruff, 1958; Boyd and Oliver, 1960) and in hypothyroid subjects (Gross et al. 1952; Malmros and Swahn, 1953; Boyd and Oliver, 1960). In the euthyroid patients serum cholesterol was usually depressed without measurably altering the basal metabolic rate (BMR). However, continuation of the dose at a constant level often led to the return of plasma cholesterol to the pretreatment level

(Boyd and Oliver, 1960). Increasing the dose to maintain the serum cholesterol lowering effect may then increase the BMR. Treatment of hypothyroid subjects with thyroxine or its analogues decreases serum cholesterol levels within a day or two and with some of the analogues reduction occurs within a few hours of administration (Boyd and Oliver, 1960).

In contrast to the effect on cholesterol, serum triglycerides are only slightly reduced by thyroxine (Best and Duncan, 1964; 1966).

To study the mechanism of action of clofibrate and to compare its effect with that of thyroxine, three investigations were carried out into:-

a) The effect of clofibrate on the conjugation of biliary bile acids in patients with hypercholesterolaemia (p. 168).

b) The effect of clofibrate on serum and faecal lipids in humans (p. 174), and

c) A comparison of the effects of clofibrate

and L-thyroxine on the serum and faecal lipids in hypothyroid subjects (p. 197).

THE EFFECT OF ORAL CALCIUM

Epidemiological investigations in different countries have shown an inverse relationship between the hardness of drinking water and the mortality from cardiovascular disease (Kobayashi, 1957; Schroeder, 1960; Morris et al. 1961; Biörck et al. 1965). Atheroma has been shown to be more extensive in young subjects (aged 30-44 years) living in Glasgow, a soft water area, compared to subjects of the same age group living in London, a hard water area (Crawford and Crawford, 1967). As elevated serum cholesterol and triglyceride levels are well established as high risk factors in the development of ischaemic heart disease (Dawber et al. 1962; Morris et al. 1966) depression of these lipids by increased calcium intake (Yacowitz et al. 1965) might explain the relationship between the hardness of drinking water and atherosclerosis. The

mechanism by which calcium produces a reduction in the serum cholesterol is not clear, but it has been suggested that calcium may interfere with cholesterol absorption (Wells and Cooper, 1958; Iacono et al. 1960).

The role of magnesium - the other principle cation of hard water - in the pathogenesis of ischaemic heart disease is less certain (see Schroeder, 1960; Morris et al. 1962).

An investigation was made into the effect of oral calcium on the excretion of cholesterol and its metabolites (p. 207).

THE EFFECT OF DIETARY CHOLESTEROL

In 1965, Keys et al. showed that decreasing the cholesterol content of the diet had little effect on serum cholesterol concentrations. Earlier experiments (Mayer et al. 1954) had similarly suggested that dietary cholesterol intake and plasma cholesterol levels were unrelated. This was supported by Bhattathiry and Siperstein (1963) who found that although

the addition of cholesterol, in the form of eggs, to the diet depressed cholesterol synthesis in the human liver, the level of plasma cholesterol remained unchanged.

However, Connor et al. (1960, 1964) found that by increasing the dietary intake of cholesterol by the addition of egg-yolk, the serum cholesterol levels were markedly increased and when the subjects were fed a cholesterol-free diet, the serum cholesterol levels decreased.

At present, there are two alternative mechanisms which are thought to protect man from hypercholesterolaemia of dietary origin. The first, is the negative feedback mechanism in which the level of dietary cholesterol controls the hepatic synthesis of cholesterol. This has been found to operate in many experimental animals such as rat (Frantz et al. 1954), dog, rabbit (Gould, 1951) and chicken (Sakakida et al. 1963) and is discussed in more detail in Part IB p. 37 of this thesis.

The second protective mechanism is the limited capacity of the human intestine to absorb cholesterol (Karvinen et al. 1957; Kaplan et al. 1963; Taylor et al. 1965; Taylor and Ho, 1967).

The effect of dietary cholesterol on serum and faecal lipids was investigated in one subject (p. 224).

EXPERIMENTAL

1) The effect of feeding taurine on bile acid conjugation and serum cholesterol levels in man

Four experiments were carried out in three men. Each experiment consisted of a 2 week control period, a period of 10-15 days in which taurine was added to the diet and a further 2 week control period. J.M., 26 years old, was receiving physiotherapy after a mild right hemiparesis due to cerebral arterial thrombosis. J.C. was a clinically normal volunteer aged 62 years. C. McL., aged 34 years, was convalescing after medical treatment for a duodenal ulcer.

The subjects were admitted to a metabolic ward where each received a constant diet throughout the experiment. J.M. was studied during two dietary regimens in which the fat content was 50 g. of butter in the first experiment and 5.0 g. of sunflower seed oil in the second. Two weeks were allowed for adjustment between the two regimens. The other subjects were given mixed diets based on estimated consumption at home. Dietary fat in these subjects was given in saturated form. Taurine, 1.5 g. per day, was given in divided doses with meals during the 10-15 days treatment period. Body weight changed by less than 3 kg. during the three experimental periods.

Fasting serum cholesterol levels were measured daily during the experiments by the method of Abell *et al.* (1952). On two occasions in each subject (before and towards the end of the taurine period) the duodenum was intubated and bile collected and the conjugated biliary

bile acids analysed as described in Part III (p. 104).

a) Serum cholesterol

Results of the four experiments are shown in Table XVIII in which mean serum cholesterol levels from the 8th day to the end of the taurine periods are compared with means of all the determinations in each control period.

Table XVIII

The effect of taurine on serum cholesterol concentrations.

Subject	Mean serum cholesterol (mg./100 ml.)		
	Control	Taurine	Control
J.M. (butter diet)	214.7	206.5	210.5
J.M. (sunflower seed oil diet)	168.1	160.3	148.6
J.C.	228.8	231.1	227.2
C. McL.	185.0	185.0	197.1
Mean	199.2	195.7	195.9

Feeding taurine had no consistent effect

on serum cholesterol despite the considerable reduction in the glycine/taurine ratio of conjugated biliary bile acids (Table XIV).

Table XIV

The effect of taurine on glycine/taurine ratio of conjugated biliary bile acids.

Subject	Glycine/Taurine Conjugation Ratios	
	Control	Taurine
J.M. (butter diet)	4.8	1.1
J.M. (sunflower seed oil diet)	3.4	0.8
J.C.	4.9	0.6

2) The effect of clofibrate

a) The effect of clofibrate on the conjugation of biliary bile acids in patients with hypercholesterolaemia.

Clofibrate (0.5 g. q.i.d.) was given to five patients with hypercholesterolaemia or ischaemic heart disease. Further details are given in Table XX.

Table XX. Patients and Serum Cholesterol Changes.

Patient No.	Age (years), sex	Type of hypercholesterolaemia, fasting serum triglycerides and any xanthomata	Ischaemic heart disease	Serum cholesterol (mg./100 ml.) mean before treatment	Serum cholesterol (mg./100 ml.) after 1 month's CPIB (% change)
1	35,M	high normal cholesterol, normal triglycerides	effort angina	253	208(-18%)
2	38,M	familial with slightly raised triglycerides	myocardial infarct 16 months before	351	356(+2%)
3	35,M	idiopathic with some hypertriglyceridaemia and xanthoma planum	nil	459	196(-57%)
4	60,F	familial, with normal triglycerides and tendon xanthoma	myocardial infarct 33 days before	408	364(-11%)
5	38,M	idiopathic with normal triglycerides	angina after heavy meals	324	235(-27%)

None of the patients was judged on clinical grounds to have thyroid disease and none was receiving any other cholesterol-lowering treatment. Patients 1 and 4 were each given a constant diet of mixed foods (35% of calories from fat) in the metabolic ward. Patients 2, 3 and 5 were asked to continue their usual diet at home while clofibrate was given.

Serum cholesterol was measured by the method of Abell et al. (1952). Bile was collected before starting clofibrate and after two weeks treatment in three patients and after one month's treatment in two of the outpatients (Nos. 2 and 3). The chromatographic separation of the conjugated bile acids and the determination of the glycine/taurine ratio was carried out as described in Part III (p. 104). The significance of changes in serum cholesterol and G/T ratio from their mean pretreatment levels was determined by the Student "t" test for paired values.

A. Changes in serum cholesterol

One month's treatment led to an average drop in serum cholesterol of 22% (Range +2 to -57%) but the changes were not significant.

B. Changes in bile acid conjugation

During clofibrate treatment the G/T ratio of biliary bile acid conjugates showed little change in four patients and a decrease in patient No. 5 (an out-patient). Table XXI.

Table XXI.

The effect of clofibrate on the glycine/taurine ratio of biliary bile acid conjugates.

Patient No.	G/T ratio	
	Control	Clofibrate
1	1.4	1.1
2	2.9	2.3
3	4.3	4.0
4	2.5	2.9
5	2.3	1.1
Mean	2.68	2.28

The mean G/T ratio changed from 2.68 to 2.28.

The ratio on treatment was not significantly different from the control ratio.

On inspection of the thin-layer chromatoplates of hydrolyzed bile there was no obvious change in the relative proportions of cholic, chenodeoxycholic and deoxycholic acids. Chromatograms from the first 4 patients, before and during treatment, are shown in Figure 18 Bile acids from the fifth patient gave a similar result.

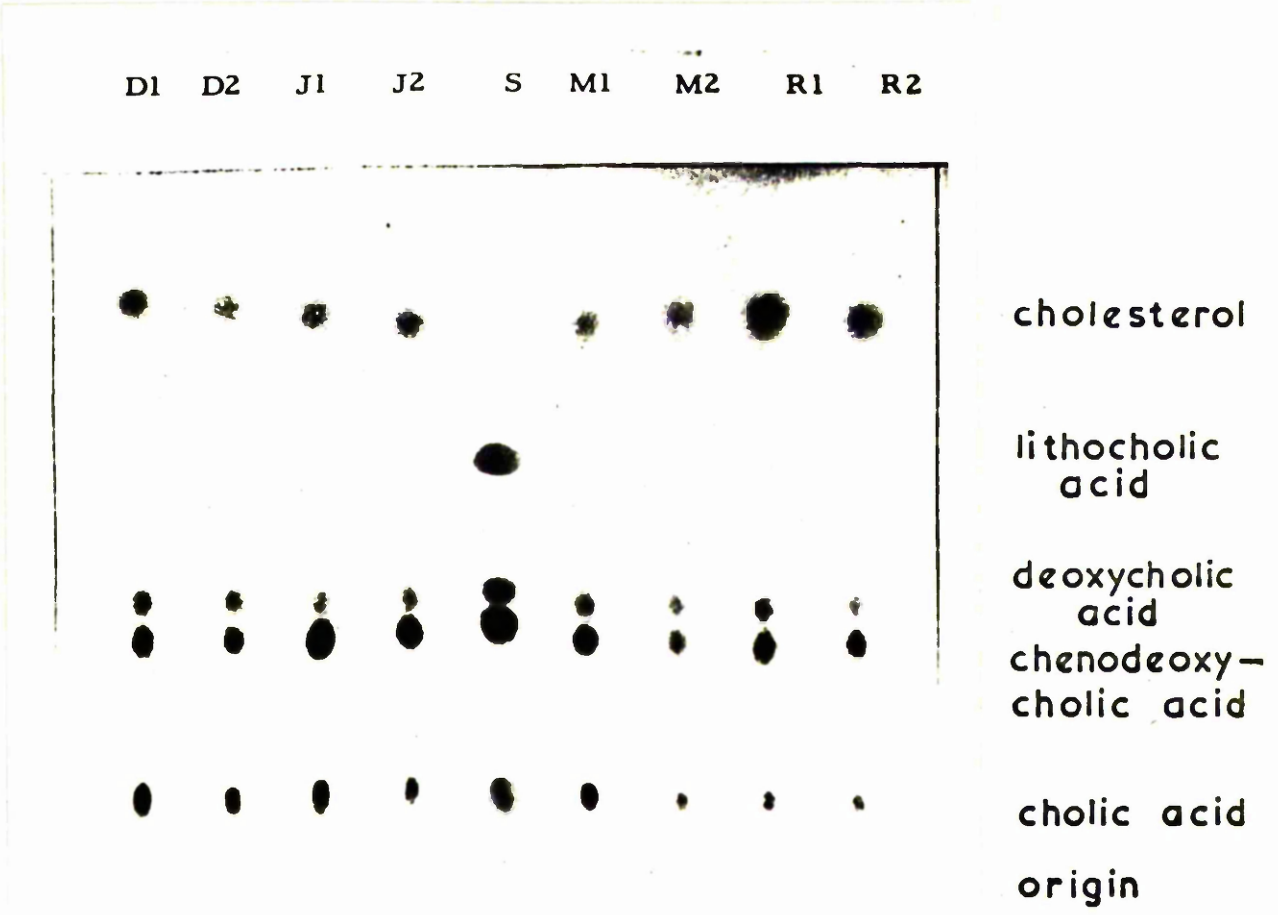


Figure 18. Thin-layer chromatoplate of free bile acids in hydrolyzed biles of first 4 patients. Origin at bottom. From left to right: patient 1 control, patient 1 treatment, patient 2 control, patient 2 treatment, standards (from below upwards: cholic, chenodeoxycholic, deoxycholic and lithocholic acids), patient 3 control and treatment, patient 4 control and treatment. The major spots in the bile samples are, from below upwards, cholic, chenodeoxycholic and deoxycholic acids, with cholesterol running to the front.

b) The effect of clofibrate on serum and faecal lipids in humans

Details of the 21 subjects investigated are presented in Table XXII. Eighteen were ambulant patients admitted to the ward for clofibrate therapy. Of these, nine had had a myocardial infarction from six months to ten years previously, eight had other forms of occlusive arterial disease, and one had familial hypercholesterolaemia. Two of the normal subjects were members of the scientific staff, and the third was convalescing from an attack of acute rheumatism.

After a preliminary period of three to six days, clofibrate was given for nine to fifteen days at the dosage indicated in Table XXII.

Table XXII. Clinical Details of Subjects.

Subject	Age (years)	Sex	Diagnosis	Dose of Clofibrate	Pre-treatment serum lipid levels: Cholesterol mg./100 ml.	Pre-treatment serum lipid levels: Triglyceride mg./100 ml.
A.W.	49	M	Peripheral arterial disease	2 g./day	258	150
R.M.	50	M	Peripheral arterial disease	2 "	220	67
J.H.	45	M	Peripheral arterial disease: myocardial infarction	2 "	252	154
A.C.	58	F	Angina pectoris	2 "	293	147
E.D.	58	F	Angina pectoris: peripheral arterial disease	2 "	264	247
D.M.	28	M	Normal subject	2 "	154	33
M.D.	23	M	Normal subject	2 "	174	51
J.D.	23	M	Convalescent	2 "	230	87
M.R.	37	M	Myocardial infarction	2 "	351	93
T.M.	50	M	Myocardial infarction	2 "	210	70
J.P.	60	F	Myocardial infarction	1 "	303	179
W.B.	49	M	Peripheral arterial disease: myocardial infarction	1 "	248	188
J.E.	53	F	Aortic arch atheroma	1 "	216	71
J.G.	18	M	Familial hypercholesterolaemia	1 "	320	126
M.M.	63	F	Angina pectoris: mild diabetes mellitus	1 "	370	93
J. McA.	58	F	Angina pectoris	1 "	299	81
F.R.	57	M	Peripheral arterial disease: myocardial infarction	1 "	274	143
A.K.	56	F	Peripheral arterial disease: myocardial infarction	1 "	243	125
M. McD.	48	M	Angina pectoris	1 "	293	110
M.W.	63	F	Myocardial infarction	1 "	400	127
J.M.	44	M	Myocardial infarction	1 "	369	187

Fasting blood samples were obtained three times per week for serum cholesterol and triglyceride estimations, and the values presented are therefore the mean of two or three blood samples taken during each faecal collection. Faeces were collected (three-day collections in ten subjects and six-day collections in eleven subjects) before clofibrate administration, and again between the ninth and fifteenth days of clofibrate therapy. With one exception, the subjects were given an unrestricted ward diet throughout their hospital stay. In order to assess any effect due to dietary change on admission to hospital, 1 subject (M.W.) was maintained throughout the experimental period on a constant diet, approximating as closely as possible to her habitual diet. The composition of this diet per 24 hours was as follows:- total calories 951, total fat 51 g., cholesterol 900 mg., carbohydrate 83 g., and protein 40 g.

Ten subjects were readmitted several months

later (range 1 to 9 months, mean 4.6 months) for a further 6-day collection of faeces. Clofibrate had been continued during the interval in a dosage of 0.75 g. twice daily in 7 subjects and 1.0 g. twice daily in 3 subjects.

Serum total cholesterol was measured by an autoanalyzer method (Technicon N.24P), and serum triglyceride by the method of van Handel and Zilversmit (1957).

Faecal specimens were stored in weighed containers in a deep freeze until processed as described in Part III (p. 87). Faecal bile acids and neutral steroids were measured as described in Part III (p. 97). In addition, since the above technique does not separate the parent neutral steroids from their analogues of plant origin, gas-liquid chromatography (Eneroth et al. 1964) was used to separate these compounds in the neutral steroid extracts of 11 subjects, and the percentage of total neutral steroids formed by plant sterols was determined

before and during clofibrate treatment.

Faecal fat (neutral fat plus free fatty acid) estimations were carried out on freeze dried specimens by Soxhlet extraction of approximately 1.0 g. of the dry matter with diethyl ether for 6 hours (Harrison, 1949).

The significance of changes in serum lipids and faecal constituents from their mean pretreatment levels was determined by the Student "t" test for paired values.

Serum Cholesterol

Within nine to fifteen days of commencement of clofibrate therapy serum cholesterol levels had fallen significantly (Table XXIII, Figure 19), 18 of the 21 subjects showing a fall of between 2 and 130 mg. per 100 ml. serum. In 12 of these, cholesterol fell by more than 15% of the initial level. Two subjects (E.D. and J.M.) showed no significant change, and in the remaining subject (J.H.) the serum cholesterol increased by 41 mg. per 100 ml. over the mean basal level.

The greatest reduction in serum cholesterol level occurred in those subjects with the highest pretreatment levels, and a significant correlation was observed between the height of the initial cholesterol level and the magnitude of the fall ($r = -0.48$, $p < 0.05$). Both the high and low dosage groups showed a reduction in serum cholesterol level. The decrease was, in fact, greater in the group on 1 g. clofibrate daily (50 mg. per 100 ml. $p < 0.05$) than in the group on 2 g. daily (15 mg. per 100 ml.), the former group having higher pretreatment levels.

Table XXIII

Serum Lipids

(Mean values ± S.D.)

