STUDIES IN CHOLESTEROL METABOLISM

by

The work reported in this thesis has not been previously submitted for any other degree or diploma and is my own original work.

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STATEMENT

I should like to thank Professor M.J. Whyte and Dr. P.J. Hewatt, whose constant interest and advice has been of great assistance during the conduct of these studies, and to Mr. V. Craig for his assistance in writing the computer programmes.

The method for measuring the rate of aspiration of CO₂ was developed by Ronald Carroll of the Department of Biochemistry, London, Ontario, Canada.

This work was performed during the tenure of an Australian National University Medical Scholarship. Mrs. M.L. Blackmore typed the final manuscript.

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I should like to thank Professor H.M. Whyte and Dr. P.J. Nestel, whose constant interest and advice has been invaluable during the preparation of this thesis. I am also grateful to Dr. A. Irvine, Medical Director of Upjohn Pty. Ltd., for his co-operation during the conduct of these studies, and to Mr. W. Craig for his assistance in writing the computer programmes.

The method for measuring the rate of expiration of $^{14}$CO$_2$ was developed by Professor K.K. Carroll of the Department of Biochemistry, London, Ontario, Canada.

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The subject of human cholesterol metabolism has assumed increasing importance in recent years in view of its possible relationship to atherosclerosis and gall stone disease. This thesis describes several studies dealing with the effects of drugs on cholesterol transport and metabolism in man. The investigations have broadly taken two forms, those in which the object has been to learn how the drug affects cholesterol metabolism, and those in which drugs with known modes of action have been used as investigative tools in the study of normal and abnormal cholesterol metabolism.

The following chapters summarize the literature related to this field of study, some emphasis has been given to studies performed on human subjects, but since these cannot be divorced from the more extensive literature concerning other species observations on animals have also been described. Those aspects of this literature which are of particular relevance to each individual study are discussed in the relevant chapter. In the third chapter a brief discussion is given of the major investigational techniques employed.

Chapter 4 records an evaluation of a new bile acid-sequestering resin, colestipol (S-76, 597A), as a cholesterol-lowering agent in patients with hypercholesterolemia. The therapeutic value of combining colestipol with other agents has also been examined. Chapter 5 describes studies in which the effects of colestipol on cholesterol metabolism have been examined in more detail, using the techniques of compartmental isotope dilution analysis, and isotopic steroid balance. The opportunity was taken to compare estimates of cholesterol turnover obtained simultaneously with these two techniques. Having established its effects on human cholesterol metabolism, colestipol was then used to investigate the participation of different plasma lipoproteins in the transport of cholesterol (Chapter 6). These findings with colestipol were subsequently compared with those obtained with another drug, clofibrate. In addition, the effects of colestipol in subjects with different types of hyperlipoproteinemias were studied in the hope that the results might contribute towards an understanding of the metabolic defects responsible for these conditions.

CHAPTER 1

INTRODUCTION
The subject of human cholesterol metabolism has assumed increasing importance in recent years in view of its possible relationship to atherosclerosis and gall stone disease. This thesis describes several studies dealing with the effects of drugs on cholesterol transport and metabolism in man. The investigations have broadly taken two forms: those in which the object has been to learn how the drug affects cholesterol metabolism, and those in which drugs with known modes of action have been used as investigational tools in the study of normal and abnormal cholesterol metabolism.

The following chapter reviews the literature related to this field of study. Emphasis has been given to studies performed on human subjects, but since these cannot be divorced from the more extensive literature concerning other species observations on animals have also been described. Those aspects of the literature which are of particular relevance to each individual study are discussed in the relevant chapters. In the third chapter a brief discussion is given of the major investigational techniques employed.

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Chapter 7 describes the effects of colestipol on the activity of the plasma cholesterol esterification reaction, in an attempt to define its relationship to cholesterol turnover.

In Chapter 8 the effects of phenobarbitone therapy on cholesterol and bile acid metabolism have been examined in normal human subjects, using the techniques of steroid balance and bile acid isotope dilution analysis. The reason for this study was the knowledge that phenobarbitone induces several hepatic enzyme systems, including some that regulate cholesterol metabolism in experimental animals.

Chapter 9 summarises the results of these studies and suggestions are made for future research.
CHOLESTEROL SYNTHESIS

Sites of cholesterol synthesis

Most, if not all, body tissues appear capable of synthesizing cholesterol from acetate (Krook et al., 1950; Dietschy and Wilson, 1968). It has been demonstrated that such endogenous synthesis is a major source of body cholesterol in man (Kaplan et al., 1963; Wilson and Lindsay, 1968; Grundy and Ahrens, 1969). Less than half of plasma cholesterol being of dietary origin even during the consumption of high cholesterol diets. Moreover, marked differences exist in the rate at which various tissues incorporate acetate into sterols. By far the most active organs of such synthesis are liver in both the rat (Dietschy and Roberfroid, 1966) and the monkey (Dietschy and Wilson, 1965) and the liver and the small intestine. Intestinal cholesterol synthesis has been mostly localized to the crypt cells of the terminal ileum in both animals (Shear, Wessar, Loper et al., 1972; Dietschy, 1965a) and man (Dietschy and Canal, 1971). Cholesterol synthesized in the intestinal wall reaches the circulation...
Cholesterol is an essential structural component of all body cells, and exists in plasma as a constituent of plasma lipoproteins. With the exception of that within the nervous system, tissue cholesterol is in equilibrium with that of plasma (Chobanian and Hollander, 1962). This body pool of exchangeable cholesterol is constantly renewed throughout life, a process referred to as cholesterol turnover. New cholesterol enters by absorption of dietary cholesterol and by endogenous synthesis, while cholesterol leaves the body mainly within the faeces as cholesterol itself, together with its products of bacterial degradation, and as bile acids, the products of hepatic cholesterol catabolism. These processes will be briefly discussed in the following paragraphs, together with other aspects of cholesterol turnover and transport. Extensive reviews have recently been made by several authors (Chevallier, 1967; Taylor and Ho, 1967; Nestel, 1970a; Dietschy and Wilson, 1970; Myant, 1971).

**CHOLESTEROL SYNTHESIS**

**Sites of cholesterol synthesis**

Most, if not all, body tissues appear capable of synthesizing cholesterol from acetate (Srere et al, 1950; Dietschy and Wilson, 1968). It has been demonstrated that such endogenous synthesis is a major source of body cholesterol in man (Kaplan et al, 1963; Wilson and Lindsey, 1965; Grundy and Ahrens, 1969), less than half of plasma cholesterol being of dietary origin even during the consumption of high cholesterol diets. However, marked differences exist in the rate at which various tissues incorporate acetate into sterols. By far the most active organs of such synthesis in vitro in both the rat (Dietschy and Siperstein, 1967) and the monkey (Dietschy and Wilson, 1968) are the liver and the small intestine. Intestinal cholerasterogenesis has been mostly localised to the crypt cells of the terminal ileum in both animals (Shefer, Hauser, Lapar et al, 1972; Dietschy, 1968a) and man (Dietschy and Gamel, 1971). Cholesterol synthesized in the intestinal wall reaches the circulation
by way of the lymphatics (Wilson, 1968). Calculations that these two organs are responsible for more than 95% of total body cholesterol synthesis in the squirrel monkey (Dietschy and Wilson, 1970) have been confirmed by \textit{in vivo} studies (Wilson, 1968).

The cholesterogenic activity of different human tissues has not been comprehensively studied in a similar manner. However, high rates of \textit{in vitro} cholesterol synthesis have been demonstrated in the liver (Bhattathiry and Siperstein, 1963; Taylor et al, 1955) and distal ileum (Dietschy and Gamel, 1971) of man, while synthetic activity in other parts of the gastrointestinal tract is considerably less (Dietschy and Gamel, 1971). It is noteworthy that in obese human subjects adipose tissue, although of relatively low synthetic activity, may make an appreciable contribution to total body cholesterol synthesis by virtue of its abnormally large mass (Nestel et al, 1969; Angel and Farkas, 1971; Miettinen, 1971a; Nestel, Ahrens and Schreibman, 1973).

\section*{Biochemical Pathway of Cholesterol Synthesis}

The pathway of cholesterol biosynthesis from acetate has been the subject of considerable investigation in recent years, and has recently been summarised by Briggs and Brotherton (1970). The biosynthetic pathway is outlined below (where the number of carbon atoms at each step is indicated in parenthesis):

\begin{itemize}
  \item Acetate (2C) \rightarrow hydroxymethylglutarate (6C) \rightarrow mevalonate (6C) \rightarrow isopentenyl pyrophosphate (5C) \rightarrow farnesyl pyrophosphate (15C) \rightarrow squalene (30C) \rightarrow several steroid intermediates, including lanosterol, zymosterol, lathosterol and desmosterol \rightarrow cholesterol (27C).
\end{itemize}

This pathway appears to be present in most mammalian tissues, with the exception of the adult central nervous system (Srere et al, 1950). The rate-limiting factor in both the liver (Siperstein and Guest, 1960; Bucher et al, 1960) and intestinal mucosa (Dietschy, 1968a) appears to be the activity of the enzyme 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMG CoA reductase), which catalyzes the conversion of 3-hydroxy-3 methylglutaryl CoA to
mevalonic acid. In the liver this enzyme occurs mainly within the endoplasmic reticulum (Goldfarb, 1972), while in the crypt cells of the intestinal mucosa it occurs in both the endoplasmic reticulum and the mitochondria (Shefer, Hauser, Lapar et al, 1972). Its activity undergoes a striking diurnal variation in experimental animals (Hamprecht et al, 1969; Edwards et al, 1972), which appears to be related to the secretions of the adrenal cortex (Hickman et al, 1972).

Regulation of cholesterol synthesis

Animal and human experiments have established that the principal physiological variables regulating cholesterol biosynthesis are the dietary content of cholesterol and the integrity of the enterohepatic circulation of bile acids.

(i) Dietary cholesterol

Studies performed both in vitro (Gould, 1951; Dietschy and Wilson, 1968; Dietschy and Siperstein, 1967; Wilson, 1972a) and in vivo (Morris et al, 1957; Taylor et al, 1960; Bricker et al, 1972; Wilson, 1972a) have demonstrated that the intestinal absorption of cholesterol markedly suppresses hepatic cholesterogenesis in several animal species, and that the liver appears unique in this respect, synthesis within other tissues (including the ileum) being suppressed to a much lesser extent or not at all (Dietschy and Wilson, 1968; Wilson and Reinke, 1968; Angel and Farkas, 1971). The major biochemical site of this feedback in animals appears to be the conversion of HMG CoA to mevalonate (Siperstein and Fagan, 1966). Although early experiments employing continuous oral labelling of body cholesterol failed to confirm that such a mechanism is prominent in man (Kaplan et al, 1963; Wilson and Lindsey, 1965), recent studies employing steroid balance techniques (Grundy et al, 1969; Quintao et al, 1971a) have convincingly demonstrated that suppression of body cholesterol synthesis by absorbed cholesterol does occur in most human subjects. In vitro studies with human tissues have shown that this occurs within the liver (Bhattathiry and Siperstein, 1963; Fujiwara et al, 1965; Pawliger and Shipp, 1968), and only minimally in the
intestine (Dietschy and Gamel, 1971). However, the magnitude of this response shows individual variability, a factor which is of importance in determining the effect of cholesterol intake on body cholesterol pools (Quintao et al, 1971a).

(ii) The enterohepatic circulation of bile acids

There is unequivocal evidence that both intestinal and hepatic cholesterogenesis are regulated by alterations in the enterohepatic circulation of bile acids (reviewed by Wilson, 1972b). Thus, bile acid-sequestrant resin therapy, biliary division and ileal surgery have each been shown to enhance cholesterogenesis at these sites in experimental animals (Myant and Eder, 1961; Dietschy and Siperstein, 1965; Moutafis and Myant, 1968; Dietschy, 1968a; Shefer, Hauser, Lapar et al, 1972), associated with an apparent increased activity of HMG Co A reductase (Goldfarb and Pitot, 1972; White, 1972a; Shefer, Hauser, Lapar et al, 1972) and HMG Co A condensing enzyme (White, 1972a). However, it is not yet established whether this is a direct effect of bile acids, or an indirect effect mediated by altered cholesterol absorption. In the case of the intestine, present evidence favours the former possibility. Although a high cholesterol diet has little effect on intestinal cholesterogenesis in the rat (Dietschy and Siperstein, 1967), this was found to be very sensitive to changes in the intraluminal concentration of bile salts (Dietschy, 1968a). However, many studies have supported an indirect effect of bile acids on hepatic cholesterol synthesis. Purified bile salts added in physiological amounts to in vitro liver preparations have no specific effect on cholesterol synthesis (Dietschy, 1967). In animals with biliary division infusing chylomicrons intravenously prevents an increase in hepatic cholesterogenesis, whereas infusing bile acids does not (Weis and Dietschy, 1969). However, baboons subjected to ileal diversion have increased hepatic cholesterol synthesis despite normal cholesterol absorption (Wilson, 1972a). In addition, feeding cholic acid to rats with thoracic duct fistulae, in which no absorbed cholesterol can reach the
liver, effectively inhibits hepatic cholesterogenesis (Hamprecht et al, 1971). These observations suggest a complex mechanism of control of hepatic cholesterogenesis by the enterohepatic circulation of bile acids, involving both a direct effect of bile acids returning to the liver via the portal vein and an indirect effect, mediated by altered cholesterol absorption.

Although the role of bile salts in regulating cholesterologenesis has been less extensively studied in man than in lower animals the findings to date suggest that the mechanisms are similar. Total body cholesterol turnover in human subjects is diminished by the administration of bile acids (Grundy et al, 1966), and is stimulated by bile acid-sequestrant therapy (Goodman and Noble, 1968; Miettinen, 1970a; Moutafis and Myant, 1969a) and ileal surgery (Moutafis et al, 1968; Miettinen, 1970a; Moore et al, 1970). Studies of the specific activities of biliary and intestinal cholesterol by Grundy et al (1971) suggested that the increased turnover produced by the latter procedures involved enhanced cholesterogenesis within both the liver and intestine, and this has recently been confirmed by direct assay of in vitro cholesterogenic activity (Moutafis et al, 1971; Dietschy and Gamel, 1971).

Since cholesterol synthesis may be increased in these circumstances despite apparently normal cholesterol absorption (Grundy et al, 1971; Nazir et al, 1972) it is possible that this results from the removal of a direct inhibitory effect exerted by bile acids.

Other factors affecting cholesterol synthesis

The differences in the regulation of hepatic and extra-hepatic cholesterologenesis are also seen with starvation. This leads to a profound reduction of cholesterol synthesis in the livers of experimental animals due to a reduced activity of HMG CoA reductase (Bucher et al, 1960), but has little or no effect on that within other tissues (Dietschy and Siperstein, 1967; Dietschy and Wilson, 1968). Accordingly, although fasting subjects have a greatly diminished total cholesterol turnover (Miettinen, 1970a), intestinal cholesterologenesis in man would appear to be only moderately reduced under such circumstances (Dietschy and Gamel, 1971).
Dietary components which stimulate cholesterogenesis in animals or man include corn oil (Bortz, 1967; Hill et al, 1960; Goldfarb and Pitot, 1972), saturated fat (Hill et al, 1960) and ethanol (LeFèvre et al, 1972).

Hormones may also be concerned in the regulation of cholesterol synthesis. While thyroid hormone (Fletcher and Myant, 1958; Miettinen, 1970b; Kurland et al, 1961), insulin (White, 1972b) and oestrogens (Kritchevsky et al, 1962; Nestel et al, 1965) apparently enhance hepatic cholesterogenesis, there has been disagreement concerning the effects of adrenocorticoids (Hickman et al, 1972; Perry and Bowen, 1955; Willmer and Foster, 1960). Noradrenaline increases hepatic cholesterol synthesis by a mechanism which appears to be indirect (Bortz, 1968), and which may be secondary to an increased flux of free fatty acids to the liver (Nestel and Steinberg, 1963).

Drugs which inhibit cholesterol synthesis at different stages include clofibrate (Steinberg, 1970; Grundy, Ahrens, Salen et al, 1972) and triparanol (Avigan et al, 1960), while phenobarbitone stimulates synthesis in some species (Middleton and Isselbacher, 1969; Jones and Armstrong, 1965).

**CHOLESTEROL ABSORPTION**

The other source of new cholesterol entering miscible body pools is dietary (exogenous) cholesterol. The daily ingestion of cholesterol in Western societies varies between 500 and 2000 mg (Keys, 1965), foods rich in cholesterol including egg yolks, dairy produce and meat. Following ingestion, dietary cholesterol becomes mixed in the intestinal lumen with endogenous cholesterol derived from bile, desquamated epithelial cells and alimentary secretions. The mechanism by which cholesterol from both sources is subsequently absorbed has been the subject of considerable investigation, and has been recently summarised by Wilson (1972b). Absorption mostly occurs in the upper small intestine (Simmonds et al,1967). Cholesterol esters are first hydrolysed to free cholesterol by pancreatic cholesterol esterase (Shiratori and Goodman, 1965), an enzyme which is
stimulated by bile salts (Vahouny et al, 1959). In the presence of bile salts and other amphipaths, such as monoglycerides and fatty acids, cholesterol is solubilized by incorporation into mixed micelles (Carey and Small, 1972). The presence of glycerides and free fatty acids enhances cholesterol absorption (Sylven and Borgström, 1968). Cholesterol then diffuses into the cells of the intestinal mucosa independently of the other micellar components (Simmonds et al, 1967), where it mixes to some extent with that derived from local synthesis and from exchange with plasma cholesterol (Borgström, 1960). A fraction is then re-esterified with fatty acids, particularly oleic acid (Blomstrand et al, 1964) before being secreted into lymph as a component of chylomicrons and very low density lipoproteins (see later). Essentially no cholesterol is absorbed via the portal vein (Chaikoff et al, 1952). An obligatory role of bile salts in cholesterol absorption is well established (Siperstein et al, 1952), and there is evidence for their involvement in each of the stages described (reviewed by Wilson, 1972b).

Cholesterol absorption in man is slow and incomplete (Hellman et al, 1960). However, although limited relative to that in some other species (Dietschy and Wilson, 1970), cholesterol absorption is not so severely limited in man as was originally concluded from the isotopic steady state experiments of Kaplan et al (1963) and Wilson and Lindsey (1965). The difficulties in interpreting such data have been discussed by Quintao et al (1971a), who have demonstrated by steroid balance techniques that cholesterol absorption in man increases almost linearly with increasing intake, and this has recently been confirmed by Kudchodkar et al (1973). Fractional absorption in these studies ranged from 25 to 50%. Quintao et al (1971b) also reported that the percent absorption of a single large dose of cholesterol was greater when the preceding diet had been of low cholesterol content. Other factors which influence cholesterol absorption are the presence of dietary fat and plant sterols, which respectively enhance and suppress cholesterol absorption (Wilson, 1962; Grundy et al, 1969).
CHOLESTEROL EXCRETION

The excretion of cholesterol occurs mainly through the gastrointestinal tract as neutral steroids and acidic steroids (bile acids), the latter being the products of hepatic cholesterol catabolism.