	Whole Group (n = 21)		Group on 2 g./day (n = 10)		Group on 1 g./day (n = 11)		Prolonged Treatment Group (n = 10)	
	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 1-9 months' Clofibrate
Cholesterol mg./100ml. serum	273.3 ±64.6	240.3** ±57.1	240.6 ±57.1	225.9 ±60.1	303.2 ±58.1	253.6** ±53.7	311.9 ±53.1	280.8 ±39.4
Triglyceride mg./100ml. serum	120.4 ±53.1	91.2*** ±52.6	110.0 ±64.5	93.5* ±68.9	130.0 ±41.1	89.2*** ±35.2	135.9 ±38.1	130.2 ±99.9

Significant difference from pretreatment levels * $p<0.05$, ** $p<0.01$, *** $p<0.001$

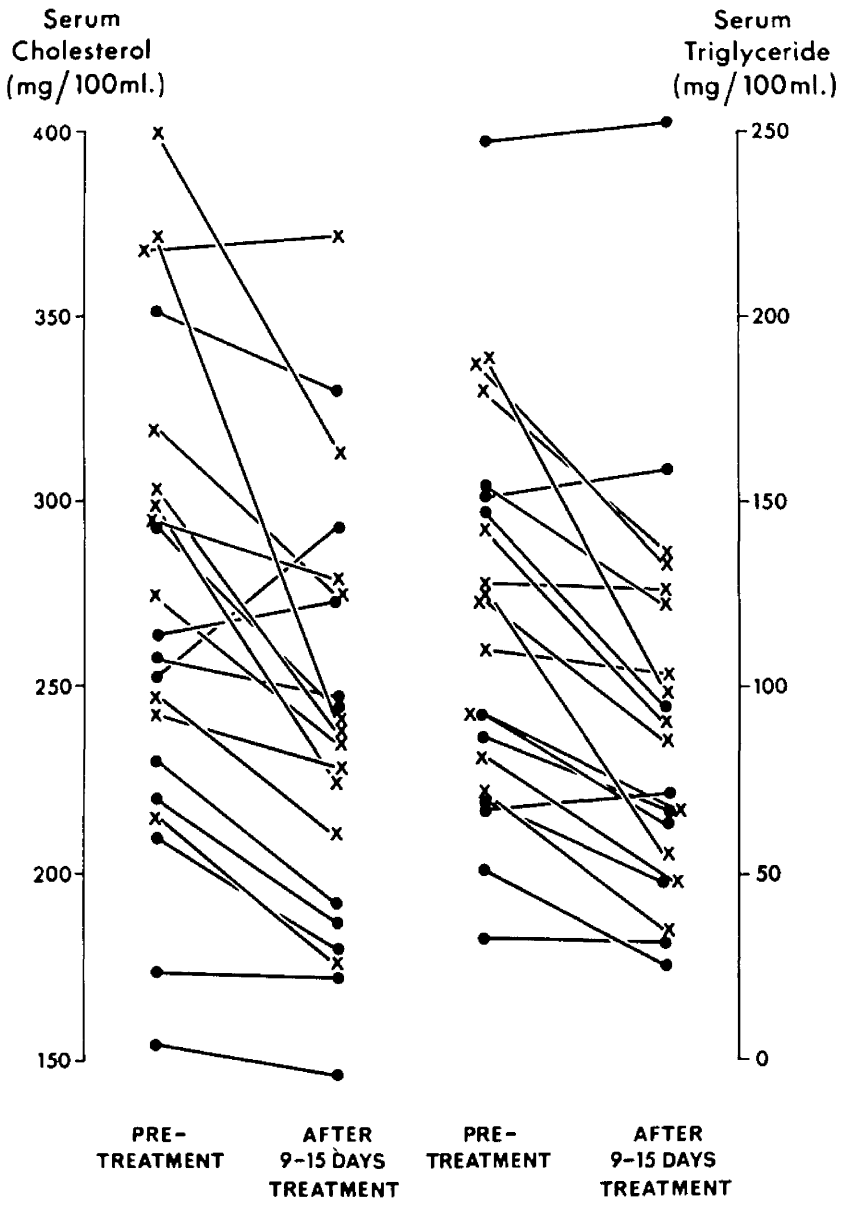


Figure 19. The effect of clofibrate, 1 g. (x-x) and 2 g. (0-0) daily for nine to fifteen days, on serum cholesterol and serum triglyceride levels.

The decrease in cholesterol levels in the 13 male subjects (18 mg. per 100 ml.) was significantly less than that seen in the 8 females (57 mg. per 100 ml., $p < 0.05$) in whom there was a greater proportion with high initial cholesterol levels.

When the estimation was repeated in 10 subjects between one and nine months later, a significant reduction of serum cholesterol had not been maintained, although seven of the subjects showed serum cholesterol levels which were between 14 and 127 mg. per 100 ml. below their pretreatment levels. In the remaining three subjects (A.K., F.R., and W.B.) increases of 10 mg., 29 mg., and 71 mg. per 100 ml. were observed relative to their pretreatment values. The mean decrease for the whole group of 31 mg. per 100 ml. was not statistically significant (Table XXIII).

Serum Triglyceride

Clofibrate led to a significant reduction of serum triglyceride levels within nine to

fifteen days (Table XXIII, Figure 19). Sixteen of the subjects showed decreases of 6 to 89 mg. per 100 ml. and in fifteen of these patients the fall was 15% or more of the initial level. No appreciable change was seen in the remaining five subjects. Although the pretreatment values were similar in the two groups, the subjects on clofibrate 1 g. daily showed a greater decrease in serum triglyceride (mean decrease 42 mg. per 100 ml. $p < 0.05$) than the group on 2 g. clofibrate daily (mean decrease 17 mg. per 100 ml.). The Serum triglyceride changes did not differ in male and female subjects. There was no significant correlation between the height of the pretreatment triglyceride level and the magnitude of the decrease occurring during clofibrate therapy. Of the subjects who showed no triglyceride change in response to clofibrate, only one (E.D.) also failed to show a decrease in cholesterol. There was no significant correlation between the pretreatment levels of

cholesterol and triglyceride in the 21 subjects, nor between the magnitude of the decreases obtained in cholesterol and triglyceride on clofibrate therapy.

When serum triglyceride estimations were repeated in ten subjects between 1 and 9 months later (Table XXIII), seven had levels between 6 and 70 mg. per 100 ml. below their pretreatment levels, while two (M.W. and W.B.) had small increases of 7 mg. and 17 mg. per 100 ml. The remaining subject (J.P.) showed a marked increase in triglyceride from her mean pretreatment level of 179 mg. per 100 ml. to 385 mg. per 100 ml., although her serum cholesterol at this time was 22 mg. per 100 ml. below its pretreatment value.

Faecal Excretion

Clofibrate led to a significant increase in the wet-weight of faeces excreted per 24 hours. The increase occurred in nine of the ten subjects on 2 g. clofibrate daily, the tenth subject (A.W.) showing no change. In the group on 1 g.

Table XXIV. Faecal Excretion (Mean Values ±S.D.)

	Whole Group (n = 21)		Group on 2 g./day (n = 10)		Group on 1 g./day (n = 11)		Prolonged Treatment Group (n = 10)	
	Pre-Treatment	After 9-15 days'	Pre-Treatment	After 9-15 days'	Pre-Treatment	After 9-15 days'	Pre-Treatment	After 1-9 months'
Weight of faeces								
Dry faeces g./24 hr.	31.7 ±17.5	42.2 ±36.9	41.0 ±18.7	66.3^{+} ±40.7	23.4 ±11.6	20.2 ±10.4	22.0 ±11.2	20.6 ±8.6
Wet faeces g./24 hr.	75.5 ±36.3	104.9** ±54.1	64.5 ±18.4	114.9** ±43.2	85.5 ±45.9	95.8 ±63.2	85.0 ±48.2	75.9 ±41.2
Faecal fat								
Faecal fat g./100 g. dry faeces	6.5 ±2.16	6.9 ±3.09	5.6 ±1.54	5.9 ±2.45	7.3 ±1.42	7.8 ±2.60	7.5 ±1.32	8.1 ±3.35
Faecal fat g./24 hr.	1.94 ±0.84	2.58* ±1.63	2.28 ±0.94	3.84** ±1.48	1.66 +0.67	1.55 ±0.83	1.63 ±0.70	1.64 ±0.86

Significant difference from pre-treatment level $^{*}p < 0.05$, $^{+}p < 0.02$, $^{**}p < 0.01$.

clofibrate daily only two subjects (J.E. and J.G.) showed an appreciable increase.

The changes in faecal wet-weight were associated with changes in the weight of material obtained after freeze-drying of the faeces (expressed as grams per 24 hours of dry faeces), a significant increase being observed only in the group on 2 g. clofibrate per day (Table XXIV).

During the first week of clofibrate therapy there was also a significant increase in the number of stools passed per week (mean = 6.6, $p < 0.02$), as compared with the number recorded during the pretreatment period (mean = 5.4 stools per week). By the second week of medication the number of stools passed was no longer significantly greater than before treatment (6.0 stools per week). Records of the number of stools passed were available for only eight of the ten subjects on 2 g. clofibrate daily. Of the remaining two subjects, who were staff members, one (D.M.) complained of mild

diarrhoea while taking clofibrate. No significant difference was observed between the two groups in the number of stools passed during clofibrate therapy.

Faecal Fat

The mean faecal fat values are shown in Table XXIV. During the pretreatment period, the fat content of the faeces in all subjects was within the range quoted as normal for this method of fat analysis (3.6 to 22.8 g. per 100 g. dry faeces) (Foweather, 1926). The small mean increase in percentage of faecal fat during clofibrate medication was not statistically significant.

There was a small but significant increase in fat excretion (g. per 24 hours) after nine to fifteen days' clofibrate (Table XXIV). This effect was confined to patients taking the larger dosage of the drug, three of whom (A.W., E.D. and D.M.) showed levels at or above the upper limit of normal (5.0 g., 5.1 g. and 5.7 g. per

24 hours respectively) (Hendry, 1960). The small dosage group showed an insignificant decrease in daily fat excretion.

One to nine months later, the values obtained for fat content of the stool and daily fat excretion were not significantly different from those observed in the pretreatment period (Table XXIV).

Faecal Neutral Steroids

Values for the faecal excretion of individual and total neutral steroids are shown in Table XXV. and Figure 20. These are reported as faecal neutral steroid content (mg. per 100 g. dry faeces) and as daily neutral steroid excretion (mg. per 24 hours). In the group given clofibrate 2 g. daily, the daily neutral steroid output showed a significant increase after nine to fifteen days' treatment. This effect was not seen in the low dosage group, in which a small decrease in faecal neutral steroid output was observed. A significant correlation was observed between the change in daily neutral steroid output

Table XXV. Faecal Neutral Steroids (Mean Values ±S.D.)

	Whole Group (n = 21)		Group on 2 g./day (n = 10)		Group on 1 g./day (n = 11)		Prolonged Treatment Group (n = 10)	
	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 1-9 months' Clofibrate
Faecal neutral steroid content (mg./100 g. dry faeces)								
CHOLESTEROL	399.5 ±221.9	378.0 ±192.1	286.3 ±198.8	331.1 ±228.4	502.5 ±196.0	420.8 ±150.3	501.4 ±206.6	331.7 ±234.3
COPROSTANOL	1739.0 ±746.8	1684.4 ±819.8	1643.4 ±788.4	1424.2 ±670.1	1825.9 ±733.8	1921.0 ±900.3	1872.9 ±755.9	1833.1 ±691.5
COPROSTANONE	335.8 ±303.9	315.4 ±322.5	276.0 ±342.8	353.3 ±408.6	390.2 ±268.6	280.9 ±235.0	414.0 ±270.6	378.2 ±271.2
TOTAL	2474.3 ±898.6	2377.8 ±1046.4	2205.7 ±994.5	2108.6 ±1020.3	2718.6 ±766.2	2622.7 ±1055.9	2788.3 ±770.1	2543.0 ±628.9
Daily neutral steroid excretion (mg./24 hours)								
CHOLESTEROL	120.5 ±91.0	151.9 ±175.4	123.8 ±101.9	223.0 ±232.4	117.6 ±84.8	87.2 ±57.0	110.2 ±85.8	76.3* ±88.9
COPROSTANOL	484.7 ±202.0	562.1 ±346.1	581.1 ±189.6	774.8* ±335.7	397.1 ±177.5	368.7 ±227.3	386.3 ±183.3	363.3 ±148.0
COPROSTANONE	114.8 ±152.5	117.6 ±165.3	130.5 ±182.2	173.8 ±209.8	100.5 ±126.9	66.6 ±95.1	104.9 ±133.0	87.6 ±95.3
TOTAL	720.0 ±406.1	831.6 ±545.5	835.4 ±444.6	1171.6** ±529.0	615.2 ±355.4	522.5 ±348.0	601.4 ±371.5	527.2 ±268.0

Significant difference from pre-treatment levels * $p < 0.05$, ** $p < 0.01$.

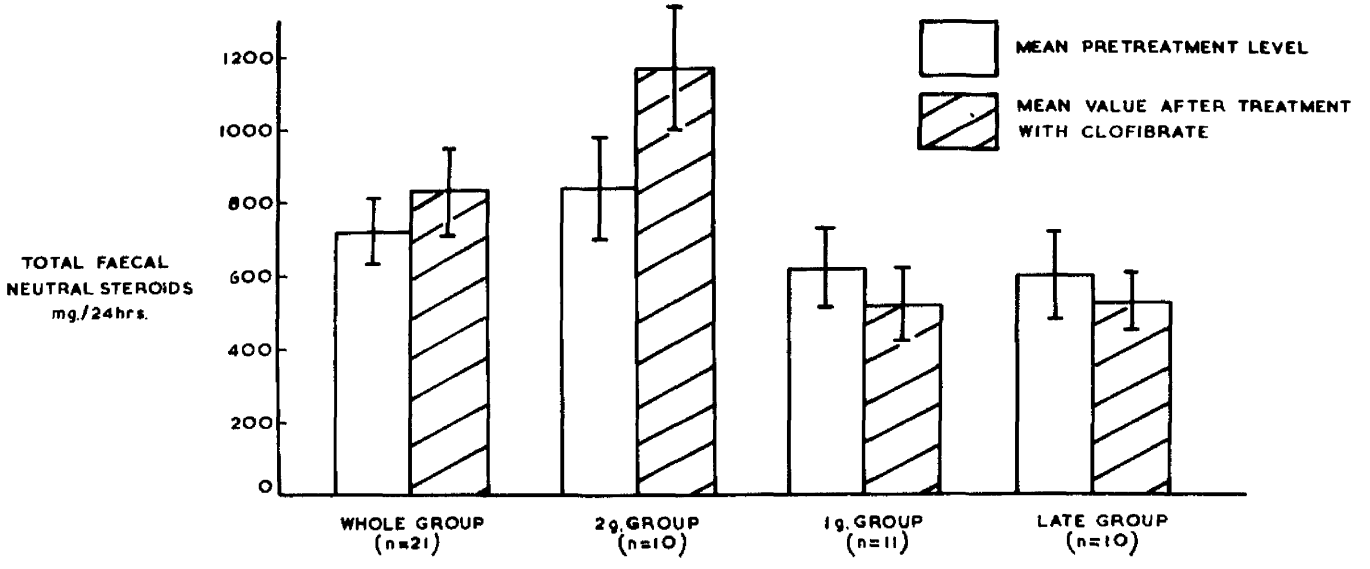


Figure 20. The effect of clofibrate on the daily excretion of faecal neutral steroids. Results are plotted as means ± standard errors. The number of subjects in each group is given in parentheses.

during clofibrate therapy, and the change in serum cholesterol level ($r = -0.45$, $p < 0.05$). Clofibrate had little effect on the total neutral steroid content of faeces although values tended to decrease. This trend was observed also in subject M.W. whose dietary intake of cholesterol was constant throughout the experiment.

The pattern of individual faecal neutral steroids showed a significant change only in the case of D.M., in whom there was an increased proportion of faecal cholesterol during clofibrate medication. In the pretreatment period the percentage of faecal neutral steroids excreted as cholesterol in this subject was 15% as compared with 16% for the remaining subjects. After nine to fifteen days' clofibrate therapy, however, an increase to 44% was seen in D.M., while no significant change was observed in the other subjects.

After one to nine months' treatment with

clofibrate, the only difference in faecal neutral steroid excretion from that seen in the pretreatment period was a small but significant decrease in daily faecal cholesterol output (Table XXV.). No significant change was observed in the percentage of total neutral steroids formed by plant sterols after nine to fifteen days' treatment with clofibrate (11.7 ± 5.8%) as compared with that in the pretreatment period (12.7 ± 6.4%).

Faecal Bile Acids

The values for the faecal excretion of individual and total faecal bile acids are shown in Table XXVI and Figure 21. They are expressed in two ways: as faecal bile acid content (mg. per 100 g. dry faeces) and as daily bile acid excretion (mg. per 24 hours). A significant decrease in faecal content of both deoxycholic acid and lithocholic acid (expressed as mg. per 100 g. dry faeces) was seen after nine to fifteen days' treatment with

Table XXVI. Faecal Bile Acids (Mean Values ±S.D.)

	Whole Group (n = 21)		Group on 2 g./day (n = 10)		Group on 1 g./day (n = 11)		Prolonged Treatment Group (n = 10)	
	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 9-15 days' Clofibrate	Pre-Treatment	After 1-9 months' Clofibrate
Faecal bile acid content (mg./100 g. dry faeces)								
DEOXYCHOLIC ACID	223.7 ±167.4	150.6** ±102.5	183.0 ±109.5	130.0+ ±86.5	260.9 ±205.2	169.3* ±120.6	281.8 ±199.3	194.8* ±133.3
LITHOCHOLIC ACID	135.6 ±99.1	96.2+ ±96.0	108.3 ±85.3	80.3 ±107.5	160.5 ±108.1	110.9* ±86.7	176.5 ±99.2	91.2** ±62.7
TOTAL	359.3 243.7	252.9**(1) ±183.1	291.3 ±183.1	223.3(1) ±180.7	421.4 ±283.6	280.2* ±176.2	458.3 ±281.5	286.0** ±179.6
Daily bile acid excretion (mg./24 hr.)								
DEOXYCHOLIC ACID	60.7 ±51.0	54.2 ±72.8	75.5 ±66.8	80.6 ±99.0	47.3 ±27.7	30.3* ±21.5	52.0 ±24.1	37.7 ±27.8
LITHOCHOLIC ACID	34.6 ±26.6	23.8 ±29.5	40.1 ±34.1	28.4 ±37.5	29.6 ±17.5	19.8* ±20.8	32.5 ±15.3	20.1** ±20.4
TOTAL	95.3 ±71.6	86.7(2) ±110.8	115.6 ±93.7	125.2(2) ±150.3	76.9 ±39.7	50.1* ±37.1	84.5 ±32.1	57.8* ±46.1

Significant difference from pre-treatment levels * $p < 0.05$, + $p < 0.02$, ** $p < 0.01$.
1) includes cholic acid 130.5 mg./100 g. dry faeces in subject D.M.
2) includes cholic acid 162.4 mg./24 hours in subject D.M.

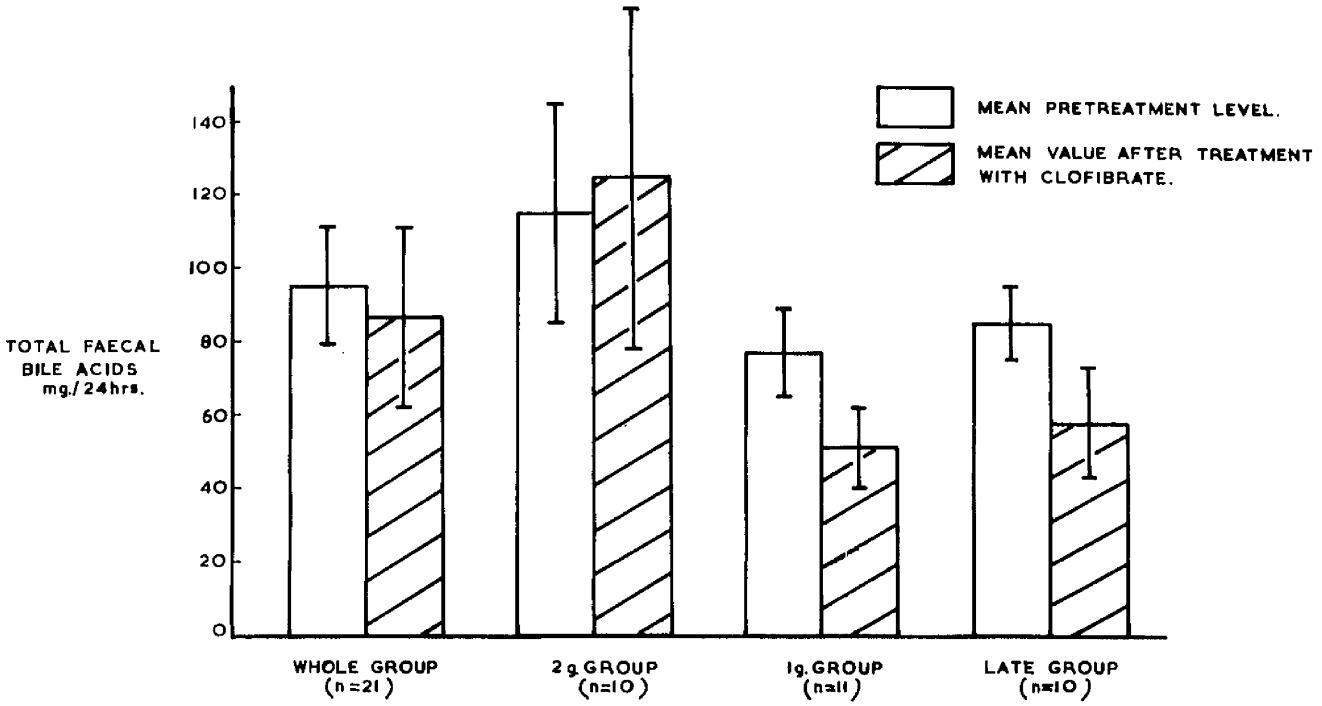


Figure 21. The effect of clofibrate on the daily excretion of faecal bile acids. Results are plotted as means ± standard errors. The number of subjects in each group is given in parentheses.

clofibrate (Table XXVI.). The decrease in bile acid output was more marked in the group taking 1 g. clofibrate daily, and this group also showed a significant decrease in daily bile acid excretion. When the group taking clofibrate 2 g. daily were analysed separately, the decrease in faecal bile acid content was found to be less marked, and not statistically significant, and no decrease in daily bile acid excretion was observed.