A. Excretion as faecal neutral steroids

Neutral steroids within faeces are a mixture of unabsorbed dietary cholesterol, dietary plant sterols and endogenous cholesterol (derived from miscible body pools), together with their products of bacterial degradation. Endogenous cholesterol enters the intestinal lumen within bile and from the gut wall (Cheng and Stanley, 1959). Cholesterol is secreted into bile by the liver at the rate of 15 to 85 mg/hr (Grundy and Metzger, 1972; Grundy, Metzger and Adler, 1972) and is solubilized by molecular association with bile salts and lecithin to form mixed micelles (Admirand and Small, 1968). The presence of adequate amounts of bile salts and lecithin appears critical in this respect, a fall in their concentration relative to that of cholesterol predisposing to the formation of cholesterol gall stones (Admirand and Small, 1968; Small and Rapo, 1970). Cholesterol from the intestinal wall appears to be derived from glandular secretions and desquamated epithelial cells, and includes absorbed and locally synthesized cholesterol, as well as that derived from exchange with the plasma (Wilson and Reinke, 1968; Danielsson, 1960). A proportion of the endogenous cholesterol is absorbed along with that from the diet, and it has been suggested that biliary cholesterol is more extensively re-absorbed than that from other sources (Dietschy and Wilson, 1970). Cholesterol thus undergoes an enterohepatic circulation. Unabsorbed cholesterol is excreted within the faeces partly as cholesterol itself, but mainly as its derivations coprostanol (66-95%) and coprostanone, the latter being products of bacterial activity within the intestine (Danielsson and Gustafsson, 1959; Rosenfeld et al, 1954; Kudchodkar et al, 1972a). During formula-feeding, additional degradation, involving presumed ring cleavage, also appears to occur (Grundy et al, 1968).
The elimination of cholesterol as faecal neutral steroids accounts for more than 50% of total endogenous cholesterol excretion in most adults (Quintao et al, 1971a; Grundy and Ahrens, 1969; Grundy et al, 1969; Grundy and Ahrens, 1970; Grundy, Ahrens, Salen et al, 1972; Connor et al, 1969; Miettinen, 1971a).

Factors affecting faecal neutral steroid excretion

Faecal endogenous neutral steroid excretion is affected by any factor influencing cholesterol absorption. It is accordingly increased by dietary plant sterols (Grundy et al, 1969), by ileal by-pass surgery (Grundy et al, 1971) and in the malabsorption syndrome (Miettinen, 1970a). Enhanced excretion may also be due to augmented secretion of cholesterol within bile, as during the consumption of cholesterol-rich diets (Quintao et al, 1971a) and clofibrate therapy (Grundy, Ahrens, Salen et al, 1972). Faecal endogenous neutral steroid excretion is also enhanced in obesity (Nestel, Schreibman and Ahrens, 1973; Miettinen, 1971a), and, in some individuals, during diets rich in polyunsaturated fatty acids (Connor et al, 1969; Wood et al, 1966; Grundy and Ahrens, 1966; Moore et al, 1968; Nestel, Havenstein, Whyte et al, 1973). It is diminished in myxoedema (Miettinen, 1970a, 1970b).

B. Cholesterol Catabolism to Bile Acids

The catabolism of cholesterol to bile acids within the liver was first described by Bloch et al (1943) and constitutes the second major pathway of cholesterol elimination. The biochemical sequence has been recently reviewed by several authors (Lindstedt, 1970; Elliott and Hyde, 1971; Mosbach, 1972). The principal steps are summarised below:

\[
\text{CHOLESTEROL} \downarrow \rightarrow \text{7a-HYDROXYCHOLESTEROL} \downarrow \rightarrow \text{CHOLIC ACID} \quad \text{CHENODEOXYCHOLIC ACID} \ \text{GLYCINE} \quad \text{TAUROCHOLIC ACID} \quad \text{TAUROCHENODEOXYCHOLIC ACID} \ \text{GLYCINE}
\]

\[
\text{GLYCOCHOLIC ACID} \quad \text{TAUROCHOLIC ACID} \quad \text{TAUROCHENODEOXYCHOLIC ACID} \ \text{GLYCOCHENODEOXYCHOLIC ACID}
\]
Although this sequence was elucidated as the result of animal work, recent experiments suggest that it applies to man (Anderson et al, 1972; Hepner et al, 1972a; Hepner et al, 1972b; Hepner et al, 1973; Hanson et al, 1973). The two primary bile acids in man are cholic acid and chenodeoxycholic acid, and these are conjugated with the amino-acids glycine and taurine (for form "bile salts") before being secreted into the bile. After absorption of water by the gall bladder, the final concentration of total bile salts is usually within the range 1 to 9g/100 ml (Haslewood, 1967). This concentration, together with that of lecithin, is critical to the micellar solubilization of biliary cholesterol (Admirand and Small, 1968). After entering the intestine, where it plays important roles in fat digestion and absorption (Holt, 1972), most of the bile salt is reabsorbed. This occurs predominantly by active transport in the terminal ileum, although some is also reabsorbed by passive diffusion in both the ileum and the colon (Dietschy, 1968b). Unabsorbed bile salts undergo bacterial modification within the distal ileum and colon, the principal alterations being deconjugation (to yield the free bile acids) and 7-dehydroxylation (to yield the secondary bile acids, deoxycholic acid from cholic acid, and lithocholic acid from chenodeoxycholic acid) (Tyor et al, 1971; Lewis and Gorbach, 1972). Further microbial metabolism, however, results in a complex mixture of other derivatives (Danielsson et al, 1963), and may have an important influence on bile acid turnover (Bergström and Danielsson, 1963).

Reabsorbed bile salts and bile acids return to the liver via the portal vein (Reinke and Wilson, 1967), where most is secreted into the bile by active transport, bile acids being reconjugated in transit (Wheeler, 1972). Thus, bile acids undergo an extensive enterohepatic circulation. Estimates for total bile acid secretion in normal adults range from 300 to 1900 mg per hour (Grundy and Metzger, 1972; Grundy, Metzger and Adler, 1972), in comparison with a rate of synthesis which usually lies between 100 and 500 mg per day (Moore et al, 1968; Connor et al, 1969; Vlahcevic et al, 1971; Miettinen, 1970c; Lewis and Myant, 1967).
Regulation of bile acid synthesis

The overall rate-limiting step in bile acid synthesis appears to be the first in the biosynthetic sequence, namely the 7α-hydroxylation of cholesterol (Danielsson et al., 1967; Shefer et al., 1968). As with hepatic cholesterol synthesis, the rate-limiting enzyme for cholesterol catabolism resides within the endoplasmic reticulum (Boyd et al., 1969) and demonstrates a diurnal variation in activity (Danielsson, 1972). The rate of synthesis is regulated homeostatically by the amount of bile acid returning to the liver via the portal vein (Shefer et al., 1969), and both in vitro (Boyd et al., 1969) and in vivo (Shefer et al., 1970) studies have demonstrated that this is due to an inhibition of cholesterol 7α-hydroxylation. Accordingly bile acid synthesis and excretion are enhanced when the enterohepatic circulation of bile acids is interrupted by resin therapy (Moutafis and Myant, 1969a; Grundy et al., 1971; Nazir et al., 1972; Miettinen, 1970c; Garbutt and Kenney, 1972), ileal surgery (Grundy et al., 1971; Miettinen, 1970a; Moore et al., 1970; Moutafis et al., 1968) or terminal ileal disease (Hofmann, 1967). Bile acids also appear to have a minor inhibitory effect on steps after 7α-hydroxylation (Shefer et al., 1970; Moir et al., 1970).

Although dietary cholesterol does not appear to affect human bile acid metabolism (Quintao et al., 1971a), it stimulates bile acid turnover in both the rat and the dog (Wilson, 1964; Quintao et al., 1971a) due to an effect on cholesterol 7α-hydroxylase (Boyd et al., 1969). In the squirrel monkey the response of bile acid synthesis to cholesterol feeding shows genetic variation, and this appears to be important in determining the effect of cholesterol feeding on the plasma cholesterol level in this species (Lofland et al., 1972).

Bile acid excretion has been shown to be altered under a number of circumstances in man. It has been reported to be increased during carbohydrate feeding (Whyte et al., 1973), in obesity (Nestel, Schreibman and Ahrens, 1973; Miettinen, 1971a) and in some subjects with endogenous hypertriglyceridaemia (Miettinen, 1970a; Miettinen, 1971b; Sodhi and
and Kudchodkar, 1973). Patients with thyrotoxicosis may also have high rates of bile acid excretion (Miettinen, 1970b), a finding which is in accord with the observation that bile acid production is increased in animals by treatment with thyroid hormones (Bergström and Danielsson, 1963). There has been disagreement concerning the effects of polyunsaturated fatty acids on bile acid metabolism. While some investigators have reported an increased excretion (Wood et al, 1966; Moore et al, 1968; Connor et al, 1969; Nestel, Havenstein, Whyte et al, 1973), others have failed to observe such an effect (Wigan and Steinberg, 1965; Spritz et al, 1965; Grundy and Ahrens, 1970). Patients with type II hyperlipoproteinaemia have excretion rates which are either similar to (Lewis and Myant, 1967; Grundy and Ahrens, 1969) or less than (Miettinen, 1970a; Miettinen, 1970c; Miettinen et al, 1967) those of normal subjects.

Bile composition

Cholic, chenodeoxycholic, deoxycholic and lithocholic acids exist in normal human bile in the ratio 40:37:22:1 (van der Linden and Nakayama, 1969; Wood et al, 1972; Garbutt and Kenney, 1972). Altered bile composition occurs under a number of circumstances in man. The cholic acid/chenodeoxycholic acid ratio is increased during cholestyramine resin therapy, as is the ratio of primary to secondary bile acids (Garbutt and Kenney, 1972; Thistle and Schoenfield, 1969; Wood et al, 1972; Abaurre et al, 1969). The normal glycine/taurine ratio of 2-4/1 appears to be increased by factors enhancing bile acid synthesis, such as cholestyramine therapy (Garbutt and Kenney, 1972; Wood et al, 1972) and ileal disorders (Garbutt et al, 1969), while the opposite effect is produced by dietary taurine (Sjövall, 1959). The bile acid/cholesterol ratio may be decreased when biliary cholesterol secretion is enhanced by cholesterol feeding (Quintao et al, 1971a) or clofibrate therapy (Grundy, Ahrens, Salen et al, 1972), when bile acid content is diminished (Vlahcevic et al, 1970; Dowling et al, 1972) or when both of these factors are operative (Grundy, Metzger and Adler, 1972). Such considerations are important in the context of cholelithiasis.
PLASMA CHOLESTEROL ESTERIFICATION

It is well established that most plasma cholesterol exists as cholesteryl ester and that the free cholesterol/esterified cholesterol ratio differs in different lipoproteins (see later). The composition of plasma cholesteryl esters in man is very similar in the various lipoproteins, about 40% being linoleate, with the exception that very low density lipoprotein (VLDL) contains slightly more mono-unsaturated esters than other lipoproteins (Shiratori and Goodman, 1965; Nestel and Couzens, 1966a). That this may result from esterification of free cholesterol within plasma rather than within the liver was first demonstrated by Sperry (1935). Glomset (1968) showed that the reaction principally involves the transfer of a fatty acid from lecithin to cholesterol forming esterified cholesterol and lysolecithin. The enzyme responsible has, therefore, been termed plasma lecithin: cholesterol acyltransferase (LCAT). Further studies have shown that the human enzyme reacts predominantly with linoleic acid (Monger and Nestel, 1967a), and that cholesterol in high density lipoprotein (HDL) is more readily esterified than that in other lipoproteins (Glomset et al, 1966; Goodman, 1964; Akanuma and Glomset, 1968). Cholesterol esters are subsequently transferred from HDL to VLDL in exchange for triglyceride (Akanuma and Glomset, 1968; Nichols and Smith, 1965).

Nestel and Monger (1967) have suggested a method for measuring cholesteryl ester turnover in vivo by analysing the specific activity-time curves of plasma free and esterified cholesterol after the intravenous administration of radioactive mevalonic acid. Using this technique they have demonstrated that in vivo cholesteryl ester turnover approximates quantitatively to in vitro cholesterol esterification, suggesting that most, if not all, plasma cholesteryl esters derive in man from the plasma LCAT reaction. This conclusion is supported by comparisons of the specific activities of hepatic and plasma esterified cholesterol after an infusion of radioactive cholesterol (Nestel and Couzens, 1966b), by study of in vitro and in vivo patterns
of cholesterol esterification within the major lipoprotein classes (Goodman, 1964; Akanuma and Glomset, 1968), and by the extremely low levels of plasma cholesteryl esters in subjects with familial LCAT deficiency (Glomset et al, 1970). Thus, although the liver contains esterified as well as free cholesterol in association with a cholesterol esterifying enzyme (Monger and Nestel, 1967a), these may not be quantitatively important as precursors of plasma esterified cholesterol. This is in contrast with the rat, in which the liver appears to be an important source of plasma esterified cholesterol (Roheim et al, 1963).

Although the esterification of cholesterol by the intestinal mucosa is known to be important for cholesterol absorption (see previously), the physiological roles of hepatic and plasma cholesterol esterification in man have not been established. Reports of high rates of cholesteryl ester turnover in conditions of enhanced lipid transport suggest an important role in cholesterol and/or triglyceride turnover (McKenzie and Nestel, 1968; Nestel, 1970b; Nestel and Monger, 1967). Glomset (1968) has postulated that plasma cholesterol esterification is related to the transfer of cholesterol from peripheral tissues to the liver for elimination, a concept supported by studies of cholesterol efflux from erythrocytes (Murphy, 1962; Glomset, 1970). Boyd (1962) has suggested that the reaction is related to the metabolism of cholesterol to bile acids. Recent in vitro studies have indicated that the plasma LCAT reaction may play a role in VLDL conversion to low density lipoprotein (LDL) (Norum et al, 1971), a possibility that had been suggested previously on the basis of theoretical physio-chemical considerations (Schumaker and Adams, 1970). Hepatic cholesterol esterification may provide a mechanism for storing cholesterol within the liver.

Some possible mechanisms that may regulate cholesteryl ester turnover were reviewed by Goodman (1965), but at the present time the factors controlling LCAT activity have not been defined. Although the early work of Sperry and Stoyanoff (1938) suggested that the plasma bile salt concentration
might be involved, more recent work has excluded this possibility (Jones et al, 1971; Calandra et al, 1971). Since it has been shown that HDL cholesterol is the preferred substrate for the LCAT reaction, it is not surprising that LCAT activity is relatively high in neonates (Lacko et al, 1972) and low in patients with Tangier disease (Clifton-Bligh et al, 1972), in whom HDL levels are relatively high and low relative to those found in normal adults (Kwiterovich et al, 1973; Fredrickson et al, 1967). Poly-peptides having marked effects on LCAT activity have been isolated from HDL (Fielding et al, 1972a), and these may be important in the regulation of cholesteryl ester turnover.

In accord with animal work suggesting that plasma LCAT is secreted into plasma by the liver (Osuga and Portman, 1971; Brot et al, 1962), it has been shown that the increased ratio of plasma free to esterified cholesterol observed in human liver disease is associated with diminished plasma cholesterol esterifying activity (Calandra et al, 1971; Jones et al, 1971; Simon and Scheig, 1970). LCAT deficiency may occur as a rare familial disorder (Norum and Gjone, 1967; Gjone and Norum, 1968); the affected members have virtually no cholesteryl esters in the plasma and, interestingly, have mild hypertriglyceridaemia and diminished amounts of HDL.

**CHOLESTEROL TURNOVER IN MAN**

Studies of the overall turnover of exchangeable cholesterol in man have been made predominantly by the techniques of steroid balance and isotope dilution analysis. These methods will be discussed in the following chapter. Recent reviews of the subject have been made by Nestel (1970a), Myant (1971) and Miettinen (1970a).

Steroid balance techniques have indicated that cholesterol turnover is increased in obesity (Nestel, Schreibman and Ahrens, 1973; Miettinen, 1971a), cholestyramine therapy (Moutafis and Myant, 1969a; Nazir et al, 1972; Grundy et al, 1971) and after ileal surgery (Grundy et al, 1971; Moutafis et al, 1968; Miettinen, 1970a). Increased turnover has also been observed by some workers in
hypertriglyceridaemia (Sodhi and Kudchodkar, 1973; Miettinen, 1971b); however, other studies have failed to demonstrate this (Grundy and Ahrens, 1969). Diminished steroid excretion has been noted in myxoedema and hepatic cirrhosis and during starvation (Miettinen, 1970a), while subjects with type II hyperlipoproteinaemia appear to have either normal or low excretion rates (Miettinen, 1969; Miettinen, 1970a; Miettinen, 1971b; Lewis and Myant, 1967; Grundy and Ahrens, 1969).

The effects of dietary fatty acid composition on faecal steroid excretion have been investigated in several laboratories. Most recent studies have reported an increased steroid excretion, reflected in both the neutral and acidic fractions, on changing from a diet rich in saturated fatty acids to one rich in polyunsaturated fatty acids (Moore et al, 1968; Wood et al, 1966; Hellman et al, 1957; Connor et al, 1969; Grundy and Ahrens, 1966; Nestel, Havenstein, Whyte et al, 1973). In contrast, Avigan and Steinberg (1965), Spritz et al (1965), and Grundy and Ahrens (1970) could detect no consistent effect on faecal steroid excretion. The latter investigators attributed the plasma cholesterol-lowering action of polyunsaturated fatty acids to the transfer of plasma cholesterol into tissue stores, a process which has also been reported in rats (Gerson et al, 1961).

The isotope dilution technique has been extensively employed to measure the parameters of cholesterol turnover and distribution in normal subjects (Goodman and Noble, 1968; Nestel et al, 1969; Samuel and Perl, 1970; Sandhofer et al, 1972). Its use has confirmed the conclusions drawn from steroid balance studies that cholesterol turnover is increased in obesity (Nestel, Schreibman and Ahrens, 1973), during cholestyramine therapy (Goodman and Noble, 1968) and after ileal by-pass surgery (Moore et al, 1970). It has also provided further evidence that it may be decreased in chronic liver disease (Sandhofer et al, 1973), and increased in endogenous hypertriglyceridaemia (Sodhi and Kudchodkar, 1971). The method has failed, however, to detect altered cholesterol turnover in most subjects with type II hyperlipoproteinaemia (Goodman and Noble, 1968; Nestel et al, 1969;
Samuel and Perl, 1970; Samuel et al, 1972). Diminished turnover has been demonstrated during clofibrate therapy (Grundy, Ahrens, Salen et al, 1972), while it appears to be unaffected by neomycin (Samuel et al, 1968).

The isotope dilution technique also permits estimation of the size of body cholesterol pools. The pool of cholesterol which equilibrates rapidly with plasma cholesterol (see later) appears to be increased in familial type II hyperlipoproteinaemia (Samuel et al, 1972; Samuel and Perl, 1970), possibly due to the inclusion within it of the expanded pool of plasma cholesterol. The more slowly equilibrating pool, which probably contains much of the adipose tissue cholesterol, was reported to be increased in obesity (Nestel, Schreibman and Ahrens, 1973). The effect of antihypercholesterolaemic therapy on body cholesterol pools is also of considerable interest. Neomycin (Samuel et al, 1968), clofibrate (Grundy, Ahrens, Salen et al, 1972) and ileal by-pass surgery (Moore et al, 1970) have all been reported to reduce cholesterol pools in hypercholesterolaemic subjects, but this may not be true for cholestyramine (Goodman and Noble, 1968).

**BILE ACID TURNOVER IN MAN**

The metabolism of bile acids in man has been investigated most commonly by measuring their excretion in the faeces, by the technique of steroid balance. The results obtained in different patients and under varying circumstances are referred to in other sections. Additional information can be gained by isotope dilution techniques, which measure pool size as well as production rate. The technical aspects of this approach are discussed later. Values for the production rates and pool sizes of both primary bile acids in normal subjects have been defined by a number of investigators (Lindstedt and Ahrens, 1961; Danielsson et al, 1963; Vlahcevic et al, 1971; Lindstedt, 1957). Pool size has been reported to be diminished in patients with gall stone disease (Vlahcevic et al, 1970), myxoedema (Hellström and Lindstedt, 1964), type II hyperlipoproteinaemia (Einarsson and Hellström, 1972), and hepatic cirrhosis (Vlahcevic et al,
1972), due to a decreased synthesis of one or both primary bile acids. After ileal resection pool size is reduced due to a failure of hepatic synthesis to adequately compensate for increased faecal loss (Abaurre et al, 1969). This does not appear to occur during cholestyramine therapy, however, when bile acid pool size is maintained by enhanced cholesterol catabolism (Garbutt and Kenney, 1972). An expanded bile acid pool due to augmented synthesis has been reported in subjects with hyperlipoproteinaemia of types IV and V (Kottke, 1969; Einarsson and Hellström, 1972). Pool size is also expanded by the oral administration of chenodeoxycholic acid during attempts to dissolve cholesterol gall stones (Danzinger et al, 1972). No consistent changes in cholic acid pool size or production rate were observed during the administration of nicotinic acid (Wollenweber et al, 1967) or during the consumption of diets rich in polyunsaturated fatty acids (Hellström and Lindstedt, 1966; Lindstedt et al, 1965).

PLASMA LIPOPROTEINS

Cholesterol is transported in plasma as a component of plasma lipoproteins. These are stable water-soluble complexes of lipid, protein and carbohydrate, and have been classified into four major families on the basis of density, flotation rate in the analytical ultracentrifuge, and electrophoretic mobility. There have been several comprehensive reviews of this subject and only an outline will be given here. (Scanu, 1965; Nichols, 1967; Fredrickson et al, 1967; Hatch and Lees, 1968; Skipski, 1972; Smellie, 1971). Their classification and the physical characteristics on which this is based are summarised below:

<table>
<thead>
<tr>
<th>LIPOPROTEIN</th>
<th>FLOTATION (Sf)</th>
<th>MOBILITY (Paper electrophoresis)</th>
<th>DENSITY† (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicrons</td>
<td>&gt;400</td>
<td>Origin</td>
<td>&lt;0.95</td>
</tr>
<tr>
<td>Very Low Density</td>
<td>20-400</td>
<td>Pre-β</td>
<td>0.95-1.006</td>
</tr>
<tr>
<td>Lipoprotein (VLDL)</td>
<td></td>
<td>β</td>
<td>1.006-1.063</td>
</tr>
<tr>
<td>Low Density</td>
<td>0-20</td>
<td>α</td>
<td>1.063-1.21</td>
</tr>
<tr>
<td>Lipoprotein (LDL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Density</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoprotein (HDL)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Lipoprotein flotation rate in Svedberg units (10^-13 cm/sec/dyne/g) in NaCl solution of density 1.063 g/ml at 26°C.
† Separation at these flotation rates and densities is not entirely specific as discussed later.
**Chylomicrons**

Chylomicrons are the largest and least dense of the plasma lipoproteins, and rise to the top of plasma left standing for 18-24 hours at 4°C. They stay at the origin on paper or agarose gel electrophoresis and in the loading gel on polyacrylamide gel electrophoresis. In normal fasting plasma they are absent or present in trace amounts only. They serve mostly to transport dietary (exogenous) glycerides and cholesterol from the intestine to the bloodstream via the thoracic duct, and accordingly their plasma concentration rises after a fatty meal (alimentary hyperlipaemia).

Chylomicrons consist predominantly of glycerides (80-95% dry weight), with phospholipids, protein and free and esterified cholesterol comprising only 3-6%, 1-2%, 1-3% and 2-4% respectively (Levy et al, 1971). However, like other lipoproteins, chylomicrons have a range of composition and physical properties, their diameter varying from 750 to 6000 Å and their molecular weight from (0.4-30)x10^9 (Skipski, 1972). The larger chylomicrons have a greater relative content of glyceride than the smaller particles. Morphologically they appear to be spheroid (Lindgren and Nichols, 1960), with an outer membrane of predominantly phospholipid, free cholesterol and protein and a core of triglyceride and esterified cholesterol (Zilversmit, 1968).

The removal of chylomicrons from plasma in man is rapid, with a half-time for clearance of their triglyceride of 5-15 minutes (Nestel, 1964). The first step in chylomicron metabolism appears to be the removal of triglyceride by extrahepatic tissues (Nestel et al, 1962), leaving cholesterol-rich "remnants" which are subsequently cleared by the liver (Redgrave, 1970). The majority of chylomicron cholesterol is therefore removed by the liver (Nestel et al, 1963). The removal of the triglyceride appears to involve hydrolysis by lipoprotein lipase, an enzyme present in rich supply in the capillary endothelium of adipose tissue, heart and muscle (Robinson, 1964). Although the enzyme is essentially absent from normal plasma, it is released from tissues into plasma after an injection of heparin (Korn, 1959; Robinson and Jennings,
1965). This phenomenon is frequently employed in the assay of lipoprotein lipase activity, although La Rosa et al (1972) have recently drawn attention to the release by heparin not only of lipoprotein lipase but also of a hepatic triglyceride lipase. It has been shown that peptides of VLDL and HDL are potent activators of the enzyme (Bier and Havel, 1970; Fielding et al, 1970), and that these may be transferred from HDL to VLDL and chylomicrons during alimentary lipaemia (Havel et al, 1973).

**Very low density lipoproteins**

VLDL particles are spherical in shape and range in size from 300 to 800 Å and in molecular weight from 5 to 10 million (Levy et al, 1971; Forte et al, 1968). They transport the bulk of endogenously synthesized plasma triglyceride (Havel, 1961), and are synthesized predominantly in the liver (Windmueler and Levy, 1967) and to a lesser extent in the intestine (Windmueller and Levy, 1968; Ockner and Jones, 1970). In the rat at least, it has been shown that VLDL also transports newly synthesized cholesterol from the liver to the plasma (Roheim et al, 1963). The predominant lipid of VLDL is glyceride (50-70%), with phospholipids, free cholesterol, esterified cholesterol and protein accounting for 15-25%, 10%, 5% and 10% respectively. Gustafson et al (1965) examined the composition of several subfractions of VLDL. They observed that with increasing particle size (and decreasing density) the relative content of triglyceride and the ratio of free to esterified cholesterol increased, while the relative content of total cholesterol decreased. VLDL are rapidly metabolized with a plasma half-time for the protein component of 6 to 12 hours (Gitlin et al, 1958) and one of 1 to 3 hours for the triglyceride component (Havel, 1961; Farquhar et al, 1965). Although VLDL triglyceride has a slower fractional turnover than that of chylomicrons, the mechanisms for VLDL and chylomicron clearance appear to be similar (Levy et al, 1971). Accordingly, heparin-induced lipolysis *in vivo* causes VLDL concentrations to fall (Levy et al, 1966).
There is a growing body of evidence that VLDL metabolism results in a progressive conversion to smaller lipoproteins of increasing density. Thus, a precursor-product relationship has been demonstrated between the triglyceride fatty acid moieties of different subclasses of VLDL (Barter and Nestel, 1972), while a similar relationship exists between VLDL and LDL with respect to their triglyceride (Havel, 1961; Quarfordt et al, 1970) and protein moieties (Langer et al, 1970; Gitlin et al, 1958; Bilheimer et al, 1972; Eaton et al, 1973). The conversion of human VLDL protein to LDL protein has been demonstrated in monkeys by immunological techniques (Gulbrandsen et al, 1971). The conversion of whole VLDL into LDL has been observed both in vitro (Shore and Shore, 1962) and in subjects with abetalipoproteinaemia (Gulbrandsen et al, 1972). Therapy with nicotinic acid, which diminishes lipoprotein turnover (Langer and Levy, 1970), produces sequential falls in VLDL and then LDL lipids (Carlson et al, 1968), while heparin-induced lipolysis produces reciprocal changes in VLDL and LDL (Nichols et al, 1968). No evidence has been presented to date, however, that such a process is important for the transport of plasma cholesterol.

It should be noted that the distinction between chylomicrons and VLDL that is made on the basis of ultracentrifugal separation must take into account their considerable overlap. Thus, under conditions of enhanced triglyceride synthesis during carbohydrate feeding (Quarfordt et al, 1970) there is an increase in the plasma of endogenous particles having some of the physical characteristics of chylomicrons (Barter and Nestel, 1970), whereas during alimentary lipaemia dietary lipid may be transported within particles of VLDL proportions (Bierman and Strandness, 1965; Bergström et al, 1972).

Low density lipoproteins

Cholesterol exists in human plasma mostly within LDL, comprising about 45% of the molecule by weight. Protein, triglyceride and phospholipid account for about 25%, 10% and 20% respectively. Lee and Alaupovic (1970) isolated
6 subfractions of LDL, and reported that the relative contents of cholesteryl ester and protein increased with increasing density, while that of triglyceride decreased. The esterified to free cholesterol ratio increased from 2.9:1 in the subfraction of density 1.006-1.009 to 5.7:1 in the 1.053-1.063 subfraction. The molecular weight varies according to the method of determination but lies between 2 and 3 million (Shore and Shore, 1970). The molecules appear to be spherical with a diameter of 215 to 220 Å (Forte et al, 1968).

Studies of the kinetics of the triglyceride and protein moieties of lipoproteins have suggested that LDL is derived at least in part from the metabolism of VLDL (see previously). Bersot et al (1971) were able to demonstrate lipoproteins similar to VLDL and HDL, but not LDL, in the Golgi apparatus of rat liver homogenates. However, these findings do not completely exclude the possibility of LDL being formed to some extent within the liver (Margolis and Capuzzi, 1972). Protein-labelled LDL has a plasma half-life of 2.5 to 7 days (Hurley and Scott, 1970; Volwiler et al, 1955; Walton et al, 1965; Langer et al, 1972). By contrast, the half-times for turnover of the constituent lipids vary from 30 to 60 days for cholesterol (Lewis and Myant, 1967; Goodman and Noble, 1968; Nestel et al, 1969) and 8-12 hours for triglyceride (Havel, 1961). The sites and mechanisms of LDL clearance have not been clearly defined.

**High Density Lipoprotein**

HDL have a density range of 1.063 to 1.20 g/ml and are composed mainly of phospholipid (30%), cholesterol (17%) and protein (45-55%), with triglyceride composing only about 3%. The HDL spectrum is usually partitioned into 2 subclasses referred to as HDL₁ and HDL₂ (HDL₁ is recovered in the d<1.063 fraction and comprises the Sf0-3 cut). The former contains a higher proportion of protein than the latter and is of a lower molecular weight (150,000-180,000 compared with 340,000-380,000) (Shore and Shore, 1970). Glomset (1966) has pointed out that HDL comprise a wide spectrum
of molecules with the smaller ones probably having been secreted more recently into plasma before alterations in their structure have been brought about through interaction with plasma LCAT (see below).

There is evidence suggesting an interrelationship between HDL and VLDL: the acute fall in VLDL after an injection of heparin is accompanied by a reciprocal increase in HDL (Levy et al., 1966), and HDL levels also increase during the in vitro incubation of VLDL with post-heparin plasma (Shore and Shore, 1962); reciprocal changes in VLDL and HDL also occur during carbohydrate feeding, weight reduction and clofibrate therapy (Wilson and Lees, 1972); HDL contains peptides which activate lipoprotein lipase and which are transferred to chylomicrons and VLDL during their metabolism (Bier and Havel, 1970; Fielding et al., 1970; Havel et al., 1973). HDL also appears to play an important role in plasma cholesterol esterification: HDL free cholesterol is the preferential substrate for LCAT activity (Akanuma and Glomset, 1968) and the lipoprotein contains peptides which have marked effects on plasma LCAT activity (Fielding et al., 1972a); in addition, in vitro studies have demonstrated that esterified cholesterol is transferred from HDL to VLDL in exchange for triglyceride (Nichols and Smith, 1965). Glomset (1968) has suggested that HDL may play a role in the transfer of cholesterol from tissues into plasma, a concept supported by the accumulation of tissue cholesterol in familial HDL deficiency (Fredrickson et al., 1967) and by in vitro studies of cholesterol transfer between erythrocytes and plasma lipoproteins (Glomset, 1970).

HDL appears to be synthesized by the liver (Haft et al., 1962), but there is also evidence that some may be derived from the intestine (Rodbell et al., 1959). Labelled HDL protein has a biological half-time in man of 3 to 6 days (Gitlin et al., 1958; Furman et al., 1964). However, as is the case with LDL, the site and route of HDL clearance have not been defined.
Lipoprotein Proteins

Plasma lipoproteins contain a number of proteins (apolipoproteins), some of which occur in more than one class. The electrophoretic mobilities of different lipoprotein fractions, as well as their water-solubility, are related to the protein components. Recent reviews of the subject have been made by Shore and Shore (1972) and Alaupovic (1971). Early immunochemical studies suggested that three specific apoproteins were distributed throughout the lipoprotein spectrum, and these were termed apolipoproteins A, B and C (Alaupovic et al, 1964; Alaupovic, 1968; Gustafson et al, 1964; Gustafson et al, 1966). However, more recent studies with column chromatography have revealed that each of these lipoproteins consists of more than one polypeptide, and these are currently characterized by their carboxy-terminal amino-acids. Such studies have indicated that a greater degree of heterogeneity exists among lipoprotein polypeptides than earlier work had suggested.

HDL contains 2 major polypeptide components, which appear to be virtually confined to HDL and which together comprise the apolipoprotein A of immunochemical studies (Shore and Shore, 1970). These are found in both HDL₂ and HDL₃ subfractions. HDL also contains at least 3 other minor polypeptide components (Brown et al, 1970). These have been identified as being major components of VLDL protein, and together comprise the apolipoprotein family C. These polypeptides undergo a rapid and non-enzymic exchange between HDL and VLDL (Bilheimer et al, 1972), and appear to move from HDL to VLDL and chylomicrons during alimentary lipaemia (Havel et al, 1973). It is these polypeptides which appear to be important in regulating the activity of lipoprotein lipase (Bier and Havel, 1970; Fielding et al, 1970).

The other major protein component of VLDL was originally termed apoprotein B, since it is immunologically identical to the predominant protein component of LDL (or beta-lipoprotein). More recent studies have established that these apoproteins are almost certainly chemically identical also (Gotto et al, 1972). Evidence has been obtained that
apoprotein B consists of more than one polypeptide, as is the case with A and C proteins (Shore and Shore, 1969). Non-enzymic transfer of apoprotein B between VLDL and LDL does not occur (Bilheimer et al, 1972). However, an explanation for the existence of the same apoprotein within both lipoprotein fractions has been provided by the demonstration that LDL may be a product of VLDL metabolism (see previously).

Shore and Shore (1972) have shown that VLDL comprise a spectrum of heterogeneous lipoproteins containing an inconstant number of polypeptides, the differences in composition and physical properties of which suggest specific and complementary roles in VLDL structure and function. However, the reason for their variety and for their differing proportions in different subclasses of VLDL is not known.

There is at present little definitive information concerning the protein component of chylomicrons although all 3 apoproteins have been isolated (Alaupovic, 1971). Alaupovic (1971) has suggested that the classification of lipoproteins should be based not on their physical properties but on the immunochemically-definable apoproteins. According to this system a lipoprotein family would be a group of lipoproteins based on a single apoprotein and may have widely varying lipid composition and physical properties.

Factors Affecting Plasma Lipoprotein and Lipid Levels in Man

Reviews of this subject have been made by Fredrickson et al (1967), Scanu (1965), and Nichols (1967).

1. Age

Plasma concentrations of cholesterol (Barnes et al, 1972; Glueck et al, 1971a; Darmady et al, 1972) and triglyceride (Barnes et al, 1972; Kaplan and Lee, 1965) are low at birth. Although experiments with monkeys have suggested that more than 40% of foetal plasma cholesterol may be of maternal origin (Pitkin et al, 1972), the cholesterol level at birth is unrelated to that of the mother at delivery (Kaplan and Lee, 1965;
Glueck et al, 1971a; Barnes et al, 1972). Nor are neo­
natal plasma lipid levels related to birth weight (Barnes
et al, 1972) or maternal nutrition (Whyte and Yee, 1958).
A genetic determinant does, however, appear to exist and
is discussed later. Neonatal plasma contains a higher
proportion of HDL than does that of adults (Kwiterovich

Plasma lipid levels rise rapidly in most infants soon
after birth (Kaplan and Lee, 1965; Glueck et al, 1971a;
Darmady et al, 1972), and then continue to rise much more
slowly throughout life up to the age of about 50 years
(Fredrickson et al, 1967; Keys et al, 1950; Adlersberg et
al,1956; Leren and Haabrekke, 1971). After this age
levels change little in men or may even fall (Keys et
al, 1950; Carlons and Lindstedt, 1968; Adlersberg et al,
1956), while in women they frequently continue to rise
(Adlersberg et al, 1956; Carlson and Lindstedt, 1968;
Fredrickson et al, 1967), these changes reflecting
alterations in the concentrations of both VLDL and LDL
(Nichols, 1967; Fredrickson et al, 1967). It is noteworthy,
however, that some races, such as the highland population
of New Guinea (Goldrick et al, 1970), do not show these
age-related changes.

2. Sex

At birth and during early infancy females may have
slightly higher plasma cholesterol concentrations than
males (Darmady et al, 1972; Barnes et al, 1972), whereas
plasma triglyceride levels are virtually identical
(Barnes et al, 1972). In adulthood, however, males
tend to have higher cholesterol and triglyceride levels
than females, due to relatively higher concentrations of
VLDL and LDL, although after 50 years of age this trend
may be reversed (Leren and Haabrekke, 1971; Nichols,
1967; Adlersberg et al, 1956; Carlson and Lindstedt,
1968; Fredrickson et al, 1967). Levels of HDL tend to
be higher in women than in men (Nichols, 1967; Fredrick­
son et al, 1967). These differences cannot be related
to differences in adiposity, and may be hormonal in origin
(see later).
3. Genetic factors

Genetic factors other than sex appear to have important effects on plasma lipids and lipoproteins. It is necessary to distinguish between those affecting plasma lipoproteins in healthy persons and the inheritance of lipoprotein abnormalities.

Two kinds of data indicate that inheritance plays a role in determining the cholesterol level in healthy subjects, namely parent-child correlation and studies of twins. Schaefer et al (1958) studied over 200 healthy families and found that cholesterol levels in children were positively correlated with those of their parents, while the correlation between parents was not significant. This finding was later confirmed by Johnson et al (1965). In most twin studies the variance of plasma cholesterol level has been smaller in monozygotic than in dizygotic twins (Osborne et al, 1959; Jensen et al, 1965). The present consensus from such studies is that plasma cholesterol concentration in health is inherited in a polygenic manner. LDL shows genetic polymorphism in 2 apparently independent systems. These have been termed the Lp system, which has two variants (Berg, 1963), and the Ag system, which has four pairs of variants (Blumberg et al, 1962). One of these variants (LpA) may correspond with a recently identified LDL variant possessing pre-beta mobility (Rider et al, 1970). Sodhi (1969) reported an LDL variant with similar properties of density 1.040-1.060.

Certain racial differences in plasma lipoproteins may also be genetic in origin. Thus, the Masai of Kenya have low plasma cholesterol levels despite a diet rich in animal fat (Ho et al, 1968).

There are a number of abnormalities of lipoprotein metabolism which are due to inherited metabolic defects. These include the familial hyperlipoproteinaemias, abetalipoproteinaemia and Tangier disease.