After one to nine months' clofibrate therapy, the faecal bile acid content and daily bile acid excretion remained significantly less than during the pretreatment period (Table XXVI.).

An alteration in the excretion pattern of the individual bile acids was seen in only one sample during clofibrate medication. The bile acid pattern in subject D.M's. pretreatment faecal specimen (deoxycholic acid 274.8 mg. per 100 g. dry faeces, lithocholic acid 71.1 mg. per 100 g. dry faeces) was similar to that seen in

the other subjects: after nine to fifteen days' treatment with clofibrate no lithocholic acid was detectable, and the specimen contained cholic acid 130.5 mg. and deoxycholic acid 259.0 mg. per 100 g. dry faeces.

Summary of Results

The administration of clofibrate in a dosage either of 1 g. or 2 g. daily for nine to fifteen days reduced serum cholesterol and triglyceride levels. The changes which were observed in faecal output and faecal constituents differed somewhat in the two dosage groups.

In the group on 1 g. daily, faecal weight, faecal fat, and faecal neutral steroids were unchanged, but a significant reduction in faecal bile acid excretion occurred.

In the group on 2 g. daily, there was an increase in weight of faeces excreted during the collection period. This was associated with small but significant increases in daily excretion of faecal fat and faecal neutral steroids.

The daily bile acid excretion showed no significant change, and the faecal bile acid content, in fact, tended to decrease.

Analysis of faecal lipids after several months of clofibrate medication showed continued reduction of bile acid excretion, and a slight fall in neutral steroid excretion significant only in the case of cholesterol.

Values for the individual subjects are given in the Appendix in Tables XL-LXXVI.

c) A comparison of the effects of clofibrate and L-thyroxine on the serum and faecal lipids in hypothyroid subjects

Four hypothyroid subjects were studied. Further details are shown in Table XXVII.

Table XXVII.

Subject	Sex	Age (yrs.)	Diagnosis	Treatment	Pretreatment serum levels		
					P.B.I.	cholesterol	triglyceride
L.B.	F	66	Myxoedema	L-thyroxine 0.05 mg./day " 0.10	1.9 µg.%	420 mg.%	130 mg.%
M.M.	F	50	"	clofibrate 1.5 g./day L-thyroxine 0.10 mg./day	2.9 µg.%	245 mg.%	103 mg.%
A.G.	F	61	"	clofibrate 1.5 g./day L-thyroxine 0.05 mg./day	<1.0 µg.%	429 mg.%	126 mg.%
J.B.	F	63	"	clofibrate 1.5 g./day L-thyroxine 0.05 mg./day	1.0 µg.%	560 mg.%	136 mg.%

Three (A.G., J.B. and M.M.) were treated initially with clofibrate 1.5 g. per day for twelve to eighteen days. At the end of this period six days without treatment were allowed to elapse before commencing thyroxine therapy for nine to twelve days. Two of the patients (A.G. and J.B.) were given L-thyroxine 0.05 mg. per day and a third (M.M.) was given 0.10 mg. per day. The fourth patient (L.B.) was treated only with L-thyroxine 0.05 mg. per day for five days and then with 0.10 mg. per day.

The collection of blood and estimations of serum cholesterol and triglyceride concentrations were carried out as described in the preceding section. Faecal specimens were collected and neutral steroid and bile acid extracts obtained as described in Part III (p. 87). Neutral steroids and bile acids were measured according to the method in Part III (p. 97). Faecal fat was estimated by the method of Harrison (1949).

Serum lipids

The serum lipid results are shown in Table XXVIII. In the three subjects treated with clofibrate, the serum cholesterol levels decreased. Treatment with thyroxine caused an increase in serum cholesterol in two of the three subjects previously treated with clofibrate. The subject given only thyroxine showed a decrease in serum cholesterol concentration.

During clofibrate therapy all three showed decreases in serum triglyceride levels. Treatment with thyroxine produced a marked increase in serum triglyceride levels in all three subjects. The patient treated with thyroxine only showed a slight decrease in serum triglyceride levels.

Table XXVIII. Serum Lipids (mg./100 ml.).

Lipids	Subject	Basal	Days on Clofibrate		No Treatment	Days on Thyroxine	
			12	18		12	18
Serum Cholesterol (mg./100 ml.)	M.M.	245	213	—	205	278	—
	A.G.	429	252	222	313	319	—
	J.B.	560	488	—	423	398	—
	L.B.				420	354	312
Serum triglyceride (mg./100 ml.)	M.M.	103	88	—	150	170	—
	A.G.	126	86	99	120	148	—
	J.B.	136	59	—	124	147	—
	L.B.				130	117	124

Faecal Fat and Weight

There were no significant changes in the excretion of faecal weight or faecal fat during treatment with clofibrate.

Faecal Neutral Steroids

Faecal neutral steroid excretion rates are shown in Table XXIX. During treatment with clofibrate daily faecal neutral steroid excretion increased in two of the three subjects (M.M. and A.G.) and in all three patients after thyroxine administration for 12 days. Faecal neutral steroid excretion increased in subject L.B. who was treated only with L-thyroxine.

Table XXIX. Faecal Total Neutral Steroids.

	Subject	Basal	Days on Clofibrate		No Treatment	Days on Thyroxine	
			12	18		12	18
	M.M.	651.7	1125.4	-	354.0	363.1	-
mg./24 hr.	A.G.	461.0	723.9	531.7	311.8	378.8	-
	J.B.	486.6	206.0	-	201.0	736.9	-
	L.B.				332.2	452.2	422.5

Faecal bile acids

As is illustrated by Table XXX after 12 days treatment with clofibrate two of the subjects (A.G. and J.B.) showed a decrease in daily faecal bile acid excretion while during treatment with thyroxine the same two patients had an increase in daily faecal bile acid excretion after 12 days. The daily faecal bile acid excretion of subject L.B. also increased after 12 days treatment with thyroxine but fell to just below the basal level after 18 days treatment with thyroxine.

Table XXX. Faecal Total Bile Acids.

	Subject	Basal	Days on Clofibrate		No Treatment	Days on Thyroxine	
			12	18		12	18
	M.M.	75.6	99.1	-	64.9	56.3	-
mg./24 hr.	A.G.	47.1	36.6	27.0	18.7	40.1	-
	J.B.	26.8	14.6	-	3.1	35.0	-
	L.B.				116.3	131.4	103.0

In summary, clofibrate treatment led to a decrease in serum lipid levels in all three subjects, an increase in daily faecal neutral steroids in two of the three subjects and a decrease in daily faecal bile acid excretion in two of the three subjects.

Thyroxine treatment led to an increase in serum cholesterol levels in two of the three subjects, an increase in serum triglyceride concentrations in all three subjects, an increase in daily faecal neutral steroid in all three subjects and an increase in daily faecal bile acid excretion in two of the three subjects.

3. The effect of oral calcium on the excretion of cholesterol and its metabolites

Five female and one male subject were investigated. The main clinical findings in each patient are given in Table XXXI.

Table XXXI. Clinical and dietary details of subjects studied.

Subject	Sex	Age	Diagnosis	Calcium Intake g./day			Fat Intake g./day		Sterol Intake mg./day	
				A	B	C	A & B	C	A & B	C
1	F	64	osteoporosis	0.233	1.373	-	41.26	-	96.5	-
2	F	73	osteoporosis	0.267	1.407	1.507	36.03	36.33	82.5	86.5
3	F	65	osteoporosis	0.327	1.467	1.567	44.0	44.3	82.5	86.5
4	F	70	osteoporosis	0.243	1.383	1.483	32.87	33.17	82.5	86.5
5	F	69	osteoporosis	0.349	1.489	1.589	42.66	42.96	110.5	114.5
6	M	61	cerebrovascular accident	0.733	1.873	-	65.9	-	407.0	-

Period A - basal period on diet alone. (3 to 5 weeks).

Period B - addition of 1.14 g. calcium (calcium glycerophosphate 2 g. t.i.d. to subjects 1-5 and calcium Sandoz tab. 1 t.i.d. to subject 6). (4 weeks).

Period C - substitution of calcium glycerophosphate with skimmed milk equivalent to 1.24 g. of calcium. (4 weeks).

Patients were admitted to a metabolic unit and studied under three dietary regimens. In the first (period A) the calcium intake ranged from 0.233 g./day to 0.733 g./day, the fat intake from 32.9 to 65.9 g./day and the sterol intake ranged from 82.5 to 407 mg./day with each patient having a fixed intake of calcium, fat and sterol. Each patient was investigated on this basal diet for a period of four to six weeks. In the second regimen (period B), the calcium intake was increased by 1.14 g. in each patient. Calcium glycerophosphate was given to patients 1 to 5 and calcium gluconogalactogluconate was given to patient 6. This was continued for four weeks. In the third regimen (period C), the calcium intake was maintained at this increased level in patients 2, 3, 4 and 5 by giving 1½ pints of dried skimmed milk. There were no equilibration periods between dietary regimes. The fat content of the diet remained unaltered during

period B, but when the dried skimmed milk was added, the dietary intake of fat was increased by 0.3 g./day.

Throughout the period of study, faeces were collected for 7 day periods and processed as described in Part III (p. 87). Total fat (acidic and neutral) was measured by the method of Panton and Marrick (1934). Neutral steroids and bile acids were determined by the densitometry method described in Part III (p. 97). Faecal calcium was estimated after ashing by titrating with EDTA using ammonium purpurate as an indicator (Nordin and Smith, 1965). Dietary calcium was estimated on two 24 hour samples of the diet after ashing (Nordin and Smith, 1965).

Serum cholesterol (Technicon Autoanalyser N24P) and serum triglyceride (van Handel and Zilversmit, 1957) levels were measured twice weekly on fasting blood samples.

The significance of changes from mean

basal levels was determined by the Student "t" test for paired values. The results given in the tables are the means of the four weekly values obtained during the three regimens.

Faecal neutral steroids

Faecal neutral steroid excretion data are shown in Table XXXII.

Table XXXII. Mean faecal neutral steroid output (mg./24 hr.) before (A) and during increase in calcium content of diet by 1.14 g./day of calcium as calcium glycerophosphate (B) and 1.24 g./day of calcium as 1½ pints of skimmed milk (C).

	Cholesterol mg./24 hr.			Coprostanol mg./24 hr.			Coprostanone mg./24 hr.			Total mg./24 hr.		
	A	B	C	A	B	C	A	B	C	A	B	C
1	70.5	107.1		175.2	159.1		70.0	55.1		315.7	321.3	
2	71.8	137.2	121.3	180.7	147.5	178.7	31.4	22.1	24.3	283.9	306.8	324.3
3	112.0	162.1	165.5	174.6	154.8	166.4	36.3	43.7	21.0	322.9	360.6	352.9
4	40.5	50.5	71.1	208.6	204.4	201.9	32.0	33.4	33.4	281.1	288.3	306.4
5	53.2	129.7	86.0	309.1	275.7	312.2	19.8	37.7	28.6	382.1	443.1	426.8
6	47.0	48.5	-	313.8	219.9	-	29.5	12.8	-	390.3	281.2	-
Mean (1-6)	65.83	105.85*		227.00	193.57*		36.50	34.13		329.33	333.55	
S.D.	±25.86	±47.05		±66.61	±49.74		±17.30	±15.13		±47.16	±60.63	
Mean (2-5)	69.4	119.9	111.0**	218.3	195.6	214.8	29.9	34.2	26.8	317.5	349.7	352.6**
S.D.	±31.2	±48.3	±42.0	±62.3	±59.1	±66.6	±7.0	±9.2	±5.4	±47.1	±69.6	±53.0

*$p < 0.05$

**$p < 0.01$

Significant difference from mean pretreatment level.

Faecal cholesterol increased in each subject. The average increase for the group being statistically significant during periods B and C. In period B, when calcium supplements were added there was a steady increase in faecal cholesterol output during each of the four weekly periods from a mean of 80 mg./day to a mean of 130 mg./day. During period C, when dried skimmed milk was substituted for calcium glycerophosphate, there was no significant change in the cholesterol output compared with period B, though the output remained significantly higher than that during the control period on the basic diet.

The output of coprostanol fell significantly during period B.

The faecal output of coprostanone was not significantly affected by the addition of calcium glycerophosphate or skimmed milk to the diet.

The total neutral steroid output showed no change during treatment with calcium glycerophosphate but increased significantly when dried

skimmed milk was fed.

Faecal bile acids

Following the addition of calcium glycerophosphate to the diet, there was a significant increase in the output of deoxycholic acid and lithocholic acid. Table XXXIII.

Table XXXIII. Mean faecal bile acid output (mg./24 hr.) before (A) and after increase in calcium content of diet by 1.14 g./day of calcium as calcium glycerophosphate (B) and 1.24 g./day of calcium as 1½ pints of skimmed milk (C).

	Deoxycholic Acid mg./24 hr.			Lithocholic Acid mg./24 hr.			Total mg./24 hr.		
	A	B	C	A	B	C	A	B	C
1	42.0	85.6	–	44.0	56.7	–	86.0	142.3	–
2	27.7	43.4	26.0	18.6	37.3	23.2	46.3	80.7	49.2
3	25.8	36.5	29.9	8.9	30.8	26.5	34.7	67.3	56.4
4	8.1	15.3	27.1	8.8	10.8	19.8	16.9	26.1	46.9
5	66.6	74.5	92.7	12.5	43.4	53.3	79.1	117.9	146.0
6	22.4	31.1	-	21.9	21.4	-	44.3	52.5	-
Mean 1-6	32.10	47.73*		19.12	33.40*		51.22	81.13+	
S.D.	±20.09	±26.92		±13.28	±16.25		±26.49	±42.74	
Mean 2-5	32.1	42.4	43.9	12.2	30.6	30.7	44.3	73.0	74.6
S.D.	±24.7	±24.5	±32.6	±4.6	±14.1	±15.3	±26.2	±37.9	±47.7

*$p < 0.05$

+$p < 0.02$

Significant difference from mean pretreatment level.

This increase became apparent after the end of the first week and continued throughout the period of calcium supplementation.

During period C, when skimmed milk was substituted for calcium glycerophosphate, the faecal output of bile acids did not differ from that in period B.

Faecal fat, faecal dry weight and faecal calcium

During the period of supplementation of the diet with calcium glycerophosphate, there was a significant increase in faecal acid fat, total fat and faecal dry weight (Table XXXIV.).

Table XXXIV. Mean weight of faecal dry weight, faecal fat and faecal calcium before (A) and after increase in calcium content of diet by 1.14 g./day of calcium as calcium glycerophosphate (B) and 1.24 g./day of calcium as $1\frac{1}{2}$ pints of skimmed milk (C).

	Faecal Dry Weight g./24 hr.			Faecal Acid Fat g./24 hr.			Total Faecal Fat g./24 hr.			Faecal Calcium mg./24 hr.		
Subject	A	B	C	A	B	C	A	B	C	A	B	C
1	15.5	21.2	-	1.55	3.90	-	2.36	4.36	-	197.0	1201.8	-
2	14.1	17.3	19.2	1.17	1.80	2.16	1.48	2.00	2.50	237.4	1180.0	1084.0
3	18.4	20.4	20.5	1.08	1.64	1.57	1.65	2.11	2.20	217.3	1160.0	1083.3
4	14.0	20.8	17.8	2.25	4.02	2.54	4.41	7.44	3.79	213.2	1136.8	1150.3
5	13.6	19.8	22.3	0.79	1.29	1.27	1.30	1.99	2.05	260.6	1182.0	1286.3
6	17.1	19.6	-	1.91	3.04	-	2.67	4.22	-	-	-	-
Mean 1-6	15.45	19.85**		1.46	2.62 +		2.31	3.69*		225.1	1172.1***	
S.D.	±1.94	±1.39		±0.55	±1.20		±1.15	±2.15		±24.5	±24.7	
Mean 2-5	15.0	19.6	19.9*	1.32	2.19	1.90*	2.21	3.39	2.64	232.1	1164.7	1151.0***
S.D.	±2.3	±1.3	±2.2	±0.64	±1.2	±0.54	±1.44	±2.7	±0.77	±21.9	±21.1	±95.3

* $p < 0.05$
\+ $p < 0.02$
** $p < 0.01$
*** $p < 0.001$

Significant difference from mean pretreatment level.

With the addition of calcium glycerophosphate there was also a marked increase in the faecal calcium which reached a maximum in the second week and thereafter remained at this level.

The substitution of dried skimmed milk did not affect the faecal fat, dry weight or calcium when compared with period B. The faecal dry weight and calcium remained significantly above the level of excretion during the period on the basic diet.

The patients did not complain of any gastro-intestinal symptoms, such as diarrhoea, during the experimental period.

Serum lipids

The results are shown in Figure 22, and Table XXXV.

Table XXXV. Mean serum cholesterol and serum triglyceride values before (A) and during increase in calcium content of the diet by 1.14 g./day of calcium as calcium glycerophosphate (B) and 1.24 g./day of calcium as skimmed milk (C).

	Serum cholesterol mg.%			Serum triglyceride mg.%		
	A	B	C	A	B	C
1	302.8	286.4	-	61.8	76.4	-
2	396.0	402.3	401.5	182.8	205.5	243.1
3	180.0	198.3	208.0	51.5	62.9	54.7
4	252.0	241.5	270.0	120.8	104.4	127.8
5	177.9	174.5	-	88.4	69.2	-

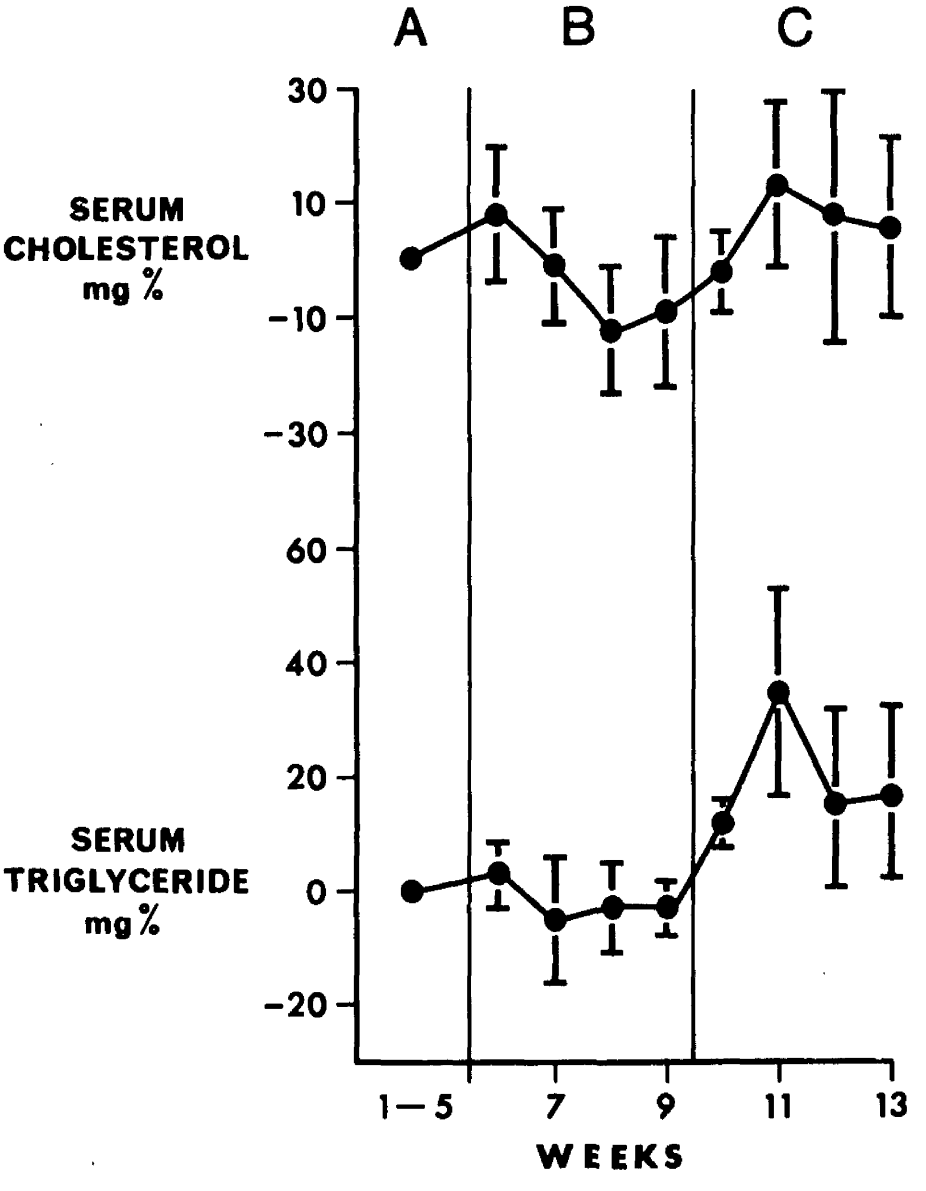


Figure 22. Change in serum cholesterol and serum triglyceride levels during calcium supplementation.

The serum cholesterol and serum triglyceride levels tended to fall during period B but the decrease was not significant. The fall was not evident until the second week and was greatest during the 3rd and 4th weeks. This pattern was similar to the changes observed in faecal excretion (Figure 23). Following the addition of the dried skimmed milk, which increased the daily fat intake by 0.3 g. there was a rise in the serum cholesterol level to above the basal levels, and an even greater rise in the serum triglyceride though again these changes were not significant.

Body Weight

A small decrease in weight occurred in all cases. The mean weight loss during the whole experiment (12 weeks) was 1.6 $\pm$ 1.2 Kg. The weight changes during periods A and B were similar while that in period C was of smaller magnitude. The weight loss cannot be attributed to the introduction of calcium into the diet

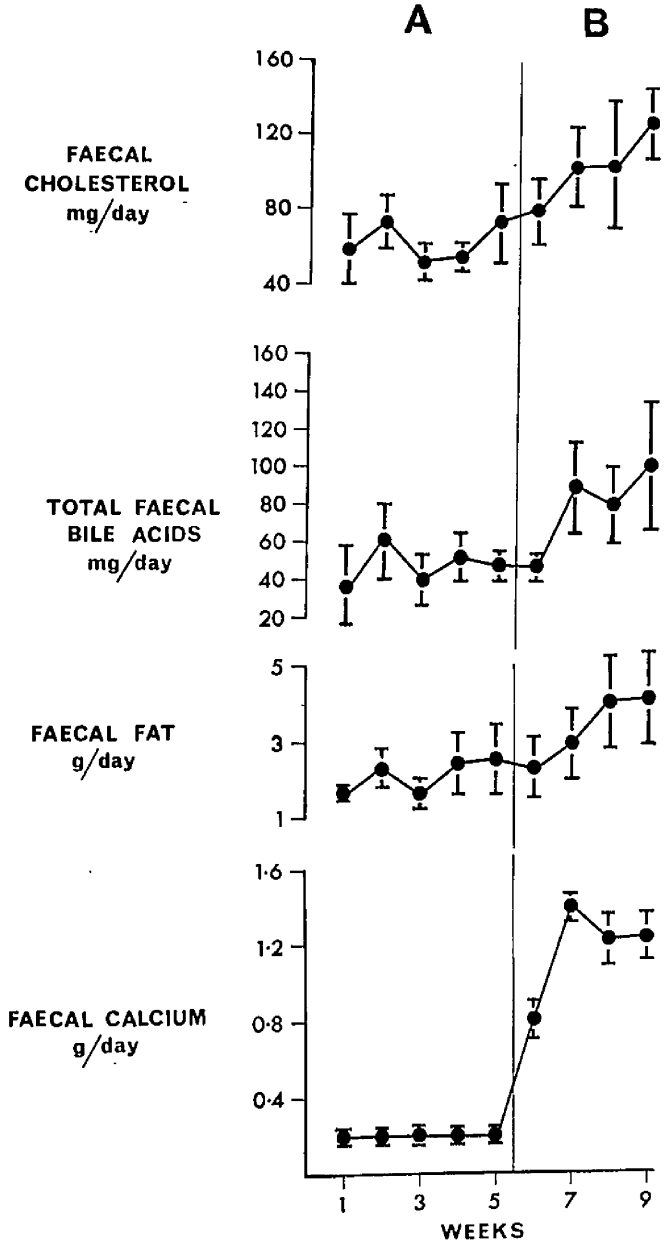


Figure 23. Changes in faecal cholesterol, bile acids, fat and calcium during first four weeks of calcium supplementation.

because it was also evident during the basal period.

4. Dietary cholesterol; its effect on serum lipids and faecal excretion of neutral steroids and bile acids

One subject only was studied, who received a diet low in cholesterol content (129 mg./day of cholesterol) and essentially free of plant sterols for fourteen days. Fasting blood samples were taken twice weekly for four weeks prior to the start of the low cholesterol diet and three times a week during the 14 days of this experimental period. Faecal specimens were obtained in five day collections during the four weeks basal period and a six day collection was obtained during the last six days of the low cholesterol diet.

The high cholesterol diet (2.058 g./day of cholesterol in the form of eggs, cheese and cream) was begun after the low cholesterol diet and was again essentially free from plant sterols. The dietary period was fourteen days. Fasting blood samples for serum lipid estimations were obtained thrice weekly throughout

the fourteen days and a faecal collection was obtained during the final six days of the high cholesterol diet.

The basal diet (0.374 mg./day of cholesterol) was the subjects normal home diet and the cholesterol content was obtained from a diet history.

The collecting, drying and extracting of faeces was carried out as described in Part III (p. 87). Faecal neutral steroids and faecal bile acids were measured by the densitometry method described in Part III (p. 97). Serum cholesterol was measured by the Technicon Autoanalyser (N.24P) and serum triglyceride by the van Handel and Zilversmit (1957) technique.

The serum lipid results are shown in Table XXXVI.

Table XXXVI.

Serum Cholesterol (mg.%)			Serum Triglyceride (mg.%)		
Basal Period	Low Cholesterol Diet	High Cholesterol Diet	Basal Period	Low Cholesterol Diet	High Cholesterol Diet
180	166	209	46	67	74

Serum cholesterol levels showed a slight decrease from the basal levels during the low cholesterol diet. During the high cholesterol diet however, there was an increase of 43 mg.% over the low cholesterol diet and an increase of 29 mg.% over the basal level.

Serum triglyceride levels increased from the basal level during the low cholesterol diet and increased even more during the high cholesterol diet.

The daily excretion of faecal neutral steroids and bile acids are shown in Table XXXVII and XXXVIII.

Table XXXVII. The effect of oral cholesterol on faecal neutral steroid excretion.

Cholesterol Content of diet	FAECAL NEUTRAL STEROIDS (mg./24 hr.) Cholesterol	Coprostanol	Coprostanone	TOTAL
Basal	592.8	520.7	69.1	1182.6
Low	196.2	Undetected	Undetected	196.2
High	1318.4	"	"	1318.4

Table XXXVIII. The effect of oral cholesterol on faecal bile acid excretion.

Cholesterol Content of diet	FAECAL BILE ACIDS (mg./24 hr.) Cholic acid	deoxycholic acid	lithocholic acid	TOTAL
Basal	-	231.6	137.5	369.1
Low	131.8	16.4	Undetected	148.2
High	403.2	75.8	"	479.0

During the low cholesterol diet, a marked decrease in total neutral steroid excretion and total bile acid excretion was observed. No coprostanol or coprostanone were detected in the neutral steroid fraction. A considerable amount of cholic acid was excreted with no apparent excretion of lithocholic acid during this period.

During the high cholesterol diet, only cholesterol was detected in the neutral steroid fraction. Cholic acid was the major faecal bile acid excreted and again no lithocholic acid was detected.

The sterol balance of the patient during the three dietary periods is shown in Table XXXIX.

Table XXXIX. The effect of dietary cholesterol on the sterol balance.

Diet	Sterol Intake mg./24 hr.	Steroid Output Neutral + Acidic mg./24 hr.	Output-Intake
Basal	374.0	961.9	+587.9
Low	129.0	344.4	+215.4
High	2058.0	1797.4	-260.6

From these results it appears that the subject was excreting more steroid than was ingested during the basal and low cholesterol diet whereas during the high cholesterol diet the converse occurred.

DISCUSSION

In all of the investigations except one the number of subjects studied was small and only tentative conclusions can therefore be drawn from the results. This is especially true of the cholesterol-feeding experiment in which only one subject was studied. However, the experiments illustrate several of the steps in the two main pathways of cholesterol excretion, i.e. excretion in bile as bile acid conjugates and in faeces as neutral steroids and bile acids, which if altered may cause changes in serum cholesterol concentrations.

1. The effect of feeding taurine

The results show that the proportion of bile acids conjugated with taurine in human bile can readily be increased by feeding 1.5 g. taurine per day. This is in agreement with the finding of Sjövall (1959). Taurine feeding for 10 to 15 days at this level, however, produced no effect on serum cholesterol concentrations, a result which is in keeping with incidental

observations by two other groups. Failey et al. (1962) mentioned that in preliminary studies, feeding taurine as a dietary supplement to man appeared to have no effect on the serum cholesterol concentration while Hellström and Sjövall (1961) showed that although the G/T ratio fell serum cholesterol continued to rise in a similar experiment in which a patient with mild hypothyroidism was given 3 g. taurine per day for two months.

Amongst mammals two groups may therefore be distinguished. The first, represented by man and the rabbit, in which taurine has no effect on serum cholesterol levels and the second, including rats, mice and monkeys in which taurine produces a distinct effect. It is of some interest that the former conjugate their biliary bile acids mainly with glycine and the latter with taurine.

2. The effect of clofibrate

a) On biliary bile acids and serum cholesterol

The mean change in the glycine/taurine ratio during clofibrate treatment was small (-0.4) and insignificant. The changes in individual subjects between control and treatment periods were of the same order as variations found by Sjövall (1960) between repeat bile collections in healthy men. There was no change of G/T ratio in the two patients under metabolic ward conditions and the small changes in the three out-patients could have resulted from variations in dietary taurine as shown in the preceding section and by Sjövall (1959).

The number of patients studied was not large enough to permit detection of small changes in the G/T ratio, but it seems likely that clofibrate does not produce major changes in the conjugation pattern of biliary bile acids. This is in contrast to hypothyroid patients treated with thyroid hormone. The mean decreases in the G/T ratios of these subjects

reported by Hellström and Sjövall (1961) and Hellström and Lindstedt (1964) were 5.4 and 4.3 respectively.

It is of interest that in the five subjects treated with clofibrate (p. 168) there appears to be a relationship between the serum cholesterol concentration and the G/T ratio. However when additional data from the section on taurine feeding is considered then the relationship no longer holds ($r = -0.15$). The serum cholesterol levels and G/T ratios of a group of nine hypothyroid subjects (Hellström and Lindstedt, 1964) before and after treatment with thyroid hormone were also analysed but again there was no correlation between the concentration of the serum cholesterol and the G/T ratio. (untreated $r = 0.015$, treated $r = 0.406$).

There did not appear to be any change in the proportions of the bile acids during clofibrate therapy.

Thorp (1963) has suggested, among other possibilities, that clofibrate might act by displacing thyroxine from its binding by plasma albumin resulting in increased free thyroxine in the liver. If this is the mechanism that lowers plasma cholesterol during clofibrate treatment one might expect a reduction in the glycine/taurine conjugation ratio of biliary bile acids because large reductions have occurred consistently when hypothyroid patients were treated with thyroid hormones (Hellström and Sjövall, 1961; Hellström and Lindstedt, 1964). The finding that clofibrate produces no significant change in the G/T conjugation ratio is suggestive evidence against the thyroxine mechanism. It cannot, however, be considered conclusive because the patients in this study were euthyroid and it is not known at present what effect thyroxine has on the G/T conjugation ratio in euthyroid human subjects. Furthermore a true dissociation between the

cholesterol lowering and G/T conjugation effects of clofibrate has not been demonstrated because the cholesterol changes were not statistically significant.

b) The effect of clofibrate on serum and faecal lipids

Changes in plasma lipids with clofibrate

Clofibrate reduced serum cholesterol and triglyceride in the majority of the subjects (p. 178). The ability of the drug to lower the serum levels of both cholesterol and triglyceride is now well established (Acheson and Hutchinson, 1963; Berkowitz, 1963(a); Green et al. 1963; Oliver, 1963). As noted in the present series, the hypolipidaemic effect of clofibrate occurred within one to two weeks of treatment (Carlson et al. 1963; Denborough, 1963) although in some cases the maximum effect may not be attained for several months (Laubinger, 1964). The observation that the greatest fall in cholesterol occurs in those patients with the highest pretreatment levels has also been

made by Counihan and Keelan (1963). Similarly, dietary treatment, d-thyroxine, and clofibrate were all found to depress the serum cholesterol in proportion to its initial level (Hansen, 1963). It has been stated by some workers (Hellman et al. 1963) that females show a greater reduction in serum cholesterol levels than males in response to clofibrate medication, while others (Acheson and Hutchinson, 1963; Green et al. 1963; Knuchel, 1964) have observed no sex differences in the magnitude of fall in serum cholesterol. The greater response shown by the females in the present series may be due to the fact that they had a greater proportion of high pretreatment serum cholesterol levels than the males. In contrast to the findings of Oliver (1962) it was found that a daily dose of 1 g. clofibrate was sufficient to produce a hypolipidaemic effect over a two-week period. The fall in serum cholesterol and triglyceride in the group taking 1 g. clofibrate daily was, in fact, larger than

that obtained in the group given clofibrate in an initial dosage of 2 g. daily. This observation also is probably due to the higher pretreatment serum lipid levels in the small dosage group.

After several months of clofibrate treatment most of the 10 subjects had maintained serum cholesterol and triglyceride levels below the pretreatment values. The large increase, after a transient fall, in the already elevated triglyceride in subject J.P. is unexplained, although substantial increases in serum triglyceride levels during treatment with clofibrate have been reported in a minority of subjects by other authors (Oliver, 1963; Fasoli and Cesana, 1963; Cramér, 1963). The increase in serum triglyceride level in this patient was not associated with factors previously shown to cause elevation in this value such as weight gain (Albrink et al. 1962), or changes in dietary intake of fat (Antonis and Bersohn,

1961) or carbohydrate (Ahrens et al. 1961).

Changes in faecal excretion

The increase in number of stools passed during the first week of clofibrate medication was presumably a side-effect of the drug, which appeared to be mild and transient when a small initial dose of clofibrate was used. In the higher dosage group the effect was of longer duration, and was reflected in the increased weight of faeces excreted after nine to fifteen days of clofibrate therapy. One of the subjects reported mild diarrhoea after commencement of clofibrate (2 g. daily), and other workers (Oliver, 1963; Prior and Campbell, 1963) have noted that diarrhoea or intestinal hurry might occur when clofibrate was given in an initial dosage of 2 g. to 3 g. daily. There was no evidence of steatorrhoea during treatment. The small increase in daily faecal fat excretion is well within the normal range, and is probably the result of intestinal hurry in the subjects

on the larger dose. This is in accord with the finding that reduction of serum lipids during treatment with a combination of clofibrate and androsterone is not associated with steatorrhoea when cholesterol is lowered by means of a bile-sequestering resin (Berkowitz, 1963(b).

A change in the pattern of excretion of faecal neutral steroids and bile acids occurred only in subject D.M. This subject, in whom clofibrate caused mild diarrhoea, also showed increases in weight of faeces excreted and in daily fat excretion which were greater than those seen in the other subjects. The increased proportion of cholesterol and the presence of cholic acid both reflect diminished bacterial degradation of these compounds, and are presumably the result of intestinal hurry in this subject.

Mechanism of serum lipid changes

A reduction in serum cholesterol level following clofibrate therapy might, in theory,

be achieved by several means; firstly, by increased excretion of cholesterol into the gastro-intestinal tract via the liver and bile and secondly, by reduced absorption of cholesterol from the gastro-intestinal tract. Both these effects would be manifested by increased excretion of neutral steroids in the faeces. A third possibility is increased degradation in the liver of cholesterol to bile acids, which would result in increased faecal bile acid excretion. Fourthly, the synthesis of cholesterol in the liver and gastro-intestinal tract might be diminished by clofibrate. In such an event the excretion of cholesterol and bile acids into the gastro-intestinal tract would tend to fall, resulting in decreased faecal excretion of neutral steroids and bile acids.

In the low-dosage group, the marked fall in serum lipids was accompanied by a significant reduction in faecal bile acids, and a similar,

though smaller, change in faecal neutral steroids. These results are compatible with the last-mentioned mechanism; namely, a decreased rate of cholesterol synthesis reflected in diminished faecal excretion of cholesterol and its end-products. After several months of clofibrate therapy, the faecal excretion of bile acids and, to a lesser degree, neutral steroids was still lower than in the pretreatment period, suggesting that this mechanism continues to operate. In the high-dosage group however, these faecal lipid changes were partly obscured by increased faecal lipid output resulting from intestinal hurry. The fact that the serum lipid changes in this group were smaller than those in the low-dosage group confirms that the increased faecal lipid output in the high-dosage group was not causally related to the fall in serum lipids which occurred at the same time.. The suggestion that the fall in serum lipid levels and in faecal lipid output both result

from diminished lipid synthesis is further supported by the observation that subjects with the largest fall in serum cholesterol tended to show the greater reduction in faecal neutral steroid output.

Any theory of the mechanism of action of clofibrate must account for the fact that it reduces the serum levels of both cholesterol and triglyceride. One explanation which has been advanced is that binding of clofibrate to the plasma albumen reduces its affinity for other acids, including thyroxine and non-esterified fatty acids (Thorp, 1963). This is said to result in a preferential localisation in the liver of thyroxine and its active metabolites, which in turn leads to decreased hepatic synthesis of cholesterol and triglyceride, while decreased peripheral availability of thyroxine reduces lipolysis in adipose tissue. However, a study of bile acid conjugates before and during clofibrate therapy did not confirm

an increased thyroid-like activity, in that the characteristic change in the glycine; taurine ratio did not occur (Part IV, p. 168). Moreover clofibrate administration was found by Danowski and Alley (1967) to reduce cholesterol and triglyceride levels in a subject with complete primary myxoedema, indicating that availability of thyroid hormone is not essential for the hypolipidaemic effect of clofibrate.

Although these findings suggest that clofibrate does not exert its hypolipidaemic action in the manner described above, there is some evidence that it reduces lipid synthesis in the liver. A diminished hepatic content of cholesterol and triglyceride has been found in rats given clofibrate (Azarnoff et al. 1965). Further animal studies have indicated that clofibrate inhibits cholesterol biosynthesis at some stage prior to the formation of mevalonate (Azarnoff et al. 1965; Avoy et al.

1965). It has been established that there is no accumulation in the plasma of humans of cholesterol precursors such as desmosterol, which would indicate a block in the final stage of cholesterol synthesis. Other investigations have produced, in studies of serum lipoproteins, indirect evidence of inhibition of synthesis of triglyceride (Walton et al. 1963) and of the very-low-density lipoproteins which transport triglyceride (Strisower et al. 1965). While the experimental observations in the present study can provide no direct evidence for or against these theories, the findings of diminished faecal output of bile acids and neutral steroids during clofibrate medication indicate diminished excretion and catabolism of cholesterol, possibly resulting from diminished cholesterol synthesis.

c) A comparison of the effect of clofibrate and L-thyroxine on serum and faecal lipids in hypothyroid subjects.

A decrease was observed in serum cholesterol levels in all three subjects on clofibrate within 12 days of treatment. The lowering of serum cholesterol by clofibrate in euthyroid patients is well known (see previous section), but there have been relatively few reports of its effect on hypothyroid subjects. Harrison and Harden (1966) failed to detect any lowering of serum cholesterol after three weeks treatment with clofibrate in two subjects with gross myxoedema although Danowski and Alley (1967) observed a marked decrease in serum cholesterol in one woman with primary myxoedema after treatment with clofibrate.