The familial hyperlipoproteinaemias were originally classified into 5 types by Fredrickson et al (1967) on the basis of the lipoprotein distribution pattern. Type II
hyperlipoproteinaemia (familial hypercholesterolaemia) is one of the most common and is characterized by an increased level of LDL, in association with a normal or only moderately elevated level of VLDL. It seems likely that the condition demonstrates genetic heterogeneity, at least one form being transmitted as an autosomal dominant (Fredrickson, 1971; Schrott et al, 1972) and another being inherited in an apparently polygenic manner (Jensen and Blankenhorn, 1972). Although the biochemical defect has not been established, there is evidence suggesting a decreased plasma clearance of LDL protein (Langer et al, 1972) and of plasma cholesterol (Myant, 1971), while bile acid synthesis appears to be decreased in some individuals (Miettinen et al, 1967; Miettinen, 1970a). The other common form of familial hyperlipoproteinaemia (type IV) is characterized by an elevated level of VLDL, and also appears to be inherited in a heterogeneous manner (Rifkind, 1971). The metabolic defect is at present uncertain. Although it appears to be associated with an enhanced turnover of cholesterol (Sodhi and Kudchodkar, 1973), cholesteryl esters (Nestel, 1970b) and bile acids (Kottke, 1969), there is disagreement concerning the status of triglyceride metabolism, some studies suggesting a decreased removal (Boberg et al, 1971; Havel, 1970; Quarfordt et al, 1970) and others an increased production (Reaven et al, 1965; Nestel, 1966; Nikkila and Kekki, 1971) of VLDL triglyceride.

The other forms of familial hyperlipoproteinaemia are less common: type I results from a defect in the mechanism for clearing chylomicrons and is associated with diminished or absent post-heparin lipolytic activity, while hepatic triglyceride lipase appears normal (Krauss et al, 1972); type II hyperlipoproteinaemia is characterized by the presence of cholesterol-rich chylomicrons (Hazzard et al, 1970) and of VLDL having an abnormally high cholesterol content (Hazzard et al, 1972) and abnormal (β) electrophoretic mobility, possibly due to impaired conversion of VLDL to LDL (Bilheimer et al, 1971); type V hyperlipoproteinaemia comprises elevations of both VLDL and chylomicrons, the cause of which has not been resolved.
Abetalipoproteinaemia is inherited as an autosomal recessive and is characterized by the absence from plasma of VLDL and LDL, possibly due to defective synthesis of apoprotein B (Barclay, 1972). Tangier disease appears to be due to impaired synthesis of HDL, or of one of the peptides of apoprotein A, a double dose of an autosomal allele resulting in a marked reduction in the plasma concentration of HDL (Fredrickson et al., 1972).

4. Hormones

Oestrogens tend to lower plasma levels of cholesterol and increase those of triglyceride (Jensen, 1959; Oliver and Boyd, 1954; Doar and Wynn, 1970). These changes reflect increases in VLDL and HDL, while levels of LDL are decreased (Wynn et al., 1969; Hazzard et al., 1969; Furman et al., 1958; Furman et al., 1967), and are associated with increased cholesterol turnover (Nestel et al., 1965). Androgens, in contrast, tend to increase levels of LDL and decrease those of HDL (Furman et al., 1958; Furman et al., 1967).

The differing effects of sex hormones on lipoprotein metabolism may explain at least in part the sex differences in lipoprotein patterns already described. Also important in this context are the effects of oral contraceptives on plasma lipoproteins. Combinations of an oestrogen and a progestagen for this purpose produce elevations of both cholesterol and triglyceride due to alterations in lipoproteins of Sf 0-12 and Sf 20-400 (Aurell et al., 1966; Wynn et al., 1969). Stokes and Wynn (1971) compared several different combinations, and concluded that the hypertriglyceridaemic effect increased with increasing oestrogen content, while the cholesterol-raising property increased with the progestagen content. These changes are associated with decreased post-heparin lipolytic activity (Bierman et al., 1970).

The effects of sex hormones on plasma lipoprotein metabolism may also be relevant to the changes in plasma lipids which occur during pregnancy and the menstrual cycle. There is general agreement that plasma levels of cholesterol,
phospholipid and triglyceride show a progressive rise during pregnancy (Oliver and Boyd, 1955; Svanborg and Vikrot, 1965a). However, whereas Oliver and Boyd reported that lipid levels reached a maximum between the 31st and 33rd weeks and then slowly declined, Svanborg and Vikrot observed a progressive increase until delivery. In accordance with the observation that these changes were most marked in triglyceride (Svanborg and Vikrot, 1965a), Pantelakis et al (1964) noted that the predominant changes were in VLDL.

During the first week after delivery there is a decline in the level of all 3 lipids (Svanborg and Vikrot, 1965b). However, Oliver and Boyd (1955) noted that cholesterol levels were still relatively high 20 weeks later.

Oliver and Boyd (1953) noted cyclical changes in the plasma levels of phospholipid and esterified cholesterol in healthy young females, which were related to the menstrual cycle. The lowest levels occurred at about the time of ovulation.

There has been disagreement concerning the effects of glucocorticoids on lipoprotein metabolism, and further work is required in this area. Whereas Adlersberg et al (1950) observed marked increases in plasma lipids during treatment with cortisone or ACTH, Conn et al (1950) reported profound reductions in cholesterol levels during ACTH administration.

Lipoprotein metabolism is also affected by catecholamines. Adrenaline stimulates the release of free fatty acids from adipose tissue (Feigelson et al, 1961), and produces a later rise in VLDL and LDL in experimental animals (Drury, 1957). This latter change is likely to be a direct effect of the increased transport of free fatty acids to the liver (Nestel and Steinberg, 1963).

Alterations in plasma lipoproteins occur in insulin-sensitive diabetes mellitus. They appear to be a direct consequence of a deficiency of insulin and are corrected by its administration.

The abnormalities in VLDL and LDL which are characteristic of myxoedema and thyrotoxicosis (see later) appear to be due to an insufficiency or excess of thyroid hormone.
Thyroxine also reduces cholesterol levels in some cases of euthyroid hypercholesterolaemia (Eisalo et al., 1963). This lipid-lowering effect is retained by the D-isomer, which has also been employed in the treatment of hyperlipoproteinaemia (Strisower and Strisower, 1964).

5. Diet

A number of dietary constituents have profound effects on plasma lipoproteins.

A. Fatty Acids

(i) Saturated Fatty Acids

The saturated fatty acids palmitic (C\text{16}) , myristic (C\text{14}) and lauric (C\text{12}) acid increase the plasma concentrations of cholesterol and LDL, but not those of VLDL, when fed by isocaloric substitution for carbohydrate or other fats (Keys et al., 1965; Grande et al., 1970). This effect appears to be twice as potent as the cholesterol-lowering effect of polyunsaturated fatty acids (see below) (Keys et al., 1965).

Saturated fatty acids with less than 12 carbon atoms and stearic acid (C\text{18}) have no effect on plasma cholesterol levels but elevate those of triglyceride (Keys et al., 1965; Hashim et al., 1960).

(ii) Monounsaturated Fatty Acids

Dietary monounsaturated fatty acids (principally oleic acid) have little or no effect in this respect (Hegsted et al., 1965; Keys et al., 1965).

(iii) Polyunsaturated Fatty Acids

Diets rich in polyunsaturated fatty acids, such as linoleic acid, lower plasma concentrations of cholesterol and LDL (Kinsell et al., 1952; Keys et al., 1957; Keys et al., 1965), and this is associated with a reduction in the cholesterol/phospholipid ratio within LDL (Spritz and Mishkel, 1969). There appears to be an important interplay between dietary fat and cholesterol in this context. Connor et al. (1964) reported that increasing the dietary content of polyunsaturated fatty acids had no effect in subjects consuming a cholesterol-free diet with P/S ratios of
up to 2.6. However, the plasma cholesterol was lowered when the ratio was over 4 (Connor et al, 1969). Macdonald (1972) has reported that the cholesterol-lowering effect is also influenced by the nature of dietary carbohydrate. The mechanism by which polyunsaturated fats lower cholesterol levels has not been clearly defined (see previously).

Polyunsaturated fatty acids also reduce the plasma concentration of VLDL, and therefore of triglyceride also (Ahrens et al, 1957; Nestel, Havenstein, Whyte et al, 1973; Macdonald, 1972). The mechanism responsible appears to be a stimulation of triglyceride clearance mechanisms (Nestel and Barter, 1973), possibly due to an effect on lipoprotein lipase (Pawar and Tidwell, 1968), as well as to the preferential incorporation of saturated fats into plasma triglyceride (Nestel and Barter, 1971).

B. Cholesterol

There have been conflicting reports as to the importance of dietary cholesterol as a determinant of plasma cholesterol level. Hegsted et al (1965) and Mattson et al (1972) observed a linear relationship between dietary and plasma cholesterol levels when the former ranged from 100 to 700 mg/day and from 0 to 317 mg/1000 kcal/day respectively. However, Keys et al (1965) noted a curvilinear relationship within the range 50 to 1450 mg/day. Connor et al (1961) and Beveridge et al (1960) reported that once the daily intake of cholesterol had reached 475 and 634 mg respectively, further increments had little additional effect on plasma cholesterol concentration.

It is apparent from a number of studies that there is an important interplay between dietary cholesterol and fat composition. The results reported by Connor et al (1964) have already been mentioned, while Brown and Page (1965) reported that in order to achieve a 20% reduction in plasma cholesterol level the requirement for polyunsaturated fatty acids increases with increasing cholesterol intake.
C. **Plant sterols**

Dietary plant sterols have been shown to exert a cholesterol-lowering effect in man (Best et al, 1958). This is observed however, only when they are consumed in abnormally large amounts (eg. 5-10 g daily). Although the mechanism responsible for this effect is not clear (Subbiah, 1971), it may be a consequence of impaired cholesterol absorption (Grundy et al, 1969).

D. **Carbohydrate**

In most normal subjects diets rich in carbohydrate produce an increase in plasma triglyceride level, which reaches a peak in 7-14 days and then partially subsides (Ahrens et al, 1957; Brown and Page, 1960; Lees and Fredrickson, 1965). However, young lean individuals, especially females, may show little change (Macdonald, 1965). In patients with pre-existing hypertriglyceridaemia the response tends to be exaggerated and persistent (Ahrens et al, 1957; Ruderman et al, 1971). The hypertriglyceridaemia reflects an increase in VLDL (Ruderman et al, 1971; Schonfeld, 1970; Barter and Nestel, 1970). The increase in VLDL triglyceride exceeds that in VLDL cholesterol and VLDL protein (Schonfeld, 1970; Ruderman et al, 1971), changes which reflect an increase in VLDL particle size (Ruderman et al, 1971; Barter and Nestel, 1970). These changes in VLDL are accompanied by reductions in LDL (Ruderman et al, 1971; Wilson and Lees, 1972) and HDL (Wilson and Lees, 1972). The increase in VLDL levels appears to be due to enhanced VLDL production. Reaven et al (1965), Nestel (1966), Quarfordt et al (1970), Wolfe and Ahuja (1972) and Nestel and Barter (1973) all reported an increased synthesis of VLDL triglyceride, while increased VLDL protein synthesis has been observed in rats (Eaton and Kipnis, 1969). However, Quarfordt et al (1970) and Nestel et al (1970) concluded that decreased triglyceride clearance is also partly responsible. The associated changes in cholesterol metabolism have been less well investigated, but early studies suggest both increased plasma cholesteryl ester turnover (Nestel, 1970b) and enhanced bile acid production (Whyte et al, 1973).
Macdonald (1972) has recently compared the effects of different carbohydrates and their relationship to dietary fats. In men, triglyceride levels were higher with fructose than with glucose when fat consisted of sunflower seed oil. This effect was not seen with cream in the diet. With both dietary fats triglyceride concentrations were higher with starch than with glucose. The important inter-relationship of the kind of fat with the type of carbohydrate has also been emphasized by Antar et al (1970).

E. Alcohol

Oral or intravenous ethanol produces endogenous hypertriglyceridaemia in a proportion of individuals (Losowsky et al, 1963; Jones et al, 1963). Although low post-heparin lipolytic activity has been reported in chronic alcoholism (Jones et al, 1963), Chait et al (1972) concluded that the mechanism is unlikely to be one of deficient removal. This is supported by the demonstration in animals of increased VLDL secretion during alcohol consumption (Mistilis and Ockner, 1972; Baraona et al, 1973). Such an effect might be secondary to increased substrate availability as a consequence of increased fatty acid esterification in the liver (Nestel and Hirsch, 1965; Wolfe, 1969), or of decreased hepatic fatty acid oxidation (Lieber and Schmid, 1961).

F. Other Dietary Components

Diminished levels of plasma cholesterol have been reported during protein malnutrition (Lewis et al, 1964), after prolonged starvation (Jackson, 1969) (which also lowers triglyceride), and during the consumption of diets rich in vegetable matter such as pectin (Keys et al, 1961), legumes (Grande et al, 1965) and Bengal gram (Mathur et al, 1968). This latter effect may be due to a bile acid-sequestering effect similar to, but weaker than, that exerted by cholestyramine resin (Eastwood and Hamilton, 1968).

Increased levels of plasma cholesterol and LDL have been noted during Vitamin D supplementation (Fleischman
et al, 1970) and during the acute phase of starvation (Ende, 1962). Elevated levels of triglyceride are produced by essential fatty acid deficiency (linoleic acid). This is due to the presence of an abnormal triglyceride-rich lipoprotein of pre-beta mobility and density 1.08-1.12 g/ml (Collins et al, 1970).

6. Other Physiological Factors

A number of physiological factors may alter plasma lipoprotein concentrations. Concentrations of both cholesterol and triglyceride are higher when the subject is in the standing position than when supine or sitting. These changes correlate with those in packed cell volume and appear to be due to haemoconcentration (Tan et al, 1972).

The consumption of 1-2 g of fat per Kg body weight is followed by the appearance in plasma of chylomicrons and a late increase in endogenous and exogenous particles having the size of large VLDL. In normal individuals peak lipaemia is reached after 2-4 hours and the hypertri-glyceridaemia persists for 6 to 8 hours (World Health Organization, 1970).

Seasonal variations in plasma cholesterol level have been reported by Thomas et al (1961), and by Carlson and Lindstedt (1968), who observed a maximum mean concentration in the winter and a minimum in the summer. These findings could not, however, be confirmed by Samuel, Lieberman, Shmase et al (1970) in patients with atherosclerosis. Nor was there any evidence of a seasonal variation in cholesterol level in the data presented by Fleischman et al (1967).

Emotional stress has been reported by several investigators to increase plasma cholesterol levels. These studies have been recently reviewed by Friedman and Rosenman (1971).

The relationship of regular physical activity to plasma lipid levels has been reviewed by Fox et al (1971) and Hunter et al (1971A). Most studies have reported either no change or a variable decrease in cholesterol concentration and a fall in that of triglyceride. Carlson and
Mossfeldt (1964) examined the response of plasma lipoproteins to prolonged heavy exercise. Although no change in the cholesterol content of any lipoprotein could be detected, the triglyceride and phospholipid content fell in all lipoproteins, especially the triglyceride within VLDL.

7. **Pathological States**

A variety of pathological conditions may be associated with abnormalities in lipoprotein metabolism.

**Hypothyroidism** is frequently associated with elevated plasma levels of LDL and cholesterol (Peters and Man, 1950; Furman et al, 1961), changes which may be due to a decreased clearance of LDL protein (Walton et al, 1965) and cholesterol (Kritchevsky, 1960; Miettinen, 1968a; Mitropoulos and Myant, 1965) from the plasma. The plasma concentrations of VLDL and triglyceride may be normal but are commonly increased (Furman et al, 1961; Malmros and Swahn, 1953). This latter change may derive from a decreased clearance of triglyceride (Tulloch et al, 1973; Nikkilä and Kekki,1972), possibly as a consequence of diminished lipoprotein lipase activity (Tulloch et al, 1973). Thus, myxoedema may be associated with secondary hyperlipoproteinaemia of type IIa, IIb or IV.

Changes in **thyrotoxicosis** are generally less prominent than those in myxoedema, but it may be associated with diminished levels of LDL (Lindgren and Nichols, 1960) due to an increased LDL catabolism (Walton et al, 1965). The changes reported in triglyceride level have been variable and generally small (Nikkilä and Kekki, 1972). Seidel and Wieland (1971) have recently identified an abnormal lipoprotein of high density, beta mobility, high protein content and low cholesterol content in patients with hyperthyroidism.

Renal disease may also be associated with secondary hyperlipoproteinaemias. This is most noticeable in the **nephrotic syndrome**, which may be associated with elevations of LDL and VLDL, and on occasions of chylomicrons also (Gitlin et al, 1958; Baxter, 1962). The lipoprotein
pattern may accordingly be of types II, IV or V. The degree of hyperlipidaemia is related to those of hypo-albuminaemia and proteinuria (Thomas et al, 1951; McKenzie and Nestel, 1968).

These abnormalities in lipoprotein levels are associated with an increased fractional catabolic rate of the protein of both HDL and lipoproteins of Sf 3-9 (Gitlin et al, 1958). Associated disturbances of lipid metabolism include increased turnover of both plasma triglyceride and plasma cholesteryl esters (McKenzie and Nestel, 1968).

Severe uncontrolled insulin-sensitive diabetes mellitus is almost invariably associated with hyperlipoproteinaemia of types IV or V (Fredrickson et al, 1967). This appears to be due to a decreased plasma triglyceride clearance (Boberg et al, 1969), secondary to diminished activity of lipoprotein lipase (Bierman et al, 1970). Both abnormalities are reversed by the administration of insulin (Bierman et al, 1970).

**Obesity**

The most prominent lipoprotein abnormality in obesity is an elevated concentration of VLDL (Wilson and Lees, 1972) with an associated hypertriglyceridaemia (Ford et al, 1968; Albrink and Meigs, 1964). Although levels of LDL and HDL tend to decrease with weight gain (Wilson and Lees, 1972), this effect appears to be quantitatively less than the rise in VLDL. Accordingly, in a recent large scale study plasma cholesterol was positively correlated, albeit weakly, with relative body weight and adiposity (Keys et al, 1972). This is in agreement with some previous reports (Tanner, 1951), although other workers have failed to detect such a relationship (Thomas and Garn, 1960). These changes are associated with an increased turnover of cholesterol (Nestel et al, 1969; Miettinen, 1971a), triglyceride and free fatty acids (Nestel and Whyte, 1968). Associated abnormalities include elevated fasting levels of plasma insulin (Kreisberg et al, 1967) and low levels of plasma growth hormone (Hunter et al, 1966).
Obstructive biliary disease is characterized by the presence of an abnormal lipoprotein (designated lipoprotein-X) of average Sf 16, which is rich in phospholipid and unesterified cholesterol. The protein moiety consists of 40% albumin and 60% apolipoprotein C. HDL concentrations are low. The plasma accordingly has increased levels of unesterified cholesterol and phospholipid, and diminished levels of esterified cholesterol (Seidel et al, 1969; Rifkind, 1971; Mills et al, 1969).

Chronic parenchymal liver disease is frequently associated with an increased ratio of free to esterified cholesterol, which appears to be due to a decrease in the net cholesterol esterifying activity of plasma (Calandra et al, 1971). This in turn may reflect impaired LCAT activity or the presence of cholesteryl ester hydrolases (Jones et al, 1971). With severe hepatocellular failure there is a lowering of all lipoprotein fractions (Fredrickson et al, 1967; Walton et al, 1965).

The plasma lipoproteins in familial LCAT deficiency are also characterized by an abnormally high content of unesterified cholesterol, lecithin and triglyceride, abnormalities which appear to be a direct consequence of diminished enzyme activity (Glomset et al, 1970; Norum et al, 1971). The abnormal lipoprotein-X has also been identified in the plasma of patients with this condition (Torsvik et al, 1972).

Other conditions sometimes associated with hyperlipoproteinaemia include pancreatitis, which may co-exist with the type I, IV and V patterns of hyperlipoproteinaemia (Fredrickson et al, 1967), acute porphyria (Lees et al, 1970), idiopathic hypercalcaemia (Kayden et al, 1962), and glycogen and lipid storage diseases (Fredrickson et al, 1967). Hypolipoproteinaemia may occur during malabsorption (Lindgren and Nichols, 1960). Certain dysproteinaemias may be associated with either elevated or depressed plasma lipid levels. These include myeloma, macroglobulinaemia and cryoglobulinaemia, which may be responsible for the sudden appearance of severe hyperlipoproteinaemia (Fredrickson et al, 1970). In the case of
myeloma, at least, this may be due to the presence within plasma of antilipoprotein antibodies, which form complexes with plasma lipoproteins (Beaumont, 1970).

Acute infections, both viral and bacterial, have been associated with a variety of disturbances of lipoprotein metabolism (Beisel and Fiser, 1970). Viral infections in particular have been reported to decrease plasma levels of cholesterol and LDL (Page and Lewis, 1969; Lees et al, 1972).

Acute myocardial infarction is followed by marked changes in plasma lipoproteins. Tibblin and Cramer (1963) reported that levels of phospholipid and cholesterol fell during the first week after an infarction, and then gradually returned to normal by 3 weeks. Triglyceride levels increased from the second day to reach a maximum after 3 weeks; concentrations then slowly decreased over the following year. Ileal by-pass surgery produces a fall in plasma cholesterol and LDL levels by increasing the catabolism of cholesterol to bile acids, and possibly also by interfering with cholesterol absorption (Grundy et al, 1971; Buchwald et al, 1970). It may be performed for this purpose in the treatment of severe type II hyperlipoproteinaemia.

8. Drugs

Hyperlipoproteinaemia of types II, III and IV is associated with an increased risk of coronary artery disease (Kannel et al, 1971; Heinle et al, 1969; Patterson and Slack, 1972; Carlson and Böttiger, 1972). For this reason drugs have been developed for the treatment of these conditions in the hope that the reduction in plasma lipid levels will be associated with a concomitant reduction in the development of atherosclerosis. The use of drug therapy in hyperlipoproteinaemia has recently been reviewed by Lees and Wilson (1971) and by Levy et al (1972). The use of D-thyroxine for this purpose has already been mentioned. The bile acid-sequestering resin cholestyramine increases the catabolism of cholesterol to bile acids by interrupting their enterohepatic circulation
(Moutafis and Myant, 1969a; Nazir et al, 1972; Grundy et al, 1971; Miettinen, 1970c). Although there is an associated increase in cholesterol synthesis, plasma cholesterol levels are generally lowered, particularly in type II hyperlipoproteinaemia (Levy and Fredrickson, 1970; Levy et al, 1972). Persons homozygous for this condition may, however, show little or no change in plasma cholesterol level (Moutafis et al, 1971; Khachadurian, 1968), and some subjects demonstrate an increase (Grundy et al, 1971). A number of trials of cholestyramine have been associated with increased levels of VLDL and triglyceride (Grundy et al, 1971; Wood et al, 1972; Jones and Dobrilovic, 1970). The effects on cholesterol metabolism are associated with an increased fractional turnover of LDL protein (Langer et al, 1969). DEAE Sephadex appears to have a similar mode of action (Miettinen, 1970a).

Inhibitors of cholesterol synthesis have also been investigated as cholesterol-lowering agents (Steinberg, 1970). Most such drugs have proved unsuitable, however, because of toxic effects, which have included the accumulation of potentially atherogenic cholesterol precursors and adrenal insufficiency (Steinberg, 1962). Two drugs in common use whose mode of action appears to include, but is not restricted to, diminished cholesterogenesis are clofibrate and nicotinic acid. Clofibrate is particularly effective in type III hyperlipoproteinaemia (Levy et al, 1969) and may also be of value in type IV (Brown and Doyle, 1967; Hunninghake et al, 1969; Strisower et al, 1968). It is of less value in type II hyperlipoproteinaemia, however, and may even lead to increased levels of LDL and cholesterol in this condition (Levy et al, 1969; Strisower et al, 1968). Although the principal effect of clofibrate on cholesterol metabolism is that of inhibition of synthesis (Avoy et al, 1965; Grundy, Ahrens, Salen et al, 1972; Fulton and Hsia, 1972), increased faecal neutral steroid excretion has also been reported (Grundy, Ahrens, Salen et al, 1972; Horlick et al, 1971). In addition, clofibrate appears to have complex effects on triglyceride, and an increase in those of LDL, HDL and free fatty acids (Sieren et al, 1970; Nichols et al, 1968).
other aspects of lipid metabolism. These may include decreased free fatty acid mobilization (Steinberg, 1970), decreased VLDL secretion by the liver (Duncan et al, 1964), inhibition of triglyceride synthesis (Pereira and Holland, 1970) and increased triglyceride clearance (Nestel and Austin, 1968).

Nicotinic acid may reduce the levels of both VLDL and LDL, and is of frequent value in treating both hypercholesterolaemia and endogenous hypertriglyceridaemia (Carlson, 1969). Evidence for diminished cholesterol synthesis has been gained principally from animal experiments (Holmes, 1964). The flattening of the plasma cholesterol specific activity-time curve which has been noted in some patients during nicotinic acid therapy (Miettinen, 1968b; Myant, 1971) is compatible with such an effect, although this would also be produced by mobilization of tissue cholesterol. Other effects of nicotinic acid on lipid metabolism include increased faecal steroid excretion (Miettinen, 1968b) and inhibition of adipose tissue lipolysis (Carlson, 1967).

Neomycin has been found to reduce the plasma cholesterol level by 10-36% (Samuel et al, 1967). The mode of action has not been clearly defined, but appears to be independent of antibacterial activity (Van den Bosch and Claes, 1967) and may derive from increased cholesterol and bile acid excretion (Powell et al, 1962).

It has been recently reported that progestagens may be effective in treating type V hyperlipoproteinaemia, possibly by increasing lipoprotein lipase activity (Glueck et al, 1969).

A number of other drugs have been demonstrated to alter plasma lipoproteins, although they are not used in the treatment of hyperlipoproteinaemia. Decreased plasma cholesterol levels have been reported during therapy with para-aminosalicylic acid, kanamycin and chlortetracycline (Samuel and Waithe, 1961). The appearance of lipolytic activity in plasma after intravenous heparin is associated with a fall in the plasma concentrations of VLDL and triglyceride, and an increase in those of LDL, HDL and free fatty acids (Bierman et al, 1970; Nichols et al, 1968).
The various laboratory procedures employed in the present work are described in detail in each Chapter as appropriate. The present section discusses the principal methods used in a general way.

**CHOLESTEROL TURNOVER IN MAN**

The available techniques for studying human cholesterol turnover have been reviewed by Mattinen (1970a), Grundy and Ahrens (1969) and Nestel (1970a). Cholesterol turnover represents the balance between the entry of new cholesterol into body pools, by absorption from the diet and by endogenous synthesis, and its output in the form of faecal neutral and acidic sterides. It can be measured by two techniques, steroid balance and isotope dilution analysis. Both methods require steady state conditions. While it is impossible to establish with certainty that such conditions exist, the criteria of unchanging body weight, constancy of diet and stable plasma lipid levels are usually accepted as indicative of at least a near steady state for such purposes.

**CHAPTER 3**

**METHODS**

**Isotope Dilution Analysis**

Analysis of the plasma cholesterol specific activity-time curve described after the intravenous infusion of a known dose of radioactive cholesterol can provide estimates of both cholesterol pool size and cholesterol turnover. The radiolabelled cholesterol is usually administered in one of the following two forms:

1. Dispersed in 0.9% saline (Spritz et al., 1963; Grundy et al., 1971; Grundy and Ahrens, 1969).

   A small volume (0.5–1.0 ml) of an ethanolic solution of radioactive cholesterol is added to 150–200 ml 0.9% saline and the mixture immediately infused over 15–20 minutes.


   A variety of techniques have been described for complexing radiolabelled cholesterol with plasma
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   A variety of techniques have been described for complexing radiolabelled cholesterol with plasma lipoproteins.
lipoproteins \textit{in vitro}. In the present studies about 0.5 ml of an ethanolic solution of radiolabelled cholesterol, shown previously to be sterile, was added dropwise to 20 ml of the subject's own freshly drawn plasma, and the mixture incubated for 1 hour at 37°C (Nestel et al., 1965).

Porte and Havel (1961) reported that radioactive cholesterol equilibrates among the lipoproteins when added to plasma \textit{in vitro}, and suggested that such preparations resembled biosynthetically labelled plasma lipoproteins. The validity of this suggestion has, however, been questioned by Rose (1968), who showed that the addition of an ethanolic solution of cholesterol to plasma produces abnormal particles composed mainly of cholesterol and \(\beta\)-lipoprotein. The size and properties of these particles varied with the concentration of the solution. The smaller particles, produced by the use of dilute ethanolic solutions, appeared to be the more "physiological" in their interaction with erythrocytes, plasma LCAT and plasma lipoproteins.

These 2 methods of infusing radioactive cholesterol have recently been compared in the rat by Nilsson and Zilver-smit (1972). These workers confirmed previous reports that cholesterol in suspension (Method i, above) is rapidly removed from the circulation and later reappears within plasma lipoproteins (Avigan, 1959; Portman and Sinisterra, 1957). It was also shown that the early disappearance of suspended cholesterol is due mainly to phagocytosis by Kupffer cells in the liver. However, the later release of cholesterol is rapid and complete, so that the subsequent decline of plasma cholesterol specific activity is identical to that obtained with an equal dose of a lipoprotein preparation. With both methods the specific activity of free cholesterol exceeds that of esterified cholesterol for the first few days, after which they are essentially the same (Kurland et al., 1961; Nestel et al., 1965; Nestel et al., 1969; Nilsson and Zilver-smit, 1972).

The resulting plasma cholesterol specific activity-time curve was originally analysed in man as a single...
However, Lewis and Myant (1967) reported values for cholesterol turnover with this method which were considerably greater than those obtained by steroid balance (see below), and suggested that this reflected a failure of such analysis to consider the effect of slowly equilibrating pools of tissue cholesterol. The existence of the latter was apparent both from early studies of tissue cholesterol specific activity (Chobanian and Hollander, 1962) and from the shape of the log plasma cholesterol specific activity-time curve, which describes a curve for the first 4-6 weeks before becoming approximately linear. Accordingly, Goodman and Noble (1968) analysed such curves into 2 exponentials and fitted them to a 2-pool model of cholesterol turnover, using the equations of Gurpide et al (1964). In this the body exchangeable cholesterol is conceived as behaving as 2 pools, one which equilibrates relatively rapidly with that of plasma (Pool A) and another which equilibrates more slowly (Pool B) (Figure 3-1).

Studies of tissue cholesterol radioactivity had indicated that most cholesterol within erythrocytes, the liver and possibly also the lung, ileum and spleen belongs to the rapidly equilibrating pool (Gould et al, 1955; Chobanian and Hollander, 1962; Lindstedt, 1962; Nestel and Couzens, 1966b), while most of that within other tissues exchanges more slowly, or in the case of the adult brain, possibly not at all.

These observations have recently been confirmed in the baboon by Wilson (1970). It should be emphasised, however, that such pools represent mathematical constructs without physical meaning, and that probably all tissues contain cholesterol belonging to more than one pool.

From knowledge of the dose of radioactivity and the characteristics of the specific activity decay curve Goodman and Noble (1968) calculated a number of model parameters, namely, the size of Pool A (M_a); the rate constant for total removal of cholesterol from Pool A (k_a), which includes transfer to Pool B (k_ab) and excretion from Pool A (k_a); the rate constant for total removal from Pool B (k_bb = k_ba + k_b); and the production rate of cholesterol in Pool A (PR_a) defined as the entry of "new" cholesterol into that
pool (from synthesis and absorption), excluding recycled material originating in Pool A. Only these parameters can be calculated without making assumptions concerning cholesterol synthesis within Pool B (PRB) and the excretion of cholesterol from Pool B (kB). Estimates of cholesterol turnover by this procedure are lower than those derived from 1-pool analysis (Grundy and Ahrens, 1969), but are nevertheless still higher than those obtained by steroid balance (Grundy and Ahrens, 1969), although the difference appears to be less in obese individuals than in subjects of average body weight (Nestel, Schreibman and Ahrens, 1973). This is partly explained by the recent demonstration that 2-pool analysis is still an oversimplification, since it ignores an early very rapid decline in plasma cholesterol specific activity (Samuel et al, 1968) and the existence of an extremely slowly equilibrating pool of cholesterol. Evidence for the latter has been gained by following plasma specific activity-time curves for a year or more (Samuel and Perl, 1970; Goodman et al, 1973; Samuel and Lieberman, 1973; Samuel et al, 1972) and by more detailed examination of tissue cholesterol specific activity than had been previously performed (Wilson, 1970; Myant, 1971; Quintao et al, 1971b; Samuel et al, 1972). Such cholesterol appears to include much of that within xanthomata, muscle, skin and colon.

Wilson (1970) has reported that 2-pool analysis gives values for the pool size of total exchangeable cholesterol (Mₐ + Mₐ) in the baboon similar to those obtained by carcass analysis. However, the calculation of Pool B by isotope dilution requires the assumption that cholesterol enters and leaves the system only via Pool A (PRₐ=0,kₐ=0), and this may not be true for man. Thus, it has recently been reported that cholesterol leaves Pool B via the human skin at a rate corresponding to about 8% of cholesterol turnover (Bhattacharyya et al, 1972), and that skin and adipose tissue may make notable contributions to total cholesterol synthesis in man (Fulton and Hsia, 1972; Angel and Farkas, 1971). For these reasons only the size of Pool A has been calculated in the present studies.
Figure 3-1
Two-pool model of cholesterol turnover.
Steroid balance

This involves the determination of the faecal excretion of endogenous neutral and acidic steroids, the sum of which is considered to represent the turnover of total body exchangeable cholesterol, on the assumption that virtually all steroid excretion occurs via this route. Although it is now known that some cholesterol is also excreted via the skin (Bhattacharyya et al, 1972) and metabolized by the adrenal glands (Borkowski et al, 1972), faecal analysis nevertheless provides a valuable means of studying cholesterol metabolism. A major advantage over isotope dilution is the requirement for a relatively short study period only (1-2 weeks compared with 10 or more weeks). In addition, steroid balance enables neutral and acidic steroids to be examined separately.

The results obtained by faecal analysis require correction for variations in faecal flow. This is achieved by simultaneously measuring the excretion of a non-absorbable inert marker, a constant dose of which is taken by the experimental subject for several days before and during the faecal collection. The most commonly used substance for this purpose is chromium oxide which is measured chemically (Davignon et al, 1968), although more recently an isotopic technique employing $^{51}$CrCl$_3$ has been used (Woodbury and Kern, 1971).

A second major correction relates to the magnitude of neutral steroid losses which may occur during intestinal transit (Grundy et al, 1968). These workers reported that during continuous oral labelling up to 60% of neutral steroid radioactivity may not be recovered from subjects consuming liquid formula diets for reasons that do not appear to be technical. Although the nature of these losses is obscure, it appears likely that they are due to degradation of the ring structure by intestinal bacteria (Ahrens, 1970). After giving radiolabelled cholesterol orally no radioactivity can be detected in CO$_2$ or urine. The possibility that the losses reflect the conversion of steroids to small volatile fragments is supported by the observation that similar losses
of cholesterol radioactivity occur during in vitro incubation of faeces (Denbesten et al, 1970). It was proposed by Grundy et al (1968) that corrections for such losses may be made by measuring the recovery of orally administered β-sitosterol, a plant sterol which although only minimally absorbed is lost to the same extent as cholesterol during intestinal transit. The suitability of β-sitosterol for this purpose was subsequently confirmed by Salen et al (1970), who demonstrated that no synthesis of β-sitosterol occurs in man and that the small fraction absorbed is mostly secreted into bile, predominantly as the free sterol although to a lesser extent as bile acids also. Recent work has suggested that such neutral steroid losses may be partially or completely avoided by the use of solid diets (Kottke and Subbiah, 1972; Kudchodkar et al, 1972a), a phenomenon possibly related to an effect of dietary cellulose on bacterial gut flora (Denbesten et al, 1970). However, further studies are required to establish these observations.

In contrast with neutral steroids no corrections are required for losses of bile acids. Recovery studies with radiolabelled bile acids, given orally either as a single dose (Grundy et al, 1965) or continually to achieve steady state conditions (Grundy et al, 1968), have demonstrated essentially complete recovery in the faeces even in the presence of considerable neutral steroid losses.

The laboratory measurement of faecal steroids is complicated by the complexity of the bacterial changes produced during intestinal transit (Chapter 2) and by the presence of other compounds of similar physico-chemical properties. The latter include a variety of plant sterols (β-sitosterol, campesterol, stigmasterol) and their derivatives in the case of neutral steroids, and fatty acids and organic acidic pigments in the case of bile acids. It is only relatively recently that reliable and adequately validated techniques have been developed for this purpose. Early attempts to measure neutral steroids by gravimetry or the Liebermann-Burchard reaction after digitonin precipitation and to measure bile acids by titration or spectrophotometry after zinc hydroxide precipitation or
column chromatography lacked specificity and sensitivity (reviewed by Grundy et al, 1965; Miettinen et al, 1965) and have been replaced by isotopic methods (isotopic steroid balance) and gas-liquid chromatographic analysis.

Both of the latter procedures involve the initial partition of neutral and acidic steroids into separate extracts. This is readily achieved for neutral steroids by extraction with petroleum spirit after mild alkaline saponification. Bile acid extraction is more difficult, however, since much is bound to bacteria and more prolonged extraction is required under acidic conditions.

In the method of isotopic steroid balance, first developed by Hellman et al (1957), no further purification is required. This procedure depends on the knowledge that 3 or more weeks after an injection of radiolabelled cholesterol the specific activities of faecal endogenous steroids are related to that of plasma cholesterol (Hellman et al, 1957; Avigan and Steinberg, 1965; Grundy et al, 1965; Grundy and Ahrens, 1966). Equilibration usually occurs within 2 days for biliary cholesterol, 3-6 days for primary bile acids and 9-12 days for secondary bile acids (Lindstedt, 1962; Rosenfeld and Hellman, 1962; Avigan and Steinberg, 1965; Wood et al, 1966). Thus, the rate of excretion of radioactivity can be converted to steroid mass by dividing by an appropriate value for plasma cholesterol specific activity.

There are a number of considerations in selecting the latter. Whereas, under normal circumstances, biliary cholesterol has the same specific activity as plasma cholesterol on any given day, the average specific activity of biliary bile acids equals that which existed in plasma cholesterol several days previously, due to the extensive enterohepatic recycling of bile acids. In addition, a finite time is required for endogenous steroids to pass through the intestine. A disadvantage of this technique is that the relationship between the specific activities of faecal steroids and plasma cholesterol will vary according to changes in cholesterol and bile acid turnover and in intestinal motility. However, this problem is minimised by delaying faecal collection until 6 or more weeks after isotopic labelling, when the plasma cholesterol specific
activity-time curve is relatively flat. In the present work neutral steroid and bile acid excretions were calculated from the plasma cholesterol specific activities which existed respectively one and 2 days before each faecal pool.

Additional problems with the isotopic technique concern the measurement of faecal radioactivity by scintillation counting, which is complicated by relatively low counting rates and colour quenching due to pigmented contaminants. The latter is reduced by prior bleaching with hydrogen peroxide.

Gas-liquid chromatographic (GLC) analysis has the advantages of avoiding the use of radioactivity, of being more sensitive and of permitting the quantitation of individual bile acids and neutral steroids, including those of plant origin. The quantitation of bile acids in this way requires the prior cleavage of the peptide bond. This may be ensured by rigorous hydrolysis under pressure, a procedure which may, however, result in considerable losses when performed in glassware owing to adsorption to silicic acid (Eneroth and Sjövall, 1969). If this is not avoided by the use of Teflon-lined bottles, corrections must be made from the fractional recovery of a radiolabelled internal standard. It is likely, however, that these problems may have been overcome by the recent development of an enzymatic method for peptide cleavage (Nair et al, 1967).