During thyroxine treatment, serum cholesterol levels tended to increase above pretreatment level in the three subjects treated initially with clofibrate.

In contrast to these findings, the subject L.B. who was treated only with L-thyroxine showed a decrease in serum cholesterol levels,

a finding also reported by Malmros and Swahn (1953) and Boyd and Oliver (1960). However, it may be that in the present study the serum cholesterol concentrations of the three subjects treated initially with clofibrate had been lowered to such an extent that the subsequent effect of thyroxine was less apparent.

Serum triglyceride

The decrease in serum triglyceride concentrations observed in all three subjects during treatment with clofibrate is in agreement Danowski and Alley (1967). During thyroxine therapy however, serum triglyceride levels increased markedly in these three subjects.

The serum triglyceride level in subject L.B. showed little change during treatment with L-thyroxine. A similar situation had been observed earlier by Best and Duncan (1964, 1966) in euthyroid subjects given D-thyroxine. Feldman and Carter (1963) however had observed

falls of 24 to 48% in four hypothyroid subjects during treatment with this compound. Their basal values ranged from 366 to 515 mg./100 ml. serum which are much higher than the basal values of the subjects in this report.

The finding by Strisower and Strisower (1964) that D-thyroxine lowers principally the $Sf^{o}0-20$ lipoprotein class whereas clofibrate lowers principally the $Sf^{o}20-400$ class, the triglyceride carrying lipoprotein, together with the relatively low basal triglyceride level might explain the failure of L-thyroxine to lower serum triglyceride levels.

From the serum lipid results, especially the serum triglyceride values, it appears that clofibrate is a more efficient hypolipidaemic agent than L-thyroxine and that the mechanism of action of clofibrate does not depend on the presence of thyroxine. This is especially borne out by the results in subjects A.G. and J.B. whose protein bound iodine concentrations

were < 1.0 μg.% and 1.0 μg.% respectively.

Faecal neutral steroids and bile acids

Treatment with clofibrate showed no consistent effect on the excretion of faecal neutral steroids but the tendency for the bile acid excretion to decrease was similar to that observed in euthyroid subjects (see previous section).

The increase in faecal neutral steroid and bile acid excretion during thyroxine therapy, also reported by Weiss and Marx (1955) and Kritchevsky (1960), suggests that thyroxine increases the catabolism and excretion of cholesterol.

It therefore appears that the mechanisms of action of clofibrate and thyroxine in lowering serum cholesterol levels are different. Clofibrate decreases the faecal excretion of neutral steroids and bile acids (Part IV) whereas the results of this study and of other workers indicate that thyroxine increases the

excretion of neutral steroids and bile acids. Unlike thyroxine, clofibrate has no effect on the glycine to taurine ratio of biliary bile acids (Part IV). Other workers have suggested that clofibrate decreases the hepatic synthesis of cholesterol (Azarnoff et al. 1965; Avoy et al, 1965). Thyroxine on the other hand has been shown to increase cholesterol synthesis (Rosenman et al. 1952).

The effect of oral calcium on cholesterol metabolism

Three main changes in faecal lipid excretion occurred during increased calcium intake. Faecal cholesterol excretion increased significantly during calcium glycerophosphate administration and was accompanied by a significant decrease in coprostanol excretion. Total neutral steroid output therefore remained constant. When the skimmed milk diet was substituted for calcium glycerophosphate, faecal cholesterol remained elevated while coprostanol excretion increased, causing a significant

increase in the excretion rate of total neutral steroids.

Faecal bile acid excretion increased during calcium supplementation attaining statistical significance during calcium glycerophosphate treatment.

The following mechanisms may be involved in these changes:-

a) The increase in cholesterol excretion following calcium glycerophosphate may result from an inhibition of coprostanol formation rather than an increased output of endogenous cholesterol. This is based on the finding that total neutral steroid excretion remained unchanged.

b) The significant increase in total neutral steroid excretion on the skimmed milk diet may on the other hand have resulted from an increase in endogenous cholesterol output during this period.

c) The increase in faecal bile acids during both calcium supplementation periods is probably

due to the formation of insoluble calcium salts of bile acids rendering them less readily reabsorbed.

d) The formation of these insoluble bile salts would then also cause an increase in neutral steroid excretion, as soluble bile salts are necessary for optimal cholesterol absorption. (Siperstein et al. 1952).

e) The presence of increased amounts of bile acids in the colon might also inhibit coprostanol formation as has been demonstrated in rats. (Wells, 1957).

f) Reduction in the efficiency of bile salt reabsorption has been shown to increase cholesterol turnover and also cholesterol and bile acid output. (Bergström and Danielsson, 1958).

The six effects listed above may be interrelated in the following way.

Calcium, in the organic forms tested, reacts with bile acids to form insoluble salts which are less easily absorbed than those bile salts

normally present in the intestine. Total bile acid excretion is therefore increased. There are then two secondary effects; firstly, on the bacterial conversion of cholesterol resulting in a decrease in coprostanol formation, and secondly, on the absorption of cholesterol resulting in an increased excretion of endogenous cholesterol.

The increase in faecal fat observed during calcium supplementation can also be explained, partly by a reduction in the availability of bile acids but mainly by the formation of calcium salts of fatty acids. This increase in faecal fat excretion together with the increase in faecal dry weight has been previously reported by Yacowitz *et al.* (1965).

The changes in serum cholesterol and triglyceride levels were not significant. While the concentration of each compound fell towards the end of the calcium glycerophosphate period, they rose again following the introduction of

skimmed milk. The form in which the calcium is given therefore appears to influence its effect on serum cholesterol and triglyceride. In this case, the small rise in fat and carbohydrate intake due to the skimmed milk may have been responsible for the differences. The interval of two weeks between commencement of calcium glycerophosphate and its maximum effect on serum lipids was the same as that for the excretion of faecal lipids (see Figures 22, 23). These results are contrary to the findings of Yacowitz et al. (1965) who gave approximately two and a half times as much calcium and observed a decrease in serum lipid levels after four days. More recently Maibach (1967) found after four weeks of oral calcium (1.5 g. per day) that serum cholesterol levels were not significantly decreased. He also found no significant change in serum beta-lipoproteins or total lipid levels.

The lack of effect of an increased excretion

rate on serum cholesterol concentration may have been due to the dose of calcium administered, which was lower than in other reported studies, or it may have been due to an increased cholesterol synthesis resulting from the malabsorption of the bile acids.

The amount of calcium administered in the present study was far greater than that normally obtained even in the hardest water, and it is difficult to believe that the lower cardiovascular mortality in hard water areas could be due to the reported serum cholesterol lowering effect of calcium.

However, there does appear to be a "water factor" influencing cardiovascular mortality. In Japan (Kobayashi, 1957), United States (Schroeder, 1960), Great Britain (Morris et al. 1961) and Sweden (Biörck et al. 1965) associations have now been shown between local drinking water and cardiovascular disease: the softer the water the higher the mortality.

Studies concerning the constituents of water and cardiovascular disease by Morris et al. (1962) showed that only the calcium content correlated with cardiovascular disease. Parsons et al. (1961) in Tasmania observed marked clinical improvement in patients with coronary artery disease when treated with magnesium sulphate but whether this is due to increased magnesium or sulphate (Schroeder (1960) showed a correlation between various anions such as sulphate, bi-carbonate and fluoride in water and deaths from cardiovascular disease)) is not known.

More recently, Crawford and Crawford (1967) suggested that the higher incidence of deaths from cardiovascular disease in the soft water areas might be due to increased susceptibility of the myocardium in the soft water area. They studied cardiac lesions found in a very soft water area (Glasgow) and in a very hard water area (London) in two comparable series of medico-legal autopsies; men who had died from an

accident and men who had died suddenly and unexpectedly from ischaemic heart disease. In the accident series the prevalence of myocardial scars (healed infarcts) was greater in the soft than in the hard water area. An important finding was that there was more atheroma and more lumen stenosis in the soft water area at ages 30-40 years but there was no difference between the two areas at ages 45-69 years, only "old occlusion" was commoner in the Glasgow area. From their results it appears that there is initially a more rapid development of cardiovascular disease in the soft water area but as time passes the development of the disease in the hard water area progresses until in the older age group there is no difference between the two areas.

The results of this study show that calcium, when taken as calcium glycerophosphate, increases faecal bile acid excretion, faecal fat and faecal cholesterol excretion but total neutral steroid

excretion remains unchanged. It appears therefore that in this form, calcium inhibits the formation of coprostanol without affecting cholesterol absorption. However, when taken as skimmed milk, total neutral steroid excretion increased as did faecal bile acid and faecal fat excretion suggesting that cholesterol absorption was inhibited. Although it appears that calcium has a similar effect on bile acid excretion as cholestyramine, a bile acid binding resin (Bergen et al. 1959), it is not so efficient as cholestyramine in lowering serum cholesterol levels. From these results it appears doubtful that oral calcium supplements can be recommended as a means of reducing serum cholesterol concentrations.

The effect of oral cholesterol on serum cholesterol concentrations

The slight decrease in serum cholesterol level during the low cholesterol diet, in the single patient studied, is in agreement with the findings of Keys et al. (1965) that

decreasing the cholesterol content of the diet has little effect on serum cholesterol concentrations. The increase in serum triglyceride during this period may have been due to the increased carbohydrate and reduced fat intake which occurred during this dietary period (Ahrens et al. 1961). Serum cholesterol changes observed during the high cholesterol diet are also in agreement with those previously reported by Connor et al. (1960, 1964) but are at variance with the findings of Bhattathiry and Siperstein (1963). However, these workers obtained their serum cholesterol values after the subject had been on the diet for only 3 days and the slight increase in serum triglyceride levels reported may have been due to the increased dietary intake of fat.

The marked decrease in daily excretion of faecal neutral steroids was probably due in part to the decreased amount of dietary cholesterol and in part, to the effect this decreased dietary

cholesterol has on cholesterol metabolism.

The decrease in excretion of faecal bile acids suggests that there is either a diminished secretion of bile acids into the gastrointestinal tract or an increased absorption of bile acids from the intestine. As bile acid absorption is normally very efficient, the former suggestion is more likely.

During the high cholesterol diet there will be an excess of cholesterol available for absorption. At present, there are two conflicting theories to account for the way in which man prevents hypercholesterolaemia of dietary origin from occurring. One of these is the negative feedback mechanism (Bhattathiry and Siperstein, 1963). This would result in a decrease in cholesterol synthesis and would be accompanied by an increase in bile acid formation. These two processes would operate in an attempt to maintain the amount of cholesterol being secreted from the liver at the basal level. The other

mechanism is that the human intestine has a limited capacity to absorb cholesterol (Karvinen et al. 1957, Kaplan et al. 1963, Taylor et al. 1965 and Taylor and Ho, 1967). If this were the mechanism, then a marked increase in faecal neutral steroid excretion with no change in faecal bile acid excretion would be observed.

The slight increase in daily faecal neutral steroid excretion and the marked increase in faecal bile excretion in the subject described here suggests that it is the negative feedback mechanism which operates during diets rich in cholesterol. This is further supported by the finding that during the high cholesterol diet less steroid was excreted than ingested whereas on the low cholesterol diet the converse took place.

It would appear therefore that dietary cholesterol has a controlling effect on cholesterol metabolism. It must be re-emphasised, however, that no firm conclusions can be drawn because of

the paucity of data available from the present study.

General discussion

From the results of these investigations it would appear that the most effective means of lowering serum cholesterol levels is by inhibiting the synthesis of cholesterol prior to the cyclisation step.

Decreasing the dietary intake of cholesterol had no appreciable effect on the serum cholesterol level although this increased when the dietary intake of cholesterol was increased. The investigation did show however, that the negative feedback mechanism may be a controlling factor in regulating hypercholesterolaemia of dietary origin. The marked increase in faecal bile acid excretion during calcium administration appeared to influence the faecal excretion of neutral steroids without having a significant effect on the serum cholesterol level. An effect similar to that produced by cholestyramine - increased faecal bile acid excretion

accompanied by a decrease in serum cholesterol level - was anticipated but it may have been that the dose of calcium given was insufficient to promote an increase in faecal bile acid excretion sufficient to overcome the effect of the resulting increase in cholesterol synthesis. From these two investigations it appears that alteration of cholesterol intake or output is not a very effective means of lowering serum cholesterol levels.

Another major route of cholesterol loss from the body is the conversion of cholesterol to bile acids in the liver. Alteration of the glycine/taurine ratio of the conjugated bile acids appeared to be a mechanism whereby serum cholesterol levels could be lowered. However, the alteration of the glycine/taurine ratio by feeding taurine was ineffective and suggests that the increased taurine conjugation of bile acids which accompanies the lowering of serum cholesterol in hypothyroid subjects after

treatment with thyroid hormones is probably an epiphenomenon. This is further supported by the finding that the bile acid conjugation ratio was unaltered by administration of clofibrate even although serum cholesterol levels decreased.

The mechanism of action of clofibrate has not been completely elucidated. It has been suggested that cholesterol synthesis is inhibited by clofibrate and the study of the faecal excretion of neutral steroids and bile acids (Part IV, p. 174) appears to support this theory. Clofibrate was originally thought to act by causing an increase of thyroxine in the liver (see Part IB, p. 59) which resulted in diminished synthesis of cholesterol and triglyceride. From the three investigations involving clofibrate and from the suggested mechanism of action of thyroid hormones on cholesterol metabolism (see Part IB, p. 56) it seems doubtful whether the availability of

thyroid hormone is necessary for the serum cholesterol lowering effect of clofibrate.

From a technical standpoint these investigations also show the effectiveness of thin-layer chromatography in lipid analysis. The quantitative densitometry technique is a particularly useful analytical tool and could be well employed in investigations of various diseases, such as diabetes mellitus, in which there is a disorder of lipid metabolism.

APPENDIX

INDEX TO TABLES

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XL.

CHOLESTEROL

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	537.4	+58.1	91.9	+47.3
W.B.	658.3	-166.8	331.4	-118.9
J.E.	513.6	-81.5	191.4	-60.2
J.G.	387.1	-61.8	72.3	+14.0
M.M.	565.4	-213.5	176.4	-118.4
J. McA.	802.4	-99.1	99.4	-0.3
F.R.	303.4	+147.5	52.2	-28.6
A.K.	816.6	-688.3	85.7	-72.7
M. McD.	304.7	+21.0	59.7	+0.2
M.W.	379.2	+14.3	80.5	-5.1
J.M.	259.7	+171.2	52.4	+10.0
Mean	502.5	-81.7	117.6	-30.3
S.D.	±196.0	N.S.	± 84.8	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLI.

COPROSTANOL

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	3425.1	+196.4	534.6	+306.4
W.B.	1532.3	+35.1	771.4	-93.8
J.E.	1356.0	-117.9	505.3	-129.4
J.G.	1450.0	+192.5	270.7	+164.8
M.M.	1484.1	+858.1	463.0	-77.0
J. McA.	2112.4	-693.4	261.8	-61.9
F.R.	2904.5	+292.4	499.5	-332.5
A.K.	1110.2	-373.1	144.0	-69.6
M. McD.	1892.6	+612.3	337.5	+79.1
M.W.	1130.5	-63.5	240.0	-35.5
J.M.	1686.9	+107.6	340.2	-63.7
Mean	1825.9	+95.1	397.1	-28.5
S.D.	± 733.8	N.S.	±177.5	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLII.

COPROSTANONE

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	521.0	-57.3	82.9	+28.1
W.B.	925.0	-105.8	465.7	-131.5
J.E.	152.4	-78.3	56.8	-34.3
J.G.	755.5	-477.9	141.0	-67.4
M.M.	373.1	-373.1	116.4	-116.4
J. McA.	144.0	+95.0	17.9	+15.8
F.R.	155.6	+129.8	26.8	-11.9
A.K.	541.4	-356.7	60.6	-41.9
M. McD.	245.8	+110.6	39.5	+24.9
M.W.	132.3	-132.3	28.1	-28.1
J.M.	346.3	+43.6	69.8	-9.8
Mean	390.2	-109.3	100.5	-33.9
S.D.	±268.6	N.S.	±127.0	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLIII.

TOTAL NEUTRAL STEROIDS

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	4483.4	+197.3	709.4	+381.8
W.B.	3115.6	-237.5	1568.5	-344.2
J.E.	2022.0	-277.7	753.5	-223.9
J.G.	2592.6	-347.2	484.0	+111.4
M.M.	2422.6	+271.5	755.8	-315.8
J. McA.	3058.8	-697.5	379.1	-46.4
F.R.	3363.5	+569.7	578.5	-372.8
A.K.	2468.2	-1418.1	290.2	-184.1
M. McD.	2443.1	+743.9	436.7	+102.2
M.W.	1642.0	-181.5	348.6	-68.7
J.M.	2292.9	+322.4	462.4	-58.5
Mean	2718.6	-95.9	615.2	-92.6
S.D.	±766.2	N.S.	±355.4	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLIV.

DEOXYCHOLIC ACID

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P	207.4	-30.2	33.9	+6.4
W.B.	77.7	-1.3	39.1	-6.0
J.E.	0	+44.4	0	+13.6
J.G.	360.7	-98.7	67.3	+2.2
M.M.	221.4	-158.8	69.3	-59.0
J. McA.	797.3	-371.6	98.8	-38.8
F.R.	239.6	-7.4	41.2	-29.0
A.K.	197.1	-21.4	21.6	+1.6
M. McD.	202.7	-202.7	31.2	-31.2
M.W.	348.2	-129.6	73.9	-32.0
J.M.	217.6	-29.9	43.9	-14.9
Mean	260.9	-91.6*	47.3	-17.0*
S.D.	±205.2	$p < 0.05$	±27.7	$p < 0.05$

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLV.

LITHOCHOLIC ACID

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	127.1	-8.3	20.5	+6.4
W.B.	70.9	-19.1	35.7	-13.3
J.E.	0	+38.2	0	+11.6
J.G.	374.8	-76.4	70.0	+9.1
M.M.	81.7	-64.2	25.6	-22.7
J. McA.	241.7	-158.6	30.0	-18.3
F.R.	194.5	+42.4	33.5	-21.1
A.K.	279.3	-154.2	32.4	-19.8
M. McD.	72.0	+8.4	10.7	-0.7
M.W.	156.2	-112.5	33.2	-24.7
J.M.	166.7	-40.5	33.6	-14.5
Mean	160.5	-49.5*	29.6	-9.8*
S.D.	±108.1	$p < 0.05$	±17.5	$p < 0.05$

Table XLVI. The effect of clofibrate 1 g. daily for 9 - 15 days.

	TOTAL BILE ACIDS			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	334.4	-38.4	54.4	+12.7
W.B.	148.6	-20.3	74.8	-19.3
J.E.	0	+82.6	0	+25.2
J.G.	735.5	-175.1	137.3	+11.3
M.M.	303.1	-223.0	94.9	-81.7
J. McA.	1039.0	-530.2	128.8	-57.1
F.R.	434.1	+35.0	74.7	-50.1
A.K.	476.4	-175.6	54.0	-18.2
M. McD.	274.7	-194.3	41.9	-13.9
M.W.	504.4	-242.1	107.1	-56.8
J.M.	384.3	-70.4	77.5	-29.4
Mean	421.3	-141.1*	76.9	-25.2*
S.D.	±283.6	$p < 0.05$	±39.7	$p < 0.05$

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLVII.

WEIGHT OF FAECES

	g./24 hr. dry faeces		g./24 hr. wet faeces	
Subject	Initial level	Change	Initial level	Change
J.P.	16.3	+7.0	80.0	+13.0
W.B.	50.3	-7.1	187.0	+12.0
J.E.	37.3	-6.9	87.0	+60.0
J.G.	18.7	+7.8	66.0	+106.0
M.M.	31.3	-14.8	64.0	-33.0
J. McA.	12.4	+1.7	42.0	-1.0
F.R.	17.2	-12.0	51.0	-26.0
A.K.	13.4	-3.3	37.0	-1.0
M. McD.	18.5	-0.2	86.0	+1.0
M.W.	21.2	-2.0	152.0	+7.0
J.M.	20.2	-5.0	88.0	-24.0
Mean	23.3	-3.2	85.5	+10.4
S.D.	±11.6	N.S.	±45.9	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLVIII.

FAECAL FAT

	g. /100 g. dry faeces		g./24 hr.	
Subject	Initial level	Change	Initial level	Change
J.P.	8.31	+0.11	1.35	+0.76
W.B.	6.59	+0.12	3.32	-0.42
J.E.	5.23	-0.61	1.95	-0.55
J.G.	6.38	+3.25	1.19	+1.36
M.M.	6.97	+7.32	2.18	+0.17
J. McA.	6.21	+3.07	0.77	+0.54
F.R.	7.47	+0.01	1.29	-0.90
A.K.	10.47	-5.66	1.76	-1.27
M. McD.	8.44	-0.44	1.58	-0.13
M.W.	6.37	-1.75	1.35	-0.47
J.M.	7.48	+0.36	1.52	-0.33
Mean	7.27	+0.53	1.66	-0.11
S.D.	±1.42	N.S.	±0.67	N.S.

The effect of clofibrate 1 g. daily for 9 - 15 days

Table XLIX.

SERUM LIPIDS

	CHOLESTEROL (mg./100 ml.)		TRIGLYCERIDE (mg./100 ml.)	
Subject	Initial level	Change	Initial level	Change
J.P.	303.0	-65.0	179.0	-43.0
W.B.	248.0	-38.0	188.0	-89.0
J.E.	216.0	-40.0	71.0	-37.0
J.G.	320.0	-46.0	126.0	-71.0
M.M.	370.0	-130.0	93.0	-26.0
J. McA.	299.0	-75.0	81.0	-33.0
F.R.	274.0	-39.0	143.0	-52.0
A.K.	243.0	-15.0	125.0	-39.0
M. McD.	293.0	-14.0	110.0	-6.0
M.W.	400.0	-87.0	127.0	-1.0
J.M.	369.0	+3.0	187.0	-52.0
Mean	303.2	-49.6**	130.0	-40.8**
S.D.	±58.1	$p < 0.01$	±41.1	$p < 0.01$

The effect of clofibrate 2 g. daily for 9 - 15 days

Table L.

CHOLESTEROL

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	306.6	-27.5	98.5	+22.3
R.M.	0	0	0	0
J.H.	730.6	-384.4	307.1	-116.5
A.C.	164.7	+223.3	18.7	+31.8
E.D.	333.6	-220.0	175.0	-56.0
D.M.	305.3	+246.8	250.4	+480.8
M.D.	128.9	+6.8	51.3	+65.5
J.D.	374.8	+83.5	185.3	+352.7
M.R.	369.6	+407.1	90.1	+169.4
T.M.	148.7	+112.7	61.2	+42.6
Mean	286.3	+44.8	123.8	+99.3
S.D.	±198.8	N.S.	±101.8	N.S.

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LI.

COPROSTANOL

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	2162.1	+511.4	694.5	+461.5
R.M.	860.0	+353.4	302.3	+190.0
J.H.	1943.1	-76.2	816.8	+210.7
A.C.	3414.7	-1104.1	388.2	-100.5
E.D.	1284.9	-52.9	674.0	+616.0
D.M.	1106.8	-411.6	907.7	+13.0
M.D.	1318.0	-348.7	524.3	+309.7
J.D.	1015.1	-324.7	501.9	+308.5
M.R.	2196.6	-669.9	535.4	-25.4
T.M.	1132.8	-69.3	466.3	-46.7
Mean	1643.4	-219.3	581.1	+193.7*
S.D.	±788.4	N.S.	±189.6	$p < 0.05$

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LII

COPROSTANONE

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	230.0	+680.8	73.9	+320.1
R.M.	0	0	0	0
J.H.	1121.6	+73.2	471.5	+186.1
A.C.	160.0	+25.3	18.2	+11.4
E.D.	0	+135.6	0	+142.0
D.M.	553.4	-553.4	453.9	-453.9
M.D.	0	+226.1	0	+194.6
J.D.	321.2	-321.2	158.9	-158.9
M.R.	147.8	+298.6	36.0	+113.1
T.M.	225.8	+208.5	93.0	+78.4
Mean	276.0	+77.4	130.5	+43.3
S.D.	±342.8	N.S.	±57.6	N.S.

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LIII.

TOTAL NEUTRAL STEROIDS

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	2698.7	+1164.7	866.9	+803.9
R.M.	860.0	+353.4	302.3	+190.0
J.H	3795,3	-387.4	1595.4	+280.3
A.C.	3739.4	-855.4	425.1	-37.3
E.D.	1618.5	-137.3	849.0	+702.0
D.M.	1965.5	-718.2	1612.0	+39.9
M.D.	1446.9	-115.8	575.7	+569.7
J.D.	1711.1	-562.4	856.1	+492.3
M.R.	2714.0	+35.8	661.5	+257.1
T.M.	1507.3	+252.0	620.5	+74.2
Mean	2205.7	-97.1	836.5	$+337.2^{**}$
S.D.	$\pm$994.5	N.S.	$\pm$140.6	$p < 0.01$

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LIV.

DEOXYCHOLIC ACID

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Charge	Initial level	Change
A.W.	260.6	-180.2	85.2	-51.3
R.M.	67.6	-7.8	23.8	+0.5
J.H.	371.7	-113.4	156.3	-14.1
A.C.	195.2	-10.3	22.2	+3.8
E.D.	103.7	-39.9	54.0	-7.1
D.M.	274.8	-15.8	225.4	+117.6
M.D.	65.3	-14.6	26.0	-4.5
J.D.	51.4	+4.8	25.4	+40.6
M.R.	264.5	-60.3	64.5	+3.7
T.M.	175.1	-92.7	72.1	-39.5
Mean	183.0	-53.0^{+}	75.5	+5.0
S.D.	±109.5	$p < 0.02$	±66.8	N.S.

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LV.

LITHOCHOLIC ACID

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	65.7	-65.7	23.1	-23.1
R.M.	87.2	-71.7	30.7	-18.0
J.H.	291.7	-70.3	122.6	-0.7
A.C.	140.0	+164.2	15.9	+24.3
E.D.	43.8	+1.2	23.4	+9.6
D.M.	71.1	-71.1	58.4	-58.4
M.D.	20.9	-20.9	8.4	-8.4
J.D.	26.7	-26.7	13.2	-13.2
M.R.	196.6	-62.7	47.9	-3.2
T.M.	139.6	-59.3	57.5	-25.7
Mean	108.3	-28.3	40.1	-11.7
S.D.	±85.3	N.S.	±34.1	N.S.

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LVI.

	TOTAL BILE ACIDS			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	326.3	-245.9	108.3	-73.4
R.M.	154.8	-79.5	54.5	-17.5
J.H.	663.4	-183.7	278.9	-14.8
A.C.	335.2	+153.9	38.1	+28.1
E.D.	147.5	-38.7	77.4	+2.5
D.M.	345.9	+43.6(1)	283.8	+221.6(2)
M.D.	86.2	-35.5	34.4	-12.9
J.D.	78.1	-21.9	38.6	+27.4
M.R.	464.1	-126.0	112.4	+0.5
T.M.	314.7	-151.8	129.6	-65.2
Mean	291.6	-68.6	115.6	+9.6
S.D.	±183.1	N.S.	±93.7	N.S.

(1) D.M. excreted 130.5 mg. cholic acid/100 g. dry faeces in this specimen during clofibrate.

(2) D.M. excreted 162.4 mg. cholic acid/24 hrs. in this specimen during clofibrate.

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LVII.

WEIGHT OF FAECES

	g./24 hr. dry faeces		g./24 hr. wet faeces	
Subject	Initial level	Change	Initial level	Change
A.W.	32.1	+11.1	70.0	-7.0
R.M.	35.0	+5.3	64.3	+26.4
J.H.	42.0	+13.0	88.3	+45.0
A.C.	11.4	+0.1	43.3	+54.1
E.D.	52.5	+52.2	56.7	+63.3
D.M.	82.0	+50.0	92.7	+120.6
M.D.	39.9	+46.1	41.0	+80.0
J.D.	49.4	+68.0	53.6	+88.7
M.R.	24.4	+9.0	52.7	+16.6
T.M.	41.2	-1.5	82.7	+16.0
Mean	41.0	$+25.3^{+}$	64.5	$+50.4^{**}$
S.D.	± 18.7	$p < 0.02$	± 18.4	$p < 0.01$

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LVIII.

FAECAL FAT

	g./100 g. dry faeces		g./24 hr.	
Subject	Initial level	Change	Initial level	Change
A.W.	7.26	+4.34	2.32	+2.68
R.M.	6.08	-0.65	2.00	+0.20
J.H.	8.46	-0.39	3.56	+0.88
E.D.	6.07	-1.23	3.18	+1.88
D.M.	4.30	0	3.53	+2.15
M.D.	4.75	-0.34	1.03	+2.77
J.D.	4.03	-0.08	1.86	+2.78
M.R.	5.55	-0.39	1.35	+0.37
T.M.	4.07	+1.10	1.67	+0.38
Mean	5.62	+0.26	2.28	+1.57**
S.D.	±1.54	N.S.	±0.94	p < 0.01

The effect of clofibrate 2 g. daily for 9 - 15 days

Table LIX.

SERUM LIPIDS

	CHOLESTEROL (mg./100 ml)		TRIGLYCERIDE (mg./100 ml.)	
Subject	Initial level	Change	Initial level	Change
A.W.	258.0	-11.0	150.0	+8.0
R.M.	220.0	-33.0	67.0	+4.0
J.H.	252.0	+41.0	154.5	-31.5
A.C.	293.0	-50.0	147.0	-52.5
E.D.	264.0	+9.0	247.0	+5.0
D.M.	154.0	-9.0	33.0	-1.0
M.D.	174.0	-2.0	51.0	-26.0
J.D.	230.0	-39.0	87.0	-20.0
M.R.	351.0	-22.0	93.0	-29.0
T.M.	210.0	-31.0	70.0	-22.0
Mean	240.6	-14.7	109.9	-16.5*
S.D.	±57.1	N.S.	±64.5	$p < 0.05$

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LX.

NEUTRAL STEROIDS

COMPOUND	mg./100 g. dry faeces		mg./24 hr.	
	Mean basal level	Change	Mean basal level	Change
CHOLESTEROL	399.5 ±221.9	-21.5 N.S.	120.5 ±91.0	+31.4 N.S.
COPROSTANOL	1739.0 ±746.8	-54.6 N.S.	484.7 ±202.0	+77.3 N.S.
COPROSTANONE	335.8 +303.9	-20.4 N.S.	114.8 ±152.5	+2.9 N.S.
TOTAL NEUTRAL STEROIDS	2474.3 ±898.6	-96.5 N.S.	720.0 ±406.1	+112.1 N.S.

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LXI.

BILE ACIDS

COMPOUND	mg./100 g. dry faeces		mg./24 hr.	
	Mean basal level	Change	Mean basal level	Change
DEOXYCHOLIC ACID	223.7 ±167.4	-73.2** p < 0.01	60.7 ±51.0	-6.5 N.S.
LITHOCHOLIC ACID	135.6 ±99.1	-39.4+ p < 0.02	34.6 ±26.6	-10.7+ p < 0.02
TOTAL BILE ACIDS	359.3 ±243.7	-106.4(1)** p < 0.01	95.3 ±71.6	-8.6(2) N.S.

(1) includes cholic acid 130.5 mg./100 g. dry faeces in subject D.M.

(2) includes cholic acid 162.4 mg./24 hr. in subject D.M.

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LXII.

FAECAL WEIGHT

	Dry faeces		Wet faeces	
	Mean basal level	Change	Mean basal level	Change
Faeces g./24 hr.	31.7 ±17.5	+10.4 N.S.	75.5 ±36.3	+29.4** p < 0.01

Table LXIII.

FAECAL FAT

	g./100 g. dry faeces		g./24 hr.	
	Mean basal level	Change	Mean basal level	Change
Faecal fat	6.53 ±2.16	+0.41 N.S.	1.94 ±0.84	+0.64* p < 0.05

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LXIV.

SERUM LIPIDS			
CHOLESTEROL (mg./100 ml.)		TRIGLYCERIDE (mg./100 ml.)	
Mean basal level	Change	Mean basal level	Change
273.3	-33.0***	120.4	-29.2***
±64.6	p < 0.001	±53.1	p < 0.001

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LXV.

NUMBER OF STOOLS/DAY

Subject	Mean basal level	Change in 1st 7 days	Change in 2nd 7 days
T.M.	0.60	+0.40	-0.18
J.D.	0.60	+0.54	+0.65
J.H.	0.80	+0.34	+0.05
A.W.	0.60	+0.25	+0.40
R.M.	1.00	0	-0.34
E.D.	0.60	-0.03	-0.18
A.C.	0.80	-0.09	-0.09
M.R.	1.00	-0.29	-0.50
W.B.	1.25	+0.03	-0.25
A.K.	0.40	+0.45	+0.45
M. McD.	0.80	+0.05	-0.09
M.M.	0.60	-0.03	+0.06
J.P.	0.80	-0.09	+0.48
M.W.	1.00	0	+0.28
J.G.	0.80	+0.34	-0.20
J. McA.	0.67	+0.47	-0.07
F.R.	1.00	+0.14	0
J.M.	0.60	+0.54	+0.97
Mean	0.77	$+0.168^{+}$	+0.08
S.D.	$\pm$0.21	$p < 0.02$	N.S.

Combined results of the two groups treated with clofibrate, 1 g. or 2 g. daily for 9 - 15 days

Table LXVI. The effect of clofibrate in males and females.

COMPOUND	Mean Change MALES	FEMALES	CHANGE
Neutral steroids mg./100 g. dry faeces	+62.6	-387.5	N.S.
Neutral steroids mg./24 hr.	+265.4	+26.6	N.S.
Bile Acids mg./100 g. dry faeces	-96.2	-126.4	N.S.
Bile acids mg./24 hr.	+30.5	-18.2	N.S.
Serum cholesterol mg./100 ml.	-18.1	-57.0	$p < 0.05$
Serum triglyceride mg./100 ml.	-29.9	-28.4	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXVII.

	CHOLESTEROL			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	658.3	+179.8	331.4	-11.5
J.G.	387.1	-147.1	72.3	+9.8
M.M.	565.4	-214.9	176.4	-119.7
J. McA.	802.4	-402.4	99.4	-42.6
F.R.	303.4	-104.5	52.2	-14.3
A.K.	816.6	-690.1	85.7	-63.6
M. McD.	304.7	-99.6	59.7	-29.4
J.P.	537.4	-408.5	91.9	-67.0
M.W.	379.2	+251.4	80.5	+14.3
J.M.	259.7	-61.7	52.4	-14.7
Mean	501.4	-169.8	110.2	-33.9*
S.D.	±206.6	N.S.	±85.8	$p < 0.05$

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXVIII.

	COPROSTANOL			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	1532.3	-170.0	771.4	-243.0
J.G.	1450.0	+117.0	270.7	+265.1
M.M.	1484.1	+2083.1	463.0	+114.0
J. McA.	2112.4	-259.8	261.8	+1.3
F.R.	2904.5	-562.0	499.5	-52.6
A.K.	1110.2	+301.5	144.0	+102.6
M. McD.	1892.6	-669.0	337.5	-157.0
J.P.	3425.1	-1878.7	534.6	-278.5
M.W.	1130.5	+375.1	240.0	-13.8
J.M.	1686.9	+266.3	340.2	+31.8
Mean	1872.9	-39.7	386.3	-23.0
S.D.	±755.9	N.S.	±183.3	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXIX

COPROSTANONE

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	925.0	-36.9	465.7	-127.5
J.G.	755.5	-517.7	141.0	-59.7
M.M.	373.1	-373.1	116.4	-116.4
J. McA.	144.0	+48.9	17.9	+9.5
F.R.	155.6	-6.5	26.8	+1.7
A.K.	541.4	-21.3	60.6	+30.2
M. McD.	245.8	+43.1	39.5	+3.1
J.P.	521.0	+46.2	82.9	+32.1
M.W.	132.3	+526.7	28.1	+70.9
J.M.	346.3	-67.3	69.8	-16.7
Mean	414.0	-35.8	104.9	-17.3
S.D.	±270.6	N.S.	±133.0	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXX.

	TOTAL NEUTRAL STEROIDS			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	3115.6	-27.1	1568.5	-382.0
J.G.	2592.6	-557.8	484.0	+215.2
M.M.	2422.6	+1495.1	755.8	-122.1
J. McA.	3058.8	-613.3	379.1	-31.8
F.R.	3363.5	-673.0	578.5	-65.2
A.K.	2468.2	-409.9	290.2	+69.3
M. McD.	2443.1	-726.5	436.7	-183.3
J.P.	4483.4	-2241.0	709.4	-313.5
M.W.	1642.0	+1153.2	348.6	+61.4
J.M.	2292.9	+137.3	462.4	+0.4
Mean	2788.3	-246.3	601.3	-75.2
S.D.	±770.1	N.S.	±371.5	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXI.

	DEOXYCHOLIC ACID			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	77.7	-30.3	39.1	-20.4
J.G.	360.7	-77.2	67.3	+29.6
M.M.	221.4	-165.8	69.3	-60.3
J. McA.	797.3	-299.1	98.8	-28.0
F.R.	239.6	+6.2	41.2	+5.7
A.K.	197.1	+62.9	21.6	+23.8
M. McD.	151.4	-21.0	31.2	-11.9
J.P.	207.4	-11.6	33.9	-2.9
M.W.	348.2	-230.8	73.9	-56.3
J.M.	217.6	-103.3	43.9	-22.1
Mean	281.8	-87.0*	52.0	-14.3
S.D.	±199.3	$p < 0.05$	±24.1	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXII.

	LITHOCHOLIC ACID			
	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	70.9	-34.8	35.7	-15.2
J.G.	374.8	-162.7	70.0	+2.6
M.M.	81.7	-75.6	25.6	-24.6
J. McA.	241.7	-118.9	30.0	-12.6
F.R.	194.5	-15.9	33.5	+0.6
A.K.	279.3	-215.3	32.4	-21.1
M. McD.	72.0	-25.3	10.7	-3.8
J.P.	127.1	-53.7	20.5	-9.0
M.W.	156.2	-72.0	33.2	-20.5
J.M.	166.7	-98.3	33.6	-20.6
Mean	176.5	-87.3^{**}	32.5	-12.4^{**}
S.D.	± 99.2	$p < 0.01$	± 15.3	$p < 0.01$

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXIII.

TOTAL BILE ACIDS

	mg./100 g. dry faeces		mg./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	148.6	-45.1	74.8	+35.6
J.G.	735.5	-239.9	137.3	+32.2
M.M.	303.1	-241.4	94.9	-84.9
J. McA.	1039.0	-418.0	128.8	-40.6
F.R.	434.1	-9.7	74.7	+6.3
A.K.	476.4	-152.4	54.0	+2.7
M. McD.	223.3	-46.2	41.9	-15.7
J.P.	334.4	-65.7	54.4	-11.9
M.W.	504.4	-302.8	107.1	-76.8
J.M.	384.3	-201.6	77.5	-42.7
Mean	458.3	-172.3^{**}	84.5	-26.7^{*}
S.D.	± 281.5	$p < 0.01$	± 32.1	$p < 0.05$

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXIV.

	WEIGHT OF FAECES			
	g./24 hr. dry faeces		g./24 hr. wet faeces	
Subject	Initial level	Change	Initial level	Change
W.B.	50.3	-11.5	187.0	-23.0
J.G.	18.7	+15.5	66.0	+58.0
M.M.	31.3	-15.1	64.0	-20.0
J. McA.	12.4	+1.8	42.0	-4.0
F.R.	17.2	+1.9	51.0	-16.0
A.K.	13.4	+4.1	37.0	+25.0
M. McD.	18.5	-3.7	85.0	-24.0
J.P.	16.3	+1.3	79.0	-14.0
M.W.	21.2	-6.2	151.0	-54.0
J.M.	20.2	-1.2	88.0	-19.0
Mean	22.0	-1.3	85.0	-9.1
S.D.	±11.2	N.S.	±48.2	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXV.