The gas chromatographic methods described by Grundy et al (1965) and Miettinen et al (1965) are the most well validated and have been employed in this work. Neutral steroids are first subjected to thin-layer chromatography which separates them into cholesterol, coprostanol and coprostanone, together with the corresponding steroids of plant origin. Further separation into individual steroids is performed during quantitation in the gas chromatograph. Bile acids are further purified as their methyl esters by thin-layer chromatography, to remove fatty acids and acidic pigments. Other acidic contaminants are separated in the gas chromatograph. When relatively large amounts of fatty acids are present prior column chromatography on Florosil is required, but this was not required in the present studies.
During these purification procedures additional specific losses of bile acids of extreme polarities may occur. The total recovery of radiolabelled internal standards (added to the original faecal aliquot) for the entire procedure averages over 90% for neutral steroids and over 80% for bile acids, and is determined for each assay.

Gas-liquid chromatography is performed as the trimethylsilyl (TMS) ethers of the neutral steroids and of the bile acid methyl esters, using 5α-cholestan as internal standard. Suitable stationary phases include Hi Eff 8B, QF-1, SE-30 and DC 560 on acid washed silanized Gas Chrom P (100-120 mesh), best separation of bile acids and neutral steroids being obtained respectively with the first two and the second two phases; DC 560 was used for both bile acid and neutral steroids in the present studies. Using a H₂-flame ionization detector a linear response is obtained with this system with loads of TMS steroids ranging from 0.06 to 120 μg. The detector response per unit mass of unsubstituted parent steroid is the same for different steroids, enabling quantification of complex steroid mixtures as a group without GLC separation and without the requirement for correction for the added mass of TMS groups.

A number of other techniques for the GLC analysis of faecal bile acids have also been developed (reviewed by Eneroth and Sjövall, 1969) but have not been as well validated. Notable variations from the described technique include the direct addition of nordeoxycholic acid as internal standard to the faecal homogenate (Evrard and Janssen, 1968) and the preparation of trifluoroacetates rather than TMS derivatives, which provides additional information concerning bacterial degradation (Ali et al, 1966).

When values for neutral steroid excretion by this technique have been corrected for unabsorbed dietary cholesterol, the calculated values for endogenous neutral steroid excretion are identical to those obtained by isotopic steroid balance (Grundy and Ahrens, 1966). Cholesterol absorption can be conveniently measured for this purpose by determining the fractional recovery in the faeces of cholesterol relative to that of β-sitosterol after their simultaneous oral
administration in radiolabelled form (Borgstrom, 1969). This method has been demonstrated to be at least as accurate as more complicated and time-consuming techniques involving continuous oral labelling (Quintao et al., 1971b). It has also been demonstrated in two laboratories that GLC analysis and isotopic steroid balance give similar values for bile acid production (Grundy and Ahrens, 1966; Kudchodkar et al., 1972b).

BILE ACID TURNOVER IN MAN

The methods for studying bile acid turnover in man have been reviewed by Hofmann et al (1970). Although the measurement of faecal bile acid excretion by either of the steroid balance techniques has been the method most commonly employed for this purpose, other techniques have also been developed.

Isotope Dilution

A method for measuring not only the production rate but also the pool size of bile acids was first described by Lindstedt (1957). A known dose of radiolabelled bile acid is administered by the oral or intravenous route, and the specific activity-time curve followed in samples of duodenal bile collected serially over the following 4 to 10 days. The pool size and production rate of the primary bile acid concerned is then calculated after analysing the specific activity curve as a single exponential (1-pool model). The validity of this technique is based on several assumptions: (i) that complete mixing of the administered bile acid occurs before an appreciable fraction is excreted in the faeces; (ii) that the duodenal aspiration affords a valid sample of the bile acid pool; and (iii) that bile acid kinetics accurately conform to a 1-pool model. Although no controlled comparisons of this technique with faecal analysis have been published, the impression is that isotope dilution gives values that are consistently higher (Einarsson and Hellström, 1972), which may mean that one or more of the above assumptions is incorrect. As with other turnover procedures steady state conditions are required.

The techniques for hydrolysing biliary bile acids and their subsequent extraction are similar to those already discussed for faecal bile acids. Although cholic acid can
be readily separated from other bile acids by thin-layer chromatography (Eneroth, 1963), the isolation of deoxycholic and chenodeoxycholic acids is more difficult. The system of Sundaram et al (1971) appears to be the most efficient for this purpose and has been employed in the present studies. Determination of bile acid mass is usually achieved by either fluorimetry (Parveliwalla et al, 1970), gas liquid chromatography of their methyl esters (Shioda et al, 1969) or spectrophotometry of their sulphuric acid chromogens (Kottke et al, 1966; Wollenweber et al, 1966). While the first two methods have the advantages of greater sensitivity and of permitting quantitation of individual conjugated bile acids as well as of free bile acids, these were unimportant for the present purposes and the simpler spectrophotometric method was employed. Although not used in the present studies, a recently developed method for bile acid assay based on enzymatic oxidation coupled with NAD reduction may combine simplicity with specificity and sensitivity (Palmer, 1969). The use of the spectrophotometric and fluorimetric methods requires the use of specially purified silica gel in order to avoid background interference.

\[ ^{14} \text{CO}_2 \text{ production from cholesterol-26-}^{14}\text{C} \]

Since neither of the foregoing methods permit the rapid quantitation of changes in bile acid turnover Myant and Lewis (1966) estimated bile acid synthesis by measuring the production of \( ^{14} \text{CO}_2 \) after an infusion of cholesterol-26-\( ^{14} \text{C} \), a technique which has also been used in animals (Chevallier and Lutton, 1966). The method is based on the knowledge that the catabolism of cholesterol to bile acids involves the removal of the terminal 3 carbon atoms of the side-chain, probably as propionyl coenzyme A, most of which appears to be oxidised to \( \text{CO}_2 \). With this technique, values in man were consistently lower than those obtained by faecal analysis (Myant and Lewis, 1966; Lewis and Myant, 1967). The possibility that bile acids are synthesized from a pool of cholesterol of lower specific activity than that of plasma has been excluded under normal circumstances (Lindstedt, 1962). A possible explanation for the discrepancy is that...
only a fraction of the released propionyl coenzyme A may be metabolized to CO₂, the remainder being incorporated into other tissue components or being excreted into the urine. In this context, Myant and Lewis (1966) showed that 70-90% of a dose of propionic acid was converted to CO₂ over a 5 hour period, during which time less than 2% of radioactivity appeared in the urine. Another potential source of error is the production of ¹⁴CO₂ by the adrenal gland (Malinow et al, 1970), although this would tend to produce an overestimation. Despite these limitations the method would appear to be of value in detecting rapid changes in bile acid turnover, and has been used in one study in the present work for this purpose. In the method of Myant and Lewis respiratory CO₂ was trapped with aqueous lithium hydroxide, the precipitate of lithium carbonate filtered and dried, and an aliquot of this counted in a scintillation gel. This approach has been modified in an attempt to simplify the laboratory procedures involved (Carroll, 1972). Carbon dioxide was trapped in a solution of a basic amine (ethanolamine), dissolved in scintillation fluid and an aliquot counted directly in a liquid scintillation counter. Extraction of CO₂ was essentially 100% by this procedure, and counting efficiency ranged from 40 to 45%. Counting rates were usually 3 times background and samples were counted for 100 minutes.

Measurement of biliary bile acid secretion by marker dilution techniques

Grundy and Metzger (1972) described a method for measuring biliary bile acid secretion, which although providing no information concerning bile acid synthesis is relevant in the context of bile acid methodology. The technique employs duodenal intubation for 24 hours or more with a double-lumen tube. A liquid formula diet is continuously infused at constant rate through the proximal outlet, while a small fraction of duodenal content is continuously aspirated through the distal outlet. A non-absorbable marker is added to the formula diet (eg, β-sitosterol), and biliary bile acid secretion estimated by marker dilution, assuming that complete mixing of the marker and duodenal bile has
occurred. The method is apparently accurate and reproducible. However, as the authors indicate, it has not been established that the hepatic secretion and duodenal output of bile acids are the same, nor that the results can be related to the hepatic secretion of bile acids under more physiological conditions.

PLASMA LIPOPROTEIN ANALYSIS

(i) Collection of blood

Blood for lipoprotein analysis was collected after a 12-14 hour overnight fast and with the subject in the supine position (see Chapter 2). The prolonged use of a tourniquet was avoided, since this may introduce large errors due to haemoconcentration (Koerselman et al, 1961). Both heparin and EDTA (1 mg/ml blood) were used as anticoagulants, the latter having the advantage of chelating metallic ions which promote oxidation of unsaturated fatty acids. Serum was not used for lipoprotein assays since this is unsuitable for chylomicron analysis. When required, LCAT activity was inhibited by immediate cooling or by the use of p-chloromercuri phenylsulphonate (Glomset et al, 1970). All samples were analysed on the same day or within 7 days of venepuncture, storage being performed at 0-4°C in the presence of EDTA (World Health Organization, 1972). Frozen samples were not used since these are unsuitable for the analysis of lipoproteins, particularly VLDL and chylomicrons which aggregate and deteriorate under such conditions.

(ii) Fractionation of plasma lipoproteins

The techniques available for separating plasma lipoproteins have been the subject of recent reviews by Hatch and Lees (1968) and Lindgren et al (1972). Those in common use for the fractionation of large numbers of samples are based on known differences in density, interaction with macromolecular reagents and net surface change. The first two permit accurate quantitation but the latter only semi-quantitative analysis.

(a) Density

Differences in density reflect predominantly differences in protein/lipid ratio (particularly the protein/triglyceride ratio), density increasing as
this ratio rises. It is possible to separate lipoproteins on the basis of these density differences by sequential preparative ultracentrifugation at adjusted densities of 1.006 g/ml for 18 hours (to separate VLDL), 1.063 g/ml for 18 hours (to separate LDL) and 1.216 g/ml for 24 hours (to separate HDL) (Havel et al., 1955), density adjustment usually being achieved by the use of NaCl and NaCl-NaBr solutions. For such purposes angle head rotors are simple to use and permit fractionation on a large scale, although they are inferior to swinging-bucket rotors for the isolation of chylomicrons and lipoprotein subclasses by density gradient procedures. When the latter are not available subfractionation may be achieved by sequential ultracentrifugation at additional densities (Havel et al., 1955), or at different speeds and different times at a fixed density (Gustafson et al., 1965). However, such procedures require longer centrifugation times, and uncertainties exist concerning lipoprotein stability under such circumstances, particularly of HDL. In the present study preparative ultracentrifugation has been used mostly to isolate VLDL, but has also been used on occasions for the subfractionation of lipoproteins of \( d < 1.063 \) g/ml.

Another procedure for separating and quantitating lipoproteins is that of analytical ultracentrifugation (de Lalla and Gofman, 1954), in which lipoprotein distribution according to flotation rate is plotted by computerised analysis of serial schlieren films. A detailed description, however, is beyond the scope of this thesis.

(b) Macromolecular interactions

VLDL and LDL can be selectively precipitated by the addition of sulphated polyanions such as dextran sulphate or heparin, and such techniques have the advantages of simplicity, speed and economy. Fredrickson et al. (1968) have developed a technique for quantitating lipoprotein lipids by the combined use of ultracentrifugation (to isolate VLDL from one plasma sample) and
precipitation with manganese chloride and heparin (to isolate HDL in the supernatant of a second sample). Values for LDL are calculated by subtraction from the lipid concentrations in whole plasma. A modification of this procedure has been used in the present studies. After the removal of VLDL by ultracentrifugation, LDL were precipitated from the reconstituted infranate by the addition of 0.05 ml 1M manganese chloride and 2 mg sodium heparin for every 1 ml of solution (Burstein and Samaille, 1960). VLDL, LDL and HDL lipids were then measured after extraction with Dole’s solution (Dole, 1956), which, although unsuitable for phospholipid extraction (for which the method of Folch et al (1957) is preferable), was demonstrated to extract essentially all cholesterol and triglyceride. This method has the advantages over that of Fredrickson et al (1968) of requiring less plasma and of avoiding the possibility of additive analytical errors. Preliminary experiments confirmed that it gave values for lipoprotein lipids identical to those obtained by sequential preparative ultracentrifugation.

A technique which also depends on macromolecular interactions, but which was not employed in the present study, is the flocculation of VLDL and chylomicrons by polyvinylpyrrolidone.

(iii) Electric charge

Differences in the net surface electric charge of lipoproteins permit their separation by electrophoresis. Such differences represent the balance between the positive and negative charges on the terminal and side-chain amino-acid residues of their apoproteins. On all supporting media other than starch granules electrophoretic mobility is modified by mechanical and chemical interactions between the medium and the lipoproteins. The former are due to the interstices of the medium offering resistance to their movement, and are particularly prominent with polyacrylamide gel in which VLDL runs in a "post-β" rather than in the "pre-β" position which it occupies on the other common media.
(the correspondence between the electrophoretic zones on paper and ultracentrifugal fractions has been given in Chapter 2). Chemical interactions are most prominent with paper electrophoresis, and although minimised by the use of EDTA and albumin (Lees and Hatch, 1963) result in definition and resolution which is poor relative to that achieved with cellulose acetate and agarose gel. In the present study cellulose acetate (Chin and Blankenhorn, 1968) has been preferred to agarose gel (Noble, 1968) in view of its relative simplicity. However, a disadvantage of cellulose acetate is that some chylomicrons run in the pre-β region.

Staining of lipoproteins with fat-soluble dyes (e.g., Oil red O, Sudan black B) may be performed before or after electrophoretic separation. In the present work the latter was used since this avoids possible alterations in lipoprotein mobility and facilitates the identification of chylomicrons at the origin. Background staining was removed with dilute acetic acid. Although for the present purposes electrophoretograms were analysed only qualitatively as a diagnostic aid, semi-quantitative analysis can be achieved by densitometry. Values obtained by densitometry can correlate well with those obtained by ultracentrifugation (Hatch et al., 1970).

Techniques have also been described for the solvent extraction of lipids and for the recovery of chylomicrons by sonication (Chin and Blankenhorn, 1968).

Other methods for separating and quantitating plasma lipoproteins, but which have not been employed, are based on differences in particle size (gel filtration) and antigenicity (immunoelectrophoresis and immunodiffusion).

(iv) **Lipid analysis**

Lipoproteins were fractionated for the determination of their content of cholesterol and triglyceride, the extraction of which has been discussed. When required, the separation of free and esterified cholesterol and triglyceride was performed by thin-layer chromatography. This has the general advantages over paper chromatography of being more versatile and of giving better separation, and is more rapid than column chromatography. The latter is, however, preferable
for separating large amounts and can also be used to concentrate components. After lipoprotein fractionation, lipid extraction, chromatographic separation and elution, recoveries of lipoprotein lipids exceeded 90% for both cholesterol and triglyceride, as estimated by comparison with whole plasma concentrations.

The methods available for the chemical analysis of cholesterol and triglyceride have been reviewed respectively by Witter and Whintner (1972) and Bartholomew (1971). Most analytical methods for cholesterol are based either on the Liebermann-Burchard reaction or use reagents containing the ferric ion, colour intensity being measured by spectrophotometry. The former method has the disadvantages of producing less colour of lower stability and different colour intensities with free and esterified cholesterol. The latter problem can, however, be overcome by prior saponification and extraction of the resulting free cholesterol (Abell et al, 1952). Interference is produced in both methods by bilirubin and steroids other than cholesterol (eg, vitamins A and D, bile acids), but these can be mostly removed by saponification and extraction or by the use of adsorbents. In the present study cholesterol was generally measured by a semi-automated procedure in a Technicon Auto Analyzer II as described by Block et al (1966). After the removal of bilirubin by adsorption to zeolite and Lloyd reagent, an isopropanol extract was mixed on-line with Liebermann-Burchard reagent (acetic anhydride: glacial acetic acid: sulphuric acid, 6:3:1, v/v), heated to 60°C, and the absorbance read in a tubular flowcell. The use of an automated procedure ensured carefully controlled operating conditions, and duplicate analyses gave consistent agreement within ±2%. However, when greater sensitivity was required for the estimation of free cholesterol during the assay of plasma LCAT activity, the ferric chloride method of Zlatkis et al (1953) was used; this gave values slightly lower than those given by the Auto Analyzer (r = +0.96, P<0.001, n = 44; regression equation: x = y (0.96)-7.6 where x = ferric chloride result and y = Auto Analyzer result in mg/100 ml).
Methods for the chemical estimation of triglyceride are mostly based on the determination of glycerol released by alkaline saponification, although alternatively the ester groups may be assayed. Methods based on glycerol determination also measure pre-existing free glycerol, glycerol released from partial glycerides (2-10% of total plasma glycerides) and (with the exception of enzymatic glycerol assays) α-glycerophosphate released from phospholipids. Phospholipids are usually removed by adsorption to zeolite, while the presence of free glycerol can be corrected for by the use of unsaponified plasma blanks. The separation of partial glycerides requires chromatography. The other principal interfering substance is glucose, which if not removed by solvent extraction is generally removed with zeolite, copper sulphate and calcium hydroxide. In the present study lipoprotein triglycerides were assayed on the Auto Analyzer as developed by Kessler and Lederer (1966). Triglyceride was saponified on-line with isopropanolic potassium hydroxide, the resulting glycerol oxidised by periodate to formaldehyde, and the latter converted to 3,5-diacyetyl-1,4-dihydrolutidine by reaction with acetyl acetone in ammonium acetate, the final product being measured by fluorimetry. Results with this procedure have been demonstrated to show good agreement with those by other methods. Because of their variable fatty acid composition, triglyceride concentrations should either be expressed as mmols/litre or be accompanied by a statement of the standard used. In the present studies this was pure triolein.

PLASMA CHOLESTEROL ESTERIFICATION

Plasma cholesterol esterifying activity was first measured by Sperry (1935) by determining the change in free cholesterol concentration on incubating plasma at 37°C. Since the reaction rate is slow relative to the sensitivity of the cholesterol assay procedures then available, incubation was performed for periods of 1-3 days. The relatively recent development of more sensitive methods has permitted incubation times to be reduced to several hours. However, the demonstration that under such conditions the reaction follows zero
order kinetics for the first 1 or 2 hours only (Stokke and Norum, 1971), has indicated that such procedures do not measure the initial reaction rate. The extent of the esterification over prolonged incubations depends more on the availability of substrates (lecithin and free cholesterol) than on enzyme activity (Glomset, 1968). However, the development of gas chromatographic techniques for detecting changes in free cholesterol concentration has enabled similar assays to be performed after an incubation of only 1 hour (Stokke and Norum, 1971). An equally sensitive but more simple technique involves the prior equilibration of autologous lipoprotein cholesterol with exogenous radioactive cholesterol in the presence of a reversible LCAT inhibitor. After re-activating the enzyme, the fractional esterification of the radioactive cholesterol during one hour's incubation is measured by liquid scintillation counting. The mass esterification rate is then calculated from knowledge of the mass of free cholesterol originally present (Stokke and Norum, 1971). The validity of the method depends on the degree of equilibration achieved between exogenous and endogenous cholesterol (which should be complete) and the absence of altered lipoprotein structure and function during the preparation of the substrate. The problems of equilibrating radiolabelled cholesterol with plasma have already been discussed. Stokke and Norum compared results obtained isotopically with those by gas chromatography, and obtained slightly higher (10%) results with the latter. However, the GLC method is technically more complicated and the isotopic technique has been employed in the present study. Such procedures probably provide a good estimate of the in vivo rate of plasma cholesterol esterification, which depends not only on enzyme activity but also on the nature and content of plasma lipoproteins and possibly of other plasma components.

Blood samples for such purposes require immediate cooling to inhibit esterification prior to assay. EDTA may be used as anticoagulant at a concentration of 2.5 mM, which has no effect on reaction rate (Stokke and Norum, 1971). Assays may be performed on samples stored at -20°C for several days,
although in the present studies assays were performed on the
day of venepuncture. Samples demonstrating haemolysis were
discarded since this inhibits the reaction (Sperry, 1935).

A method for assaying enzyme activity per se, independ­
dently of autologous substrates, has been developed by
Glomset (1968). A lipoprotein substrate is prepared by
heating plasma at 56-60°C (to inactivate LCAT) and then
equilibrating the free cholesterol with radiolabelled
cholesterol. A small volume of test plasma is added to a
much larger volume of substrate and the mixture incubated for
3-6 hours. Although this has proved a useful procedure, there
are several reasons for not regarding it as an ideal assay
system for enzyme activity. The problems concerning the
equilibration of radioactive cholesterol exist, while one
cannot exclude an effect of endogenous substrates on the
reaction rate, despite their several-fold dilution. In
addition, heated plasma is neither a well defined, stable nor
reproducible substrate, and it has not been established that
the enzyme acts equally well with pre-heated lipoproteins.
The ideal assay system would involve the incubation of a pure
preparation of the enzyme, isolated without losses from the
test plasma, with a stable and reproducible artificial sub­
strate. Purification of the LCAT enzyme is complicated by
its tendency to form complexes with HDL and apparent insta­
libility when separated from plasma lipoproteins (Glomset,
1972). Successful assays of purified enzyme preparations
with artificial substrates have recently been described by
Fielding et al (1972b). A substrate consisting of a sonicated
dispersion of lipids and radiolabelled cholesterol together
with HDL protein cofactor was demonstrated to give reaction
rates which were linear for 12 hours and which were similar
to those obtained with native HDL of similar lipid composition.
However, the purification procedures are generally too complex
and time-consuming at present for routine use, and although
they achieve up to 3000-fold purification enzyme yield is of
the order of only 15%.

An estimate of the fractional turnover rate of plasma
cholesteryl esters in vivo can be obtained by analysing the
specific activity-time curves of plasma free and esterified cholesterol for 48 hours after the intravenous infusion of radiolabelled mevalonic acid, during which time they show a precursor-product relationship (Nestel and Monger, 1967). The analysis of such curves has been discussed by Zilversmit (1960). The rate of cholesteryl ester turnover is calculated from a knowledge of the plasma cholesterol pool size, which contains about 8 times as much cholesteryl ester as the liver. Although direct comparisons of results obtained by this method with those obtained in vitro with plasma from the same subjects have not been performed, the methods appear to be in good agreement (Nestel and Monger, 1967; Glomset, 1968).
A clinical trial is described of a new anion-exchange resin, colestipol, in the management of eight patients with hypercholesterolaemia. A dose of 5g thrice daily produced significant falls in cholesterol level, which were unrelated to pre-existing diet and which compared favourably in magnitude with those usually attainable with a similar dose of cholestyramine. In contrast to cholestyramine, however, colestipol proved to be entirely acceptable to the patients and its use was not associated with gastrointestinal disturbances. A further lowering of cholesterol could be obtained in some subjects by the addition of clofibrate. Elevations in triglyceride levels were detected in all subjects, particularly in those who were already hypertriglyceridaemic, but these could usually be prevented by the coadministration of niacin. Evidence suggesting impaired absorption of fat-soluble vitamins was also observed.

The drug would appear to be a useful addition to the range of agents at present available for treating type II hyperlipoproteinaemia. It is suggested, however, that regular screening for metabolic disturbances related to malabsorption and impaired glucose tolerance be routinely practised in all patients receiving resin therapy.
ABSTRACT

A clinical trial is described of a new anion-exchange resin, colestipol, in the management of eight patients with hypercholesterolaemia. A dose of 5g thrice daily produced significant falls in cholesterol level, which were unrelated to pre-existing diet and which compared favourably in magnitude with those usually attainable with a similar dose of cholestyramine. In contrast to cholestyramine, however, colestipol proved to be entirely acceptable to the patients and its use was not associated with gastrointestinal disturbances. A further lowering of cholesterol could be obtained in some subjects by the addition of clofibrate. Elevations in triglyceride levels were detected in all subjects, particularly in those who were already hypertriglyceridaemic, but these could usually be prevented by the addition of clofibrate or by dietary carbohydrate restriction. Small increases in fasting blood sugar and changes suggesting impaired absorption of fat-soluble vitamins were also observed.

The drug would appear to be a useful addition to the range of agents at present available for treating type II hyperlipoproteinaemia. It is suggested, however, that regular screening for metabolic disturbances related to malabsorption and impaired glucose tolerance be routinely practised in all patients receiving resin therapy.
INTRODUCTION

A relationship between hypercholesterolaemia and coronary atherosclerosis has been clearly demonstrated in a number of epidemiological (Kannel et al., 1971; Carlson and Böttiger, 1972) and clinical (Heinle et al., 1969; Patterson and Slack, 1972) studies. Although it has not been established that reduction of plasma cholesterol levels in man will also diminish the likelihood of coronary disease, treatment of hypercholesterolaemia is generally recommended. In addition to instituting dietary modifications (Fredrickson et al., 1970) it is often necessary to use cholesterol-lowering drugs. Although clofibrate is frequently effective, a proportion of patients with type II hyperlipoproteinaemia (Fredrickson et al., 1967) do not respond well (Strisower et al., 1968), and in such subjects a drug that increases the excretion of bile salts, thereby stimulating cholesterol catabolism (Boyd and Percy-Robb, 1971), is usually of greater value. Cholestyramine, a resin which binds bile acids in the intestinal lumen, is the most commonly used of this group, but produces gastrointestinal disturbances in a significant proportion of patients (Bergen et al., 1959; Gherondache and Pincus, 1964; Danhof, 1966).

Colestipol (U-26, 597A) is a new non-absorbable anion-exchange resin of large molecular weight. It is a copolymer of tetraethylenepentamine and epichlorohydrin (2:5), prepared as a partial hydrochloride, and has an \textit{in vitro} affinity for bile salts similar to that of cholestyramine (0.66-1.0mg cholate/mg resin, in 0.9% saline at pH 6.3). This paper reports a controlled trial of this new resin in the treatment of hyperlipidaemia.

METHODS

Patients

Eight patients, six males and two females, were included in the trial. Seven were suffering from type II hyperlipoproteinaemia and one from type IV. No patient presented any evidence of blood clotting disorder or gastrointestinal disease. The relevant clinical and pathological features of each patient are presented in Table 4-1.
Two patients (EA, DS) had been instructed several months before entering the trial in a diet low in cholesterol and high in polyunsaturated fatty acids, with resultant lowering of plasma cholesterol levels by 80 and 100 mg/100ml respectively. The remaining patients were consuming a typical Western diet of relatively high cholesterol and saturated fatty acid content. Other causes of hyperlipoproteinaemia, due to diabetes mellitus, hypothyroidism, renal and liver disease or alcoholism, were excluded.

Clinical Trial

At the beginning of the trial, which was conducted on an out-patient basis, each patient was interviewed and examined clinically. Two patients had evidence of coronary disease (AT, EA). The commencement of active therapy was preceded by a control period of 70 to 221 days during which an inactive preparation of microcrystalline cellulose was administered. Colestipol 15g daily was then taken by each subject for 113 to 190 days. The duration of each study is shown in Table 4-2. Preparations were taken in three equal doses immediately before meals as a suspension in fruit juice or water. The effects of the following modifications of therapy were subsequently investigated in selected subjects:

1. Combining colestipol 15g daily with clofibrate 1g b.d. (7 subjects), taurine 2g daily (4 subjects) or carbohydrate restriction (1 subject), for periods as shown in Table 4-2.
2. Increasing the daily dose of colestipol to 20-30g (5 subjects).

Each patient adhered to his usual diet for the duration of the trial, and body weights remained essentially constant (Table 4-1). Any drugs previously prescribed for other conditions were continued at constant dosage throughout the study, and were taken between successive doses of colestipol to try to avoid any interference with their absorption (Edwards and McCredie, 1967). Once the trial had started no additional drugs were prescribed, except for subject EA who commenced propranolol 120mg daily for worsening angina approximately mid-way through the control period.
Each patient was seen at approximately weekly intervals after an overnight fast of 12 hours. On each occasion 10 ml venous blood was collected into a heparinised tube for lipid determinations. The total number of measurements is shown in Table 4-2. Periodically additional blood was drawn for full blood count, coagulation studies, and determination of uric acid, blood sugar, calcium, inorganic phosphorus, iron, electrolytes and conventional indices of hepatic, renal and thyroid function.

**Laboratory Procedures**

Plasma concentrations of cholesterol and triglyceride were determined simultaneously using a semi-automated method (Auto Analyzer II, Technicon Instruments Corporation). Measurement of cholesterol was based on the colorimetric method of Liebermann and Burchard, and that of triglyceride was by the fluorimetric determination of glyceride glycerol using triolein standards (Method AAII-24, Auto Analyzer Manual, 1971). Samples were prepared by extracting 0.5 ml plasma with 9.5 ml redistilled isopropanol and mixing for 30 minutes with 2 g copper sulphate:zeolite:Lloyd reagent: calcium hydroxide, 3:40:4:8 (w:w), to remove phospholipid, glucose, bilirubin and other interfering substances. After centrifugation the supernatant was decanted into sample cups and analysed on the same day. Duplicate analyses gave consistent agreement within ± 2%. Blood sugar was estimated by the neocuproine method for reducing substances (Auto Analyzer Manual, 1971).

The characterization of the hyperlipoproteinaemia was performed by separating the plasma lipoproteins by ultracentrifugation and precipitation, using a modification of the method of Fredrickson et al (1968), and by lipoprotein electrophoresis on cellulose acetate (Chin and Blankenhorn, 1968).

**RESULTS**

**Plasma Cholesterol**

No consistent trends in plasma cholesterol level were observed during the control period. Individual mean
concentrations among the 8 subjects during this time ranged from 289 to 477mg/100ml (Table 4-2). Each patient responded to colestipol 15g daily with a fall in cholesterol level, which was in all cases complete within 4 weeks of commencing therapy. Thereafter, mean concentrations remained at significantly lower levels (P<0.01) without the development of tolerance. Reductions in concentration ranged from 20 to 80 mg/100ml (mean = 51 mg/100ml) or from 7 to 22% (mean = 14%) of initial mean values.

Increasing the daily dose of colestipol to 20 and later 30g produced an additional fall of 25 mg/100ml in subject RB, but 4 other subjects failed to show any further response. In 4 out of 7 patients the addition of clofibrate lowered cholesterol levels by a further 14-45 mg/100ml, while the addition of taurine appeared to be less effective, producing falls of 13 and 23 mg/100ml in 2 subjects only.

During active therapy no changes occurred in either the angina pectoris or xanthelasma of subject EA.

**Plasma Triglyceride**

All subjects demonstrated a persistent increase in plasma triglyceride concentration during colestipol therapy (P<0.01). These results are presented in Table 4-3. Increases ranged from 32 to 249 mg/100ml (mean = 84 mg/100ml) or from 20 to 61% (mean = 41%), and were unaffected by increasing the dose or by the addition of taurine. In 6 out of 7 patients, however, these changes were completely reversed by the addition of clofibrate. In one subject (AT) clofibrate was subsequently replaced by carbohydrate restriction for a period of 10 weeks. Although triglyceride levels during this period (214 ± 14.4 mg/100ml, mean ± S.E.M.) were higher than during clofibrate administration, the marked rise seen when colestipol was taken with her usual diet was nevertheless prevented (P<0.01).

Subsequent lipoprotein studies in the same patients demonstrated that the alterations in cholesterol and triglyceride produced by colestipol reflected reciprocal changes in the concentrations of very low density and low density lipoproteins (Chapter 6).
Other Observations

All patients found the resin to be palatable and easy to take, and no gastrointestinal disturbances were reported. One patient (AL) developed a prolongation in the prothrombin time (prothrombin index = 52%) after 118 days of active therapy, which was corrected by oral vitamin K therapy. Changes were also observed in a number of other plasma components which were statistically significant on paired t-test analysis. An overall increase in serum alkaline phosphatase activity was associated with a decrease in serum calcium concentration, and increments in mean fasting blood sugar were also detected in 7 of 8 subjects (Figure 4-1). There was no clinical evidence of an interaction between colestipol and any of the drugs already being administered for other conditions.

DISCUSSION

Plasma Cholesterol

Although some investigators have reported changes in cholesterol levels with placebo alone (Acheson and Hutchinson, 1963) none was observed in the present trial. Nor were any seasonal variations in concentration, previously noted during other studies (Thomas et al, 1961), observed during the lengthy placebo periods. However, marked erratic variations in cholesterol level were observed in all subjects throughout the study (coefficient of variation for the 32 study periods = 7.6 ± 3.3%, mean ± S.D.). Such variations have been noted previously by Samuel, Lieberman, Shmase et al (1970) who have emphasised the resultant difficulties in evaluating antihypercholesterolaemic therapy.

The reductions in cholesterol achieved with colestipol alone in the patients with type II hyperlipoproteinaemia are similar to those described in other recent reports (Parkinson et al, 1970; Glueck et al, 1971b; Probstfield et al, 1972; Ryan and Jain, 1972; Nye et al, 1972) and compare favourably with those produced by similar doses of cholestyramine (Bergen et al, 1959; Hashim and Van Itallie, 1965; Howard et al, 1966; Fallon and Woods, 1968; Jones and
Dobrilovic, 1970). They were generally smaller, however, than those reported after ileal by-pass surgery, which also leads to increased losses of bile acids (Buchwald et al., 1970). Colestipol produced a further lowering in subjects who had already benefited from dietary modification (EA, DS). In contrast to subjects with type III or type IV hyperlipoproteinaemia patients with type II hyperlipoproteinaemia are unlikely to respond well to clofibrate alone (Strisower et al., 1968). However, as shown in Table 4-2 the administration of clofibrate in addition to colestipol led to a further significant lowering in plasma cholesterol concentration in 3 of the 6 treated subjects with type II hyperlipoproteinaemia. The reason for this may be as follows. Colestipol promoted an increase in faecal bile acid excretion of 300% in these subjects, which led to an increased conversion of cholesterol to bile acids and an increase in cholesterol synthesis of 100% (see Chapter 5). The secondary rise in cholesterol turnover may be partly prevented by clofibrate which inhibits cholesterol synthesis (Grundy, Ahrens, Salen et al., 1972). Clofibrate has also been shown to enhance the excretion of cholesterol (Grundy, Ahrens, Salen et al., 1972). Others have also demonstrated the effectiveness of combining clofibrate with drugs that stimulate bile acid elimination such as neomycin (Samuel, Holtzman, Meilman et al., 1970), cholestyramine and DEAE Sephadex (Howard and Hyams, 1971). However, this response contrasts with an apparently antagonistic effect observed in the subject with type IV hyperlipoproteinaemia, who ultimately achieved a reduction in cholesterol level with clofibrate alone (285 ± 4.6 mg/100ml, mean ± S.E.M.) that was greater than that with colestipol plus clofibrate (P<0.001). Possible explanations are that colestipol was interfering with the absorption of clofibrate (Edwards and McCredie, 1967) or that the rise in VLDL induced by colestipol was incompletely blocked by clofibrate in this subject.

The rationale behind adding taurine to colestipol is based on the suggestion that its availability is rate-limiting in the formation of taurine-conjugated bile acids
(Sjövall, 1959), and on the demonstration that cholestyramine has a greater affinity for taurine conjugates than for glycine conjugates (Cook et al, 1972). The possibility existed that taurine becomes unduly depleted during resin therapy, and that its addition might therefore further stimulate cholesterol catabolism (Cook et al, 1971). This was, however, not borne out by the present limited studies.

**Plasma Triglyceride**

The changes in plasma triglyceride concentration occurring during trials of cholestyramine therapy have been variable. While some workers have observed increases of similar magnitude to those encountered here (Weizel et al, 1969; Wood et al, 1972; Grundy et al, 1971), others have reported no change at all (Fallon and Woods, 1968) or even decreases (Bressler et al, 1966), although in the latter study relatively large doses were used which may have interfered with intestinal fat absorption (Danhof, 1966). Both the frequency and magnitude of triglyceride increases have been rather less in other recent trials of colestipol than reported here (Parkinson et al, 1970; Glueck et al, 1971b; Probstfield et al, 1972; Ryan and Jain, 1972; Nye et al, 1972). Possible explanations include differences in experimental design, patient selection and diet. Thus, subjects who were already hypertriglyceridaemic at the start of the present study (AH, AT, LO) experienced significantly greater elevations than did those who were normotriglyceridaemic (P<0.02). The rise in plasma triglyceride in association with a fall in cholesterol concentration reflected the differing effect of colestipol therapy on two species of lipoproteins: the cholesterol-containing low density lipoproteins were lowered while the triglyceride-containing very low density lipoproteins were raised (see Chapter 6).

In view of the recent report that hypertriglyceridaemia per se is at least as important as hypercholesterolaemia in the development of clinical coronary heart disease (Carlson and Böttiger, 1972), it would appear wise to regard any increase in triglyceride level occurring during cholesterol-lowering therapy as undesirable and to attempt to reverse it.
A recent report that carbohydrate restriction may be of value for this purpose (Kuo, 1971) is supported by its effectiveness in subject AT. The use of clofibrate, a known triglyceride-lowering drug in other circumstances (Strisower et al, 1968), was more fully investigated and proved extremely successful in all but one subject, reducing triglyceride levels in several cases to below control values.

Other Observations

The palatability of colestipol and the absence of gastrointestinal disturbances during its administration would appear to be major advantages of the drug over cholestyramine, the use of which may be associated with alimentary disorders ranging from nausea and flatulence to faecal impaction (Bergen et al, 1959; Gherondache and Pincus, 1964; Danhof, 1966).

The importance of bile salts in the digestion and absorption of fats is well established and interference with these functions constitutes a potential disadvantage of bile acid-sequestrant therapy. Frank steatorrhoea may occur during the administration of relatively large doses of cholestyramine (Hashim et al, 1961). Although none was observed during the present trial, the small but significant changes in serum calcium and alkaline phosphatase, and the prolongation of prothrombin time in one subject, may indicate a degree of malabsorption of fat-soluble vitamins. Cholestyramine has been shown to impair vitamin K absorption in dogs (Kelley et al, 1963), and its use in patients has been occasionally associated with the development of hypoprothrombinaemia (Visintine et al, 1961; Roe, 1968). Although there have been no certain reports of other vitamin deficiencies during cholestyramine therapy for hypercholesterolaemia, impaired absorption of vitamins A and D has been observed in experimental situations (Longenecker and Basu, 1965; Thompson and Thompson, 1969), and a high incidence of elevated alkaline phosphatase levels has been noted in paediatric patients receiving large doses of the resin (Levy et al, 1972). In view of these findings it would seem advisable that patients receiving resin therapy be regularly screened for vitamin deficiency, and indeed prophylactic dietary supplementation may be indicated.
Although no changes occurred in mean corpulscular haemoglobin concentration or serum iron concentration during the present trial, the observation that cholestyramine impairs iron absorption in rats (Thomas et al, 1971) suggests that these should also be monitored during long-term resin therapy.

The observed increases in fasting blood sugar were positively correlated with those in triglyceride concentration ($r = +0.80$, $p<0.02$), which is interesting in view of the well known relationship between hypertriglyceridaemia and glucose intolerance under other circumstances, such as obesity (Ford et al, 1968) and type IV hyperlipoproteinaemia (Fredrickson et al, 1967).