FAECAL FAT

	g./100 g. dry faeces		g./24 hr.	
Subject	Initial level	Change	Initial level	Change
W.B.	6.59	+1.75	3.32	-0.10
J.G.	6.38	+0.54	1.19	+1.18
M.M.	6.97	+1.94	2.18	-0.74
J. McA.	6.21	+2.41	0.77	+0.46
F.R.	7.47	-1.04	1.29	-0.06
A.K.	10.47	-5.58	1.76	-0.90
M. McD.	8.44	-1.26	1.58	-0.51
J.P.	8.31	+8.54	1.38	+1.52
M.W.	6.37	-0.84	1.38	-0.53
J.M.	7.48	-0.61	1.52	-0.27
Mean	7.47	+0.59	1.64	+0.01
S.D.	±1.32	N.S.	±0.70	N.S.

The prolonged effect of clofibrate (1.5 g./day or 2 g./day)

Table LXXVI.

SERUM LIPIDS

	CHOLESTEROL (mg./100 ml.)		TRIGLYCERIDE (mg./100 ml.)	
Subject	Initial level	Change	Initial level	Change
W.B.	248.0	+71.0	188.0	+17.0
J.G.	320.0	-54.0	126.0	-70.0
M.M.	370.0	-127.0	93.0	-6.0
J. McA.	299.0	-14.0	81.0	-28.0
F.R.	274.0	+29.0	143.0	-41.0
A.K.	243.0	+10.0	125.0	-53.0
M. McD.	293.0	-83.0	110.0	-21.0
J.P.	303.0	-22.0	179.0	+206.0
M.W.	400.0	-56.0	127.0	+7.0
J.M.	369.0	-65.0	187.0	-68.0
Mean	311.9	-31.1	135.9	-5.7
S.D.	±53.1	N.S.	±38.1	N.S.

BIBLIOGRAPHY

ABELL, L.L., B.B. LEVY, B.B. BRODIE and F.E. KENDALL. J. biol. Chem. 1952, 195, 357.

ACHESON, J. and E.C. HUTCHINSON. J. Atheroscler. Res. 1963, 3, 711.

AHRENS, E.H. (Jr.), J. HIRSCH, W. INSULL (Jr.), T.T. TSALTAS, R. BLOMSTRAND and M.L. PETERSON. Lancet 1957, 1, 943.

AHRENS, E.H. (Jr.), J. HIRSCH, K. OETTE, J.W. FARQUHAR and Y. STEIN. Trans. Ass. Amer. Phycns. 1961, 74, 134.

ALBRINK, M.J., J.W. MEIGS and M.A. GRANOFF. New Engl. J. Med. 1962, 266, 484.

ALTSCHUL, R., A. HOFFER and J.D. STEPHEN. Arch. Biochem. Biophys. 1955, 54, 558.

ANKER, H.S. and K. BLOCH. J. biol. Chem. 1949, 178, 971.

ANTONIS, A. and I. BERSOHN. Lancet 1961, 1, 3.

ANTONIS, A. and I. BERSOHN. Amer. J. clin. Nutr. 1962, 11, 142.

ATROMID, LIPIDS and THROMBOSIS. Lancet, 1963, 1, 1360.

AVIGAN, J., DE WITT S. GOODMAN and D. STEINBERG. J. Lipid Res. 1963, 4, 100.

AVIGAN, J., D. STEINBERG, M.J. THOMPSON and E. MOSETTIG. Biochem. Biophys. Res. Communs. 1960(a), 2, 63.

AVIGAN, J., D. STEINBERG, H.E. VROMAN, M.J. THOMPSON and E. MOSETTIG. J. biol. Chem. 1960(b) 235, 3123.

AVOY, D., E.A. SWYRYD and R.G. GOULD. J. Lipid Res. 1965, 6, 369.

AZARNOFF, D.L. and D.R. TUCKER. Fed. Proc. 1964, 23, (part 1) 552.

AZARNOFF, D.L., D.R. TUCKER and G.A. BARR. Metabolism 1965, 14, 959.

BAKER, R.D. and G.W. SEARLE. Proc. Soc. Exper. Biol. Med. 1960, 105, 521.

BARRETT, C.B., M.S.J. DALLAS and F.B. PADLEY, Chem. Ind. 1962, 1050.

BARRETT, C.B., M.S.J. DALLAS and F.B. PADLEY. J. Amer. Oil Chem. Soc. 1963, 40, 580.

BEHER, W.T., W.L. ANTHONY and G.D. BAKER. Proc. Soc. Exptl. Biol. Med. 1957, 95, 734.

BEIERWALTES, W.H. and G.E. RUFF. Arch. intern. Med. 1958, 101, 569.

BENNETT, R.D. and E. HEFTMANN. J. Chromatog. 1962, 9, 359.

BERGEN, S.S. (Jr.), T.B. van ITALLIE, D.M. TENNENT and W.H. SEBRELL. Proc. Soc. Exptl. Biol. Med. 1959, 102, 676.

BERGSTRÖM, S. Ciba Foundation Symposium on Biosynthesis of Terpenes and Sterols. J.A. Churchill Ltd., London, 1959.

BERGSTRÖM, S. and H. DANIELSSON. Acta Physiol. Scand. 1958, 43, 1.

BERGSTROM, S., K. PAABO and J.A. RUMPF. Acta chem. Scand. 1954, 8, 1109.

BERKOWITZ, D. J. Atheroscler. Res. 1963 (a), 3, 538.

BERKOWITZ, D. Circulation 1963(b), 28, 648.

BERSÉUS, O. and H. DANIELSSON. Acta. chem. Scand. 1963, 17, 1293.

BEST, M.M. and C.H. DUNCAN. J.A.M.A. 1964, 187, 37.

BEST, M.M. and C.H. DUNCAN. Arch. intern. Med. 1966, 118, 97.

BEVERIDGE, J.M.R. Canad. J. Biochem. Phys. 1957, 35, 257.

BHATTATHIRY, E.P.M. and M.D. SIPERSTEIN. J. clin. Invest. 1963, 42, 1613.

BIGGS. M.W., M. FRIEDMAN and S.O. BYERS. Proc. Soc. Exptl. Biol. Med. 1951, 78, 641.

BIORCK, G., H. BOSTROM and A. WIDSTROM. Acta. Med. Scand. 1965, 178, 239.

BLOCH, K., B.N. BERG and D. RITTENBERG. J. biol. Chem. 1943, 149, 511.

BLOCH, K. and D. RITTENBERG. J. Biol. Chem. 1942(a), 143, 297.

BLOCH, K. and D. RITTENBERG. J. Biol. Chem. 1942(b), 145, 625.

BLOHM, T.R. and R.D. MACKENZIE. Arch. Biochem. Biophys. 1959, 85, 245.

BLOOMFIELD, D.K. J. Lab. clin. Med. 1964(a), 64, 613.

BLOOMFIELD, D.K. Clin Research 1964(b), 12, 263.

BOLLMAN, J.L. and E.V. FLOCK. Amer. J. Physiol. 1951, 164, 480.

BORGSTRÖM, B. J. clin. Invest. 1960, 39, 809.

BOYD, G.S. and M.F. OLIVER. Brit. med. Bull. 1960, 16, 138.

BREMER, J. Biochem. J. 1956, 63, 507.

BRIGGS, T. and E. STAPLE. Fed. Proc. 1960, 19, 239.

BROOKS, C.J.W. The Society for Analytical Chemistry, 1962, Monograph No. 2. p. 18.

BROWN, J.L. and J.M. JOHNSTON. J. Lipid Res. 1962, 3, 480.

BYERS, S.O. and M.W. BIGGS. Arch. Biochem. Biophys. 1952, 39, 301.

BYERS, S.O., R.H. ROSENMAN, M. FRIEDMAN and M.W. BIGGS. J. exptl. Med. 1952, 96, 513.

CAREY, J.B. (Jr.). J. Lab. clin. Med. 1960, 56, 797.

CAREY, J.B. (Jr.) and G. WILLIAMS. J.A.M.A. 1961, 176, 432.

CAREY, J.B. (Jr.) and G. WILLIAMS. J. Lab. clin. Med. 1962, 60, 865.

CAREY, J.B. (Jr.) and G. WILLIAMS. J. clin. Invest. 1963, 42, 450.

CARGILL, D.I. The Analyst. 1962, 87, 865.

CARLSON, L.A., B. HÖGSTEDT and L. ORÖ. J. Atheroscler. Res. 1963, 3, 467.

CHAIKOFF, I.L., B. BLOOM, M.D. SIPERSTEIN, J.Y. KIYASU, W.D. REINHARDT, W.G. DAUBEN and J.F. EASTHAM. J. biol. Chem. 1952, 194, 407.

CHALVARDJIAN, A.M. Biochem. J. 1964, 90, 518.

CHALVARDJIAN, A., L.J. MORRIS and R.T. HOLMAN. J. Nutr. 1962, 76, 52.

CHEVREUL, M.E. Ann. Chim. 1815, 95, 5-50. see "Cholesterol" R.P. Cook. Academic Press Inc. New York, 1958 p. 2.

CHEVREUL, M.E. Mem. Musll. Hist. Nat. Paris 1824, 11, 239.

CLAUDE, J.R. J. Chromatog. 1965, 17, 596.

CLAYTON, R.B. Quarterly Reviews Chem. Soc. London, 1965, 19, 168.

COLEMAN, D.L. and C.A. BAUMANN. Arch. Biochem. Biophys. 1957, 66, 226.

CONNOR, W.E., R.E. HODGES and R. BLEILER. Circulation 1960, 22, 735.

CONNOR, W.E., R.E. HODGES and R.E. BLEILER. J. Lab. clin. Med. 1961, 57, 331.

CONNOR, W.E., D.B. STONE and R.E. HODGES. J. clin. Invest. 1964, 43, 1691.

COOK, R.P., D.C. EDWARDS and C. RIDDELL. Biochem. J. 1956, 62, 225.

COOK, R.P. "Cholesterol" Academic Press Inc., New York, 1958(a), p. 174.

COOK, R.P. "Cholesterol" Academic Press Inc., New York, 1958 (b), p.244.

COOK, R.P. (ed.) "Cholesterol", Academic Press Inc., New York, 1958(c), p. 289.

COPIUS-PEEREBOOM, J.W. and H.W. BEEKES. J. Chromatog. 1965, 17, 99.

COREY, E.J., W.E. RUSSEY, P.R. ORTIZ de MONTELLANO. J. Amer. Chem. Soc. 1966, 88, 4750.

COUERBE, J.P. Ann. chim. et phys. 1834, 56, 160. See "Cholesterol" R.P. Cook. Academic Press Inc. New York, 1958, p. 2.

COUNIHAN, T.B. and P. KEELAN. J. Atheroscler. Res. 1963, 3, 580.

COUNSELL, R. E., P.D. KLIMSTRA, R.E. RANNEY and D.L. COOK. J. med. pharm. Chem. 1962(a) 5, 720.

COUNSELL, R. E., P.D. KLIMSTRA and R.E. RANNEY. J. med. pharm. Chem. 1962(b) 5, 1224.

CORNFORTH, J.W. J. Lipid Res. 1959, 1, 3.

CRAMÉR, K. J. Atheroscler. Res. 1963, 3, 500.

CRAWFORD, T. and M.D. CRAWFORD. Lancet 1967, 1, 229.

CURRAN, G.L. and R.L. COSTELLO. Proc. Soc. Exptl. Biol. Med. 1956, 91, 52.

DANIELSSON, H. Acta. chem. Scand. 1960, 14, 348.

DANIELSSON, H. Arkiv. Kemi. 1961(a), 17, 373.

DANIELSSON, H. Arkiv. Kemi. 1961(b), 17, 363.

DANIELSSON, H., P. ENEROTH, K. HELLSTRÖM, S. LINDSTEDT, and J. SJÖVALL. J. biol. Chem. 1963, 238, 2299.

DANIELSSON, H. and B. GUSTAFSSON - Arch. Biochem. Biophys. 1959, 83, 482.

DANOWSKI, T.S. and R. ALLEY. Lancet 1967, 1, 854.

DAVID, J.S.K., P. MALATHI and J. GANGULY. Biochem. J. 1966, 98, 662.

DAVIES, B.H., D. JONES and T.W. GOODWIN. Biochem. J. 1963, 87, 326.

DAWBER, T.R., W.B. KANNEL, N. REVOTSKIE and A. KAGAN. Proc. roy. Soc. Med. 1962, 55, 265.

DE FOURCROY. Ann. chim. 1789, 3, 242. see "Cholesterol" R.P. Cook. Academic Press Inc. New York, 1958, p.1.

DENBOROUGH, M.A., R.R.H. LOVELL and G. TREVAKS. Med. J. Aust. 1963, 1, 498.

DE VRIES, B. Chem. Ind. 1962, 1049.

DIETSCHY, J.M. and M.D. SIPERSTEIN. J. clin. Invest. 1965, 44, 1311.

ENEROTH, P. J. Lipid Res. 1963, 4, 11.

ENEROTH, P., B. GORDON, R. RYHAGE and J. SJÖVALL. J. Lipid Res. 1966(a)7, 511.

ENEROTH, P., B. GORDON and J. SJÖVALL. J. Lipid Res. 1966(b),7, 524.

ENEROTH, P., K. HELLSTRÖM and R. RYHAGE. J. Lipid Res. 1964, 5, 245.

ERICKSON, B.A., R.H. COOTS, F.H. MATTSON and A.M. KLIGMAN. J. clin. Invest. 1964, 43, 2017.

ERIKSSON, S. Proc. Soc. Exptl. Biol. Med. 1957, 94, 582.

EYSSEN, H., E. EVRARD and H. VANDERHAEGHE. J. Lab. clin. Med. 1966, 68, 753.

FAILEY, R.B., (Jr.) E. BROWN and M.E. HODES. Circulation 1959, 20, 984.

FAILEY, R.B., (Jr.) R.H. CHILDRESS and T.P. McCONAHAY. Amer. J. clin. Nutr. 1962, 10, 158.

FASOLI, A. and A. CESANA. J. Atheroscler. Res. 1963, 3, 475.

FELDMAN, E.B. and A.C. CARTER. Metabolism 1963, 12, 1132.

FLEISCHMAN, A.I., H. YACOWITZ, T. HAYTON and M.L. BIERENBAUM. J. Nutrition 1967, 91, 151.

FOWEATHER, F.S. Brit. J. exptl. Path. 1926, 7, 15.

FRANTZ, I.D. (Jr.), M.L. MOBBERLEY and G.J. SCHROEPFER (Jr.). Progr. in cardiovasc. Disease 1960, 2, 511.

FRANTZ, I.D. (Jr.), H.S. SCHNEIDER and B.T.H. HINKELMAN. J. biol. Chem. 1954, 206, 465.

FREDRICKSON, D.S. and K. ONO. Biochim. Biophys. Acta. 1956, 22, 183.

FRIEDMAN, M., S.O. BYERS and R.H. ROSENMAN. Circulation 1952, 5, 657.

GANSHIRT, H., F.W. KOSS and K. MORIANZ. Arzneimittel-Forsch. 1960, 10, 943.

GERSON, T., F.B. SHORLAND and G.G. DUNCKLEY. Nature, 1963, 200, 579.

GERSON, T., F.B. SHORLAND and G.G. DUNCKLEY. Biochem. J. 1965, 96, 399.

GILDEA, E.F., E.B. MAN and J.P. PETERS. J. clin Invest. 1939, 18, 739.

GLOVER, J. and C. GREEN. "Biochemical Problems of Lipids". Popják and Le Breton, 1955, p. 359.

GLOVER, J. and C. GREEN. Biochem. J. 1957, 67, 308.

GOBLEY, M. J. pharm. chem. 1846, 9, 81. see "Cholesterol" R.P. Cook. Academic Press Inc. New York, 1958, p. 2.

GOLDSMITH, G.A. Fed. Proc. (Suppl. 11) 1962, 21, 81.

GOLDSMITH, G.A., J.G. HAMILTON and O.N. MILLER. Arch. intern. Med. 1960, 105, 512.

GOODMAN, L.S. and A. GILMAN. "The Pharmacological Basis of Therapeutics", Collier-Macmillan Ltd., London, 1965, 3rd Edition, p. 759.

GOOLDEN, A.W.G. Lancet 1956, 1, 890.

GOULD, R.G. Amer. J. Med. 1951, 11, 209.

GOULD, R.G. and G. POPJÁK. Biochem. J. 1957, 66, 51P.

GREEN, K.G., W.H.W. INMAN and J.M. THORP. J. Atheroscler. Res. 1963, 3, 593.

GRÉEN, K. and B. SAMUELSSON. J. Lipid Res. 1964, 5, 117.

GROSS, J., R. PITT-RIVERS and W.R. TROTTER. Lancet 1952, 1, 1044.

GRUNDY, S.M., E.H. AHRENS (Jr.) and T.A. MIETTINEN. J. Lipid Res. 1965, 6, 397.

HAAHTI, E. and T. NIKKARI. Acta Chem. Scand. 1963, 17, 536.

HAAHTI, E., T. NIKKARI and K. JUVA. Acta. Chem. Scand. 1963, 17, 538.

HAMILTON, J.G. Arch. Biochem. Biophys. 1963, 101, 7.

HAMILTON, J.G. and J.E. MULDREY. J. Amer. oil. chem. Soc. 1961, 38, 582.

HANSEN, P.F. J. Atheroscler. Res. 1963, 3, 584.

HARRISON, G.A. in Chemical Methods in Clinical Medicine. G.A. Harrison, Ed. London, J. and A. Churchill Ltd., 1949, p. 494.

HARRISON, M.T. and R. McG. HARDEN. Scot. med. J. 1966, 11, 213.

HAUST, H.L. and J.M.R. BEVERIDGE. J. Nutrition 1963, 81, 13.

HAUST, H.L. and J.M.R. BEVERIDGE. Canad. J. Biochem. 1964, 42, 251.

HEFTMANN, E. Annual Review of Plant Physiology 1965, 14, 225.

HELLMAN, L., E.L. FRAZELL and R.S. ROSENFELD. J. clin. Invest. 1960, 39, 1288.

HELLMAN, L., R.S. ROSENFELD, W. INSULL (Jr.) and E.H. AHRENS (Jr.). J. clin. Invest. 1957, 36, 898.

HELLMAN, L., B. ZUMOFF, G. KESSLER, E. KARA, I.L. RUBIN and R.S. ROSENFELD. Ann. intern. Med. 1963, 59, 477.

HELLSTRÖM, K. and S. LINDSTEDT. J. lab. clin. Med. 1964, 63, 666.

HELLSTRÖM, K. and S. LINDSTEDT. Amer. J. clin. Nutr. 1966, 18, 46.

HELLSTRÖM, K. and J. SJÖVALL. J. Atheroscler. Res. 1961, 1, 205.

HELLSTRÖM, K. and J. SJÖVALL. J. Lipid Res. 1962, 3, 397.

HENDRY, E.B. Brit. med. J. 1960, 2, 975.

HERRMANN, R.G. Circulation Res. 1959, 7, 224.

HERNANDEZ, H.H., D.W. PETERSON, I.L. CHAIKOFF and W.G. DAUBEN. Proc. Soc. Exptl. Biol. Med. 1953, 83, 498.

HOFMANN, A.F. J. Lipid Res. 1962, 3, 127.

HOFMANN, A.F. in "Biochemical Problems of Lipids" A.C. Frazer ed., Elsevier, Amsterdam, 1963, p.1.

HOFMANN, A.F. and B. BERGSTRÖM. Fed. Proc. 1962, 21, 43.

HOLMES, W.L. and N.W.Di TULLIO. Amer. J. clin. Nutr. 1962, 10, 310.

HORNING, E.C., W.J.A. VANDENHEUVEL and B.G. CREECH. Methods in Biochemical Analysis. 1963, 11, 69.

HOTTA, S. and I.L. CHAIKOFF. Arch. Biochem. Biophys. 1955, 56, 28.

HOWE, E.E., D.K. BOSSHARDT and J.W. HUFF. J. Nutrition 1960, 72, 379.

HUTTON, H.R.B. and G.S. BOYD. Communication at the Annual Meeting on the Chemistry of Lipids, Stockholm, 1963. see "Cholesterol, Bile Acids and Atherosclerosis" H. Van Belle, North-Holland Publishing Company, Amsterdam, 1965, p. 75.

IACONO, J.M., J.F. MUELLER and C.B. AMMERMAN. Fed. Proc. 1960, 19, 15.

IVY, A.C., E. KARVINEN, T.M. LIN and E.K. IVY. J. appl. Physiol. 1957, 11, 1.

JACOBSEN, E.D., R.B. CHODOS and W.W. FALOON. Amer. J. Med. 1960, 38, 524.

JANKAU, L. Arch. exptl. Pathol. Pharmakol. 1892, 29, 237.

JOLLIFFE, N. Metabolism 1961, 10, 497.

KAPLAN, J.A., G.E. COX and C.B. TAYLOR. Arch. Pathol. 1963, 76, 359.

KARP, A. and D. STETTEN(Jr.) J. biol. Chem. 1949, 179, 819.

KARVINEN, E., T.M. LIN and A.C. IVY. J. appl. Physiol. 1957, 11, 143.

KAUFMANN, H.P. and Z. MAKUS. Fette, Seifen, Anstrichmittel, 1960, 62, 1014.

KAUNITZ, H., D.C. MALINS and D.G. McKAY. J. Exptl. Med. 1962, 115, 1127.

KAYSER, F. and R. BALAT. Bull. soc. chim. biol. 1952, 34, 806.

KEYS, A., J.T. ANDERSON and F. GRANDE. Lancet 1957(a) 1, 66.

KEYS, A., J.T. ANDERSON and F. GRANDE. Lancet 1957(b), 2, 959.

KEYS, A., J.T. ANDERSON and F. GRANDE. Metabolism 1965, 14, 759.

KEYS, A., H.L. TAYLOR, H. BLACKBURN, J. BROZEK, J.T. ANDERSON and E. SIMONSON. Circulation 1963, 28, 381.

KIMM, K.S. and A.C. IVY. Amer. J. Physiol. 1952, 171, 302.

KINSELL, L.W. "Progress in the Chemistry of Fats and other Lipids" ed. R.T. Holman, W.O. Lundberg and T. Malkin, New York, 1963, p. 138.

KNUCHEL, F. Med. Welt. 1964, 47, 2530.

KOBAYASHI, J. Ber. Ohara. Inst. 1957, 11, 12.

KRELL, K. and S.A. HASHIM. J. Lipid Res. 1963, 4, 407.

KRITCHEVSKY, D. "Cholesterol" John Wiley and Sons Inc., New York, 1958(a), p. 75.

KRITCHEVSKY, D. "Cholesterol" John Wiley and Sons Inc., New York, 1958(b), p. 116.

KRITCHEVSKY, D. "Cholesterol", John Wiley and Sons Inc., New York, 1958(c), p. 188.

KRITCHEVSKY, D. Metabolism 1960, 9, 984.

KRITCHEVSKY, D. Penn. Med. J. 1961, 64, 188.

KURON, G.W. and D.M. TENNENT. Fed. Proc. 1961, 20, 268.

LACK, L. and I.M. WEINER. Amer. J. Physiol. 1961, 200, 313.

LACK, L. and I.M. WEINER. Fed. Proc. 1963, 22, 1334.

LANGDON, R.G. and K. BLOCH. J. biol. Chem. 1953, 202, 77.

LAUBINGER, G. Fortschr. Med. 1964, 82, 783.

LECANU, L.R. Ann. chim. et. phys. 1838, 67, 54, see "Cholesterol" R.P. Cook. Academic Press Inc. New York, 1958, p. 2.

LINDSEY, C.A. and J.D. WILSON. J. Lipid Res. 1965, 6, 173.

LINDSTEDT, S. Acta. chem. Scand. 1956, 10, 1051.

LINDSTEDT, S. Acta. chem. Scand. 1957(a), 11, 417.

LINDSTEDT, S. Acta. physiol. scand. 1957(b), 40, 1.

LINDSTEDT, S., J. AVIGAN, De WITT S. GOODMAN, J. SJÖVALL and D. STEINBERG. J. clin. Invest. 1965, 44, 1754.

LITTLE, H.N. and K. BLOCH. J. biol. Chem. 1950, 183, 33.

McKILLICAN, M.E. and R.P.A. SIMS. J. Amer. Oil Chem. Soc. 1963, 40, 108.

MAIBACH, E. Schweiz. Med. Wchnschr. 1967, 97, 418.

MALINS, D.C. and J.C. WEKELL. J. chem. Educ. 1963, 40, 531.

MALMROS, H. and B. SWAHN. Acta. med. Scand. 1953, 145, 361.

MALMROS, H. and G. WIGAND. Lancet 1957, 2, 1.

MANN, G.V., S.B. ANDRUS, A. McNALLY and F.J. STARE. J. exptl. Med. 1953, 98, 195.

MARTENSSON, E. Acta Chem. Scand. 1963, 17, 1174.

MAYER, G.A., W.F. CONNELL, M.S. de WOLFE and J.M.R. BEVERIDGE. Amer. J. clin. Nutr. 1954, 2, 316.

MESSINGER, W.J., Y. POROSOWSKA and J.M. STEELE. Arch. intern. Med. 1950, 86, 189.

MICHALEC, Č. J. Lipid Res. 1963, 4, 110.

MICHALEC, Č., M. ŠULC and J. MĚŠŤAN. Nature 1962, 193, 63.

MIETTINEN, T.A., E.H. AHRENS (Jr.) and S.M. GRUNDY. J. Lipid Res. 1965, 6, 411.

MILLER, O.N., J.G. HAMILTON and G.A. GOLDSMITH. Amer. J. clin. Nutr. 1960, 8, 480.

MILLER, O.N., J.G. HAMILTON and G.A. GOLDSMITH. Amer. J. clin. Nutr. 1962, 10, 285.

MOORE, R.B., J.T. ANDERSON, A. KEYS and I.D. FRANTZ. J. Lab. clin. Med. 1962, 60, 1000.

MORRIS, J.N., M.D. CRAWFORD and J.A. HEADY. Lancet 1961, 1, 860.

MORRIS, J.N., M.D. CRAWFORD and J.A. HEADY. Lancet 1962, 2, 506.

MORRIS, J.N., A. KAGAN, D.C. PATTISON, M.J. GARDNER and P.A.B. RAFFLE. Lancet, 1966, 2, 553.

MORRIS, L.J. Chem. Ind. 1962, 1238.

MORRIS, L.J. J. Lipid Res. 1963, 4, 357.

MOSBACH, E.H., J. BLUM, E. ARROYO and S. MILCH. Anal. Biochem. 1963, 5, 158.

MUELLER, J.H. J. biol. Chem. 1915, 22, 1.

MURTHY, S.K., J.S.K. DAVID and J. GANGULY. Biochem. Biophys. Acta. 1963, 70, 490.

NESTEL, P.J., E.Z. HIRSCH and E.A. COUZENS. J. clin Invest. 1965, 44, 891.

NICHOLS, C.W. (Jr.), M.D. SIPERSTEIN and I.L. CHAIKOFF. Proc. Soc. Exptl. Biol. Med. 1953, 83, 756.

NORDIN, B.E.C. and D.A. SMITH. "Diagnostic Procedures in Calcium Metabolism" (London) J.A. Churchill Ltd. 1965, p. 166.

NORMAN, A. Acta physiol. Scand. 1955, 33, 99.

NORMAN, A. and J. SJÖVALL. J. biol. Chem. 1958, 233, 872.

NUNN, S.L., W.N. TAUXE and J.L. JUERGENS. Circulation 1961, 24, 1099.

NUTRITION REVIEWS 1964, 22, 326-328.

OLIVER, M.F. Lancet 1962, 1, 1321.

OLIVER, M.F. J. Atherosc1er. Res. 1963, 3, 427.

OLIVER, M.F. and G.S. BOYD. Lancet 1956, 2, 1273.

PANTON and MARRICK "Clinical Pathology", (London), J.A. Churchill Ltd. 1934.

PARSONS, R.S., T.C. BUTLER and E.P. SELLARS. Lancet 1961, 2, 213.

PARSONS, W.B. (Jr.). Circulation 1961, 24, 1099.

PETERSON, P.W. Proc. Soc. Exper. Biol. Med. 1951, 78, 143.

PETERS, J.P. and E.B. MAN. J. clin. Invest. 1950, 29, 1.

PIHL, A. Acta physiol. Scand. 1955(b), 34, 183.

PIHL, A. Acta physiol. Scand. 1955(a), 34, 206.

PITT-RIVERS, R. and J.R. TATA in "The Thyroid Hormones". London, Pergamon Press, 1959, pg. 79.

POLLAK, O.J. Circulation 1953, 7, 696.

POLONOVSKI, M. and R. BOURRILLON. Bull. soc. chim. biol. 1952, 34, 703.

POPJÁK, G. and J.W. CORNFORTH. Biochem. J. 1966, 101, 553.

PORTMAN, O.W. Arch. Biochem. Biophys. 1958, 78, 125.

PORTMAN, O.W. and G.V. MANN. J. biol. Chem. 1955, 213, 733.

PORTMAN, O.W. and F.J. STARE. Physiol. Rev. 1959, 39, 407.

POWELL, R.C., W.T. NUNES, R.S. HARDING and J.B. VACCA. Amer. J. clin Nutrition 1962, 11, 156.

PREZIOSI, P. "Lipid Pharmacology" ed. R. Paoletti, New York/Basel, 1964, vol. 2. p.442.

PRIOR, I.A.M. and D.G. CAMPBELL. N.Z. Med. J. 1963, 62, 538.

RANNEY, R.E. and R.E. COUNSELL. Fed. Proc. 1962, 21, 96.

REID, J. in "Drugs Affecting Lipid Metabolism" S. Garattini and R. Paoletti eds., Elsevier, Amsterdam, 1960, p. 423.

RODBARD, S., C. BOLENE, R. PICK and L.N. KATZ. Circulation 1950, 2, 479.

ROSENFELD, R.S., D.K. FUKUSHIMA, L. HELLMAN and T.F. GALLAGHER. J. biol. Chem. 1954, 211, 301.

ROSENFELD, R.S. and L. HELLMAN. Arch. Biochem. Biophys. 1962, 97, 406.

ROSENHEIM, O. and H. KING. Chem. and Industry 1932, 51, 954.

ROSENHEIM, O. and T.A. WEBSTER. Biochem. J. 1943(a), 37, 513.

ROSENHEIM, O. and T.A. WEBSTER. Biochem. J. 1943(b), 37, 580.

ROSENMAN, R.H., M. FRIEDMAN and S.O. BYERS. Circulation 1952, 5, 589.

RYAN, W.G. and T.B. SCHWARTZ. J. Lab. clin. Med. 1964, 64, 1001.

SACHS, B.A. and L. WOLFMAN. Circulation 1962, 26, 669.

SACHS, B.A. and L. WOLFMAN. Arch. intern. Med. 1965, 116, 366.

SAKAKIDA, H., C.C. SHEDIAC and M.D. SIPERSTEIN. J. clin. Invest. 1963, 42, 1521.

SAMUEL, P. and A. STEINER. Proc. Soc. Exptl. Biol. Med. 1959, 100, 193.

SAMUEL, P. and M.S. WAITHE. Circulation 1961, 24, 578.

SCHOENHEIMER, R., H. BEHRING, R. HUMMEL and L. SCHINDEL. Z. physiol. Chem. 1930, 192, 73.

SCHOENHEIMER, R. and F. BREUSCH. J. biol. Chem. 1933, 103, 439.

SCHROEDER, H.A. J. Amer. med. Ass. 1960, 172, 1902.

SEIDEL, J.C., N. NATH and A.E. HARPER. J. Lipid Res. 1960, 1, 474.

SEMENUK. G. and W.T. BEHER. J. Chromatog. 1966, 21, 27.

SETTY, C.S.N. and A.C. IVY. Amer. J. Physiol. 1960, 199, 1003.

SIPERSTEIN, M.D., I.L. CHAIKOFF and W.O. REINHARDT. J. biol. Chem. 1952, 198, 111.

SIPERSTEIN, M.D. and I.L. CHAIKOFF. J. biol. Chem. 1952, 198, 93.

SIPERSTEIN, M.D. and V.M. FAGAN. J. biol. Chem. 1966, 241, 602.

SIPERSTEIN, M.D., V.M. FAGAN and J.M. DIETSCHY. J. biol. Chem. 1966, 241, 597.

SIPERSTEIN, M.D. and M.J. GUEST. J. clin. Invest. 1960, 39, 642.

SIPERSTEIN, M.D., F.M. HAROLD, I.L. CHAIKOFF and W.G. DAUBEN. J. biol. Chem. 1954, 210, 181.

SIPERSTEIN, M.D., C.W. NICHOLS (Jr.) and I.L. CHAIKOFF. Circulation 1953(a), 7, 37.

SIPERSTEIN, M.D., C.W. NICHOLS (Jr.) and I.L. CHAIKOFF. Science 1953(b), 117, 386.

SJOVALL, J. Proc. Soc. Exptl. Biol. Med. 1959, 100, 676.

SJÖVALL, J. Clin. chim. Acta. 1960, 5, 33.

SPRITZ, N. Circulation 1965, 11 (Suppl. 31-32), 201.

SPRITZ, N., E.H. AHRENS (Jr.) and S. GRUNDY. J. clin. Invest. 1965, 44, 1482.

SRERE, P.A., I.L. CHAIKOFF, S.S. TREITMAN and L.S. BURSTEIN. J. biol. Chem. 1950, 182, 629.

STAHL, E. Pharmazie 1956, 11, 633.

STEIN, Y. and O. STEIN. J. Atheroscler. Res. 1962, 2, 400.

STEINBERG, D. and J. AVIGAN in Drugs Affecting Lipid Metabolism. S. Garattine and R. Paoletti eds., Elsevier, Amsterdam, 1960(a)p. 132.

STEINBERG, D. and J. AVIGAN. J. biol. Chem. 1960(b), 235, 3127.

STEINBERG, D., J. AVIGAN and E.B. FEIGELSON. Progr. in cardiovasc. Disease 1960, 2, 586.

STEINER, A. and B. DOMANSKI. Amer. J. med. Sci. 1941, 201, 820.

STEINER, A., E. HOWARD and S. AKGUN. Circulation 1961, 24, 729.

STEINER, A. and F.E. KENDALL. A.M.A. Arch. Pathol. 1946, 42, 433.

STOKES, W.M., W.A. FISH and F.C. HICKEY. J. biol. Chem. 1955, 213, 325.

STRISOWER, B., J.W. GOFMAN, G.F. GALIONI, A.A. ALMADA and A. SIMON. Metabolism, Clin. and Exptl. 1954, 3, 218.

STRISOWER, E.H. and B. STRISOWER. J. clin. Endoc. Metab. 1964, 24, 139.

STRISOWER, E.H., A.V. NICHOLS, F.T. LINDGREN and L. SMITH. J. Lab. clin. Med. 1965, 65, 748.

SVOBODA, J.A. and M.J. THOMPSON. J. Lipid Res. 1967, 8, 152.

SWARTWOUT, J.R., J.W. DIECKERT, O.N. MILLER and J.G. HAMILTON. J. Lipid Res. 1960, 1, 281.

SWEELEY, C.C. and B. KLIONSKY. J. biol. Chem. 1963, 238 PC. 3148.

SWELL, L., T.A. BOITER, H. FIELD (Jr.) and C.R. TREADWELL. Proc. Soc. Exptl. Biol. Med. 1954, 86, 295.

SWELL, L., D.F. FLICK, H. FIELD (Jr.) and C.R. TREADWELL. Proc. Soc. Exptl. Biol. Med. 1953, 84, 428.

SWELL, L., E.C. TROUT (Jr.), H. FIELD (Jr.) and C.R. TREADWELL. J. biol. Chem. 1959, 234, 2286.

SWELL, L., E.C. TROUT (Jr.), J.R. HOPPER, H. FIELD (Jr.) and C.R. TREADWELL. J. biol. Chem. 1958, 233, 49.

TAYLOR, C.B. and K-J. HO. Arch. Pathol. 1967, 84, 3.

TAYLOR, C.B., B. MIKKELSON, J.A. ANDERSON, D.T. FORMAN and S.S. CHOI. Exper. and Mol. Path. 1965, 4, 480.

TECHNICON AUTOANALYSER MANUAL, Method N. 24P.

TENNENT, D.M., G.W. KURON, M.E. ZANETTI and W.H. OTT. Proc. Soc. Exptl. Biol. Med. 1961, 108, 214.

TENNENT, D.M., H. SIEGEL, M.E. ZANETTI, G.W. KURON, W.H. OTT and F.J. WOLF. J. Lipid Res. 1960, 1, 469.

THORP, J.M. Lancet 1962, 1, 1323.

THORP, J.M. J. Atheroscler. Res. 1963, 3, 351.

THORP, J.M. and W.S. WARING. Nature 1962, 194, 948.

TOMKINS, G.M., H. SHEPPARD and I.L. CHAIKOFF. J. biol. Chem. 1953(a)201, 137.

TOMKINS, G.M., H. SHEPPARD and I.L. CHAIKOFF. J. biol. Chem. 1953(b),203, 781.

TREADWELL, C.R., L. SWELL and G.V. VAHOUNY. Fed. Proc. 1962, 21, 903.

TREADWELL, R., L. SWELL, G.V. VAHOUNY and H. FIELD (Jr.). J. Amer. Oil Chem. Soc. 1959, 36, 107.

TYGSTRUP, N., K. WINKLER and K. JORGENSEN in "Drugs Affecting Lipid Metabolism". S. Garattini and R. Paoletti eds., Elsevier, Amsterdam, 1960, p. 493.

TYGSTRUP, N., K. WINKLER and E. WARBURG. Lancet 1959, 1, 503.

VAHOUNY, G.V. and C.R. TREADWELL. Amer. J. Physiol. 1958, 195, 516.

VAN BELLE, H. "Cholesterol, Bile Acids and Atherosclerosis". North-Holland publishing Company, Amsterdam 1965, p. 77.

VAN DEN BOSCH, J. and P. CLAES. Progress in Biochemical Pharmacology" D. Kritchevsky, R. Paoletti and D. Steinberg eds. Basel, S. Karger, A.G. 1967, vol. 2, p. 97.

VAN HANDEL, E. and D.G. ZILVERSMIT. J. lab. clin. Med. 1957, 50, 152.

VAN TAMELEN, E.E., J.D. WILLETT, R.B. GLAYTON and K.E. LORD. J. Amer. Chem. Soc. 1966, 88, 4752.

WAINFAN, E., G. HENKIN, L.I. RICE and W. MARX. Arch. Biochem. Biophys. 1952, 38, 187.

WALTON, K.W., P.J. SCOTT, J.V. JONES, R.F. FLETCHER and T. WHITEHEAD. J. Atheroscler. Res. 1963, 3, 396.

WANG, C.I., L.E. SCHAEFER, S.R. DRACHMAN and D. ADLERSBERG. J. Mt. Sinai Hosp. N.Y. 1954, 21, 19.

WEISS, S.B. and W. MARX. J. biol. Chem. 1955, 213, 349.

WELLS, V.M. and B. BRONTE-STEWART. Brit. med. J. 1963, 1, 577.

WELLS, W.W. Arch. Biochem. Biophys. 1957, 66, 217.

WELLS, W.W. and S.B. COOPER. Arch. Biochem, Biophys. 1958, 75, 273.

WELLS, W.W. and P.A. MORES. Nature 1961, 189, 483.

WIGGINS, H.S. and I.D.P. WOOTTON. Biochem. J. 1958, 70, 349.

WILSON. J.D. J. Lipid Res. 1961, 2, 350.

WINDAUS, A. Nachr. kgl. Ges. Win. Gottingen, Math-physik. Kl. 1919, p. 237.

WINDAUS. A. Z. physiol. Chem. 1932, 213, 147.

WOOD, P.D.S., R. SHIODA and L.W. KINSELL. Lancet, 1966, 2, 604.

WOOTTON, I.D.P. and H.S. WIGGINS. Biochem. J. 1953, 55, 292.

YACOWITZ, H., A.I. FLEISCHMAN and M.L. BIERENBAUM. Brit. med. J. 1965, 1, 1352.

ZAK, B., R.C. DICKENMAN, E.G. WHITE, H. BURNETT and P.J. CHERNEY. Amer. J. clin. Pathol. 1954, 24, 1307.