The long-term effects of such resin therapy are not known. Although no potentially harmful changes in bile composition appear to occur (Wood et al, 1972), the possibility that prolonged enzyme stimulation may lead to co-factor or substrate deficiencies requires consideration (Maxwell et al, 1972).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Body weight (kg)</th>
<th>Type†</th>
<th>Pathological Features</th>
<th>Other Drug Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO</td>
<td>M</td>
<td>52</td>
<td>80.0</td>
<td>IV</td>
<td>Cerebrovascular accident; Hypertension. ECG: normal</td>
<td>Methyl dopa; Chlorothiazide</td>
</tr>
<tr>
<td>LO</td>
<td>M</td>
<td>45</td>
<td>64.7</td>
<td>IIa</td>
<td>ECG: normal</td>
<td>None</td>
</tr>
<tr>
<td>AL</td>
<td>M</td>
<td>45</td>
<td>65.7</td>
<td>IIb</td>
<td>Hypertension; Menopausal symptoms. ECG: Ventricular ectopic beats (Controlled)</td>
<td>Guanethidine; Propranolol</td>
</tr>
<tr>
<td>AT</td>
<td>F</td>
<td>48</td>
<td>52.6</td>
<td>IIa</td>
<td>ECG: normal</td>
<td>Amitryptiline</td>
</tr>
<tr>
<td>DS</td>
<td>M</td>
<td>35</td>
<td>66.7</td>
<td>IIb</td>
<td>ECG: normal</td>
<td>Propranolol (From day 51 of placebo period)</td>
</tr>
<tr>
<td>AH</td>
<td>F</td>
<td>47</td>
<td>54.0</td>
<td>IIa</td>
<td>Xanthelasma; Angina; Cholecystectomy. ECG: Old posterior myocardial infarction.</td>
<td>None</td>
</tr>
<tr>
<td>EA</td>
<td>M</td>
<td>45</td>
<td>71.0</td>
<td>IIa</td>
<td>ECG: normal</td>
<td>None</td>
</tr>
<tr>
<td>RB</td>
<td>M</td>
<td>45</td>
<td>71.7</td>
<td>IIa</td>
<td>ECG: normal</td>
<td>None</td>
</tr>
<tr>
<td>MW</td>
<td>M</td>
<td>35</td>
<td>71.0</td>
<td>IIa</td>
<td>ECG: normal</td>
<td>None</td>
</tr>
</tbody>
</table>

* Average weight for height and sex at 20-24 years of age (Society of Actuaries, 1959)
† Type of hyperlipoproteinaemia (World Health Organization, 1970).
<table>
<thead>
<tr>
<th>Patients</th>
<th>Placebo</th>
<th>Colestipol 15g</th>
<th>Colestipol 20-30g</th>
<th>Colestipol 15g plus Clofibrate 2g</th>
<th>Colestipol 15g plus Taurine 2g</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO</td>
<td>388 ± 9.2</td>
<td>361 ± 8.5</td>
<td>-</td>
<td>336 ± 5.8</td>
<td>-</td>
</tr>
<tr>
<td>AL</td>
<td>291 ± 10.7</td>
<td>241 ± 3.7</td>
<td>-</td>
<td>227 ± 3.7</td>
<td>240 ± 4.6</td>
</tr>
<tr>
<td>AT</td>
<td>477 ± 22.5</td>
<td>397 ± 6.7</td>
<td>417 ± 2.1</td>
<td>407 ± 14.3</td>
<td>-</td>
</tr>
<tr>
<td>DS</td>
<td>292 ± 7.2</td>
<td>272 ± 4.1</td>
<td>274 ± 6.3</td>
<td>227 ± 4.2</td>
<td>259 ± 6.9</td>
</tr>
<tr>
<td>AH</td>
<td>289 ± 8.6</td>
<td>256 ± 6.2</td>
<td>-</td>
<td>236 ± 6.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>(221 : 18)</em>*</td>
<td><em>(121 : 10)</em>*</td>
<td></td>
<td><em>(53 : 4)</em>*</td>
<td></td>
</tr>
<tr>
<td>EA</td>
<td>444 ± 10.1</td>
<td>369 ± 6.5</td>
<td>367 ± 8.7</td>
<td>386 ± 5.5</td>
<td>-</td>
</tr>
<tr>
<td>RB</td>
<td>304 ± 11.8</td>
<td>260 ± 4.8</td>
<td>235 ± 7.4</td>
<td>-</td>
<td>237 ± 12.4</td>
</tr>
<tr>
<td>MW</td>
<td>357 ± 11.7</td>
<td>278 ± 4.2</td>
<td>283 ± 3.7</td>
<td>280 ± 14.0</td>
<td>284 ± 6.3</td>
</tr>
</tbody>
</table>

** P<0.01  * P<0.05  All results are compared with those obtained during therapy with colestipol 15g daily.

† The numbers in brackets are respectively the duration of the study period in days and the number of determinations made.
## Table 4-3  Plasma Triglyceride Concentrations During Different Therapeutic Regimens

<table>
<thead>
<tr>
<th>Patients</th>
<th>Placebo</th>
<th>Colestipol 15g</th>
<th>Colestipol 20-30g</th>
<th>Colestipol 15g plus Clofibrate 2g</th>
<th>Colestipol 15g plus Taurine 2g</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO</td>
<td>484 ± 27.6**</td>
<td>733 ± 42.6</td>
<td>-</td>
<td>280 ± 14.7**</td>
<td>-</td>
</tr>
<tr>
<td>AL</td>
<td>168 ± 9.4**</td>
<td>202 ± 9.5</td>
<td>-</td>
<td>133 ± 7.9**</td>
<td>181 ± 12.4</td>
</tr>
<tr>
<td>AT</td>
<td>227 ± 17.8**</td>
<td>307 ± 14.6</td>
<td>268 ± 13.7</td>
<td>128 ± 6.1**</td>
<td>-</td>
</tr>
<tr>
<td>DS</td>
<td>103 ± 4.9**</td>
<td>143 ± 6.3</td>
<td>131 ± 10.3</td>
<td>95 ± 10.8**</td>
<td>127 ± 4.6</td>
</tr>
<tr>
<td>AH</td>
<td>216 ± 14.1**</td>
<td>330 ± 29.9</td>
<td>-</td>
<td>173 ± 6.6**</td>
<td>-</td>
</tr>
<tr>
<td>EA</td>
<td>118 ± 7.9**</td>
<td>191 ± 9.9</td>
<td>190 ± 11.6</td>
<td>133 ± 5.0**</td>
<td>-</td>
</tr>
<tr>
<td>RB</td>
<td>120 ± 7.0**</td>
<td>167 ± 7.0</td>
<td>141 ± 11.3</td>
<td>-</td>
<td>133 ± 11.1</td>
</tr>
<tr>
<td>MW</td>
<td>127 ± 10.1**</td>
<td>159 ± 6.8</td>
<td>159 ± 4.1</td>
<td>150 ± 7.6</td>
<td>182 ± 16.4</td>
</tr>
</tbody>
</table>

The number of days of therapy and the number of observations for each period are as in Table 4-2.

**P<0.01  All results are compared with those obtained during therapy with Colestipol 15g daily.
Figure 4-1
The effects of colestipol on serum calcium concentration, serum alkaline phosphatase activity, and fasting blood sugar in eight patients with hyperlipoproteinaemia. The values shown are the means of several determinations performed on different days during the administration of a placebo (PL) and colestipol 15g daily (CO). P values were obtained by paired t-test analysis. There was no significant change in serum inorganic phosphorus concentration.
CHAPTER 5

EFFECTS OF COLESTIPOL ON CHOLESTEROL METABOLISM IN MAN

An investigation is described into the effects of colestipol on the body pools and metabolism of cholesterol in patients with hyperlipoproteinemia, using the techniques of compartmental isotope dilution analysis and isotopic steroid balance. This study was performed in parallel with the clinical trial of colestipol described in the previous chapter, measurements being made during the placebo period and again after several months of colestipol therapy.

Colestipol therapy enhanced the fecal excretion of bile acids. This was followed by a compensatory increase in cholesterol production, which was positively correlated with the fall in the plasma concentration of cholesterol. The amount of cholesterol in the rapidly exchanging pool of tissue cholesterol was decreased in most subjects but finding probably reflecting enhanced synthesis within the liver and intestine. The rate of excretion of cholesterol from this pool was greatly increased and correlated significantly with the increased excretion of bile acids. Although the size of the larger pool of more slowly exchanging cholesterol could not be measured, the rate constant for loss of cholesterol from this pool was unchanged.

Similar values for cholesterol turnover were obtained with both techniques during colestipol treatment, but not during the placebo period when 2-pool isotope dilution analysis gave higher values. Two possible reasons for this discrepancy have been substantiated, namely the existence of a third very slowly exchanging pool of body cholesterol and the derivation of some faecal cholesterol, during states of increased cholesterol turnover, from hepatic cholesterol that is not in equilibrium with that of plasma.
ABSTRACT

An investigation is described into the effects of colestipol on the body pools and metabolism of cholesterol in patients with hyperlipoproteinaemia, using the techniques of compartmental isotope dilution analysis and isotopic steroid balance. This study was performed in parallel with the clinical trial of colestipol described in the previous chapter, measurements being made during the placebo period and again after several months of colestipol therapy.

Colestipol therapy enhanced the faecal excretion of bile acids. This was accompanied by a compensatory increase in cholesterol production, which was positively correlated with the fall in the plasma concentration of cholesterol. The amount of cholesterol in the rapidly exchanging pool of tissue cholesterol was decreased in most subjects but was unchanged or increased in two subjects, the latter finding probably reflecting enhanced synthesis within the liver and intestine. The rate of excretion of cholesterol from this pool was greatly increased and correlated significantly with the increased excretion of bile acids. Although the size of the larger pool of more slowly exchanging cholesterol could not be measured, the rate constant for loss of cholesterol from this pool was unchanged.

Similar values for cholesterol turnover were obtained with both techniques during colestipol treatment, but not during the placebo period when 2-pool isotope dilution analysis gave higher values. Two possible reasons for this discrepancy have been substantiated, namely the existence of a third very slowly exchanging pool of body cholesterol and the derivation of some faecal cholesterol, during states of increased cholesterol turnover, from hepatic cholesterol that is not in equilibrium with that of plasma.
INTRODUCTION

The recognition of the role of hypercholesterolaemia in the genesis of coronary heart disease has stimulated the development of drugs capable of reducing the plasma cholesterol concentration. Colestipol (U-26, 597A) is a recent addition to the range of drugs at present available for this purpose. It is a bile acid-sequestering resin, somewhat similar to cholestyramine, and recent trials have confirmed that it is an efficient cholesterol-lowering agent in many patients with type II hyperlipoproteinaemia (see previous chapter). The theoretical basis for the use of such resins is to interrupt the enterohepatic circulation of bile salts, which in turn might stimulate the catabolism of cholesterol to bile acids within the liver, thereby lowering the plasma cholesterol level (Boyd and Percy-Robb, 1971). In some patients, however, the plasma cholesterol is not lowered by cholestyramine therapy, despite a substantial increase in bile acid excretion (Moutafis et al, 1971). This could result from an equivalent increase in cholesterol production or from the transfer of cholesterol from tissues into plasma. These consequences of resin therapy may occasionally even lead to an increase in the plasma cholesterol level (Grundy et al, 1971). For these reasons it is desirable to establish the effects of new resins, such as colestipol, on cholesterol metabolism including the mass of cholesterol in tissue pools.

In the present study the effects of colestipol on the distribution and metabolism of cholesterol have been investigated in eight patients with hypercholesterolaemia by examining the effects of treatment on a two-pool model of cholesterol turnover (Nestel et al, 1969). In addition, a comparison has been made, both before and during resin therapy, of estimates of cholesterol turnover obtained by this technique with those obtained by isotopic steroid balance, which measures separately the faecal excretion of endogenous cholesterol and of bile acids (Grundy and Ahrens, 1966).
METHODS

Patients

Eight patients with hyperlipoproteinaemia were studied. The relevant clinical and pathological details of each subject appear in Table 5-1. Two patients had evidence of coronary heart disease. Of the two female patients studied, one (AT) had reached the menopause, while the other (AR) had previously had a hysterectomy. Subject AT was receiving ethinyl oestradiol (0.02 mg daily), a drug known to influence cholesterol metabolism (Nestel et al, 1965). All subjects had been eating their customary solid diets for several months before entering the study and continued to do so for its duration.

Two subjects (EA, DS) had been previously instructed in diets low in cholesterol and high in polyunsaturated fatty acids, with resultant lowering of plasma cholesterol levels by an average of 80 and 100 mg/100ml respectively. Body weights remained essentially constant (Table 5-1).

The nature of the investigation was fully explained and each subject gave informed consent.

Clinical Procedures and Experimental Design

The investigation was conducted on an out-patient basis. Causes of hyperlipoproteinaemia due to diabetes mellitus, hypothyroidism, renal and liver disease or alcoholism were excluded. All drugs previously prescribed for other conditions were continued at constant dosage and no additional drugs were prescribed during the study, with the exception that subject EA commenced propranolol 120 mg daily for worsening angina half-way through the placebo period.

Patient LO was given an intravenous infusion of 73 $\mu$C of DL-mevalonic acid-1-$^1\text{C}$ (specific activity = 6.85 mC/mmol)*, prepared by incubating the lactone for 1 hour at 37°C in sterile sodium bicarbonate solution of pH 8.7 (Nestel, 1970b). The remaining patients received 51 to 123 $\mu$C of cholesterol-1α-3H or cholesterol-1α,2α-3H (specific activity

* All radioactive materials were obtained from the Radiochemical Centre, Amersham, England, unless otherwise stated. Radiochemical purity exceeded 97% by thin layer chromatography.
= 500 mC/mmol) intravenously, either dispersed in 200 ml sterile 0.9% saline (DS, AH, EA) or complexed with autologous plasma lipoproteins (AL, AT, RB, MW) as described in Chapter 3. Animal experiments have demonstrated that these two methods of infusing radioactive cholesterol give identical results with 2-pool analysis (Nilsson and Zilversmit, 1972). All glassware, syringes and tubing were rinsed in absolute ethanol and the calculated doses corrected for residual radioactivity.

Each patient then entered a control period during which an inactive preparation of microcrystalline cellulose was taken three times daily immediately before meals as a suspension in fruit juice or water. Patients were seen on three occasions during the first week and thereafter at approximately weekly intervals, after a 12-14 overnight fast. On each occasion venous blood was collected into a heparinised tube for determination of plasma cholesterol specific activity and plasma concentrations of cholesterol and triglyceride.

Stools were collected from each subject (Pryke and Whyte, 1970) for 8 consecutive days, at least 35 days after the injection of the label, for the determination of faecal endogenous steroid excretion. Faeces were stored at -25°C prior to analysis. Results were corrected for variations in faecal flow by measuring the excretion of chromium oxide (Davignon et al, 1968), which was administered at a dosage of 100 mg thrice daily for 8 days before each collection and for its duration. Possible losses of neutral steroids by bacterial degradation were monitored by measuring the recovery of radioactive β-sitosterol (Ahrens, 1970), administered orally on the first day of each collection period. Subject LO was given 5μC of β-sitosterol-22,23-3H (New England Nuclear Corporation, Boston, Mass.) and the remaining subjects 2μc of β-sitosterol-4-14C.

† Faecal total endogenous steroid excretion = faecal excretion of endogenous cholesterol and its derivatives of bacterial degradation (neutral steroids) plus that of bile acids (acidic steroids).
After 68 to 98 days (179 days in the case of subject AH) colestipol 5g three times daily was substituted for the placebo. The effect on plasma cholesterol specific activity was followed in four subjects (LO, AH, EA, RB) for a further 41 to 59 days. After 55 to 60 days of colestipol therapy (323 days in the case of AL) all patients except subject AH were given a second intravenous infusion, using in each instance an injection procedure identical to that used on the first occasion. Subject LO received 150μC of DL-mevalon 2-3H (specific activity = 87 mCi/mmol) and the other 6 subjects 25 to 47μC of cholesterol-4-14C (61.7 mCi/mmol). Plasma samples were collected serially as before for a period of 82 to 149 days. Between 39 and 68 days after the second injection the stool collections were repeated, subject LO being given on this occasion 2μC of β-sitosterol-4-14C and the others 5μC of β-sitosterol-22,23-3H.

During the second collection period a fasting sample of duodenal bile was obtained by intubation from 5 subjects (LO, AT, EA, RB, MW) and stored at -25°C. Samples of bile were also collected from two normal subjects who were not receiving colestipol and who had been infused with 50μC cholesterol-26-14C (specific activity = 24.2mCi/mmol) 3 to 7 weeks previously for other reasons. Patients were weighed periodically during both study periods.

To establish that both radioactive preparations of cholesterol give similar results for 2-pool analysis and faecal steroid excretion, subject AH was given both cholesterol-4-14C (12.6μC) and cholesterol-1α,2α-3H (45.2μC) simultaneously for the second study. This was performed after 339 days of colestipol therapy. During the last 161 days she had also been taking clofibrate 2g daily, and this was continued throughout. The plasma cholesterol specific activity-time curves for 3H and 14C fell in parallel over a period of 65 days. The ratio of 3H specific activity/14C specific activity averaged 3.58 ± 0.16 (mean ± SD) compared to a value of 3.59 for 3H dpm infused/14C dpm infused. There was no significant difference (P>0.1) between the values for faecal steroid excretion determined simultaneously with the two isotopes. These results are presented in Figure 5-1.
Laboratory Procedures

Characterization of hyperlipoproteinaemia was achieved by separating plasma lipoproteins by ultracentrifugation and precipitation and by electrophoresis on cellulose acetate. Cholesterol and triglyceride concentrations were determined with a Technicon Auto Analyzer II. The details of this procedure have been described in Chapter 4.

Cholesterol turnover: 2-pool studies

Radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer (model 574) using PPO-dimethyl POPOP in toluene as scintillator solvent. Counting efficiency was monitored by automatic external standardization. Double isotope counting was employed for all samples containing both $^{14}$C and $^3$H (Kobayashi and Maudsley, 1970).

For measurement of plasma cholesterol specific activity 1.0 - 5.0 ml plasma was extracted with Dole's solution (Dole, 1956). Aliquots of the heptane phase were assayed for total cholesterol mass and radioactivity. During the first two weeks following each infusion, however, the specific activities of free and esterified cholesterol were determined separately after thin layer silicic acid chromatography of the lipid extract (Kieselgel G; hexane:diethyl ether:methanol:acetic acid, 180:40:6:4, solvent). Each plasma cholesterol specific activity-time curve was resolved with the aid of a PDP-8 series computer into two exponential components and fitted to a 2-compartment model of cholesterol turnover as described by previous workers (Nestel et al, 1969; Goodman and Noble, 1968; Gurpide et al, 1964). During the early part of the curves, when free cholesterol specific activity was greater than that of esterified cholesterol, the former was used for kinetic analysis. Thereafter, when the specific activities of free and esterified cholesterol were equal, that of total cholesterol was used (Figure 5-2). In the case of subject L0, who was given mevalonic acid, only those compartmental parameters not requiring knowledge of dose were calculated, since it cannot be established what fraction of the infused mevalonic acid was converted to cholesterol.
Cholesterol turnover: isotopic steroid balance

Faecal collections were divided into four 2-day pools, and the excretion of endogenous neutral and acidic steroids estimated by the method of isotopic steroid balance (Grundy and Ahrens, 1966). The laboratory procedures and calculations were performed exactly as described by Grundy and Ahrens (1966). In this method the faecal neutral and acidic steroids are extracted separately from aliquots of homogenized faeces after prior saponification. The excretion of radioactivity within each fraction is then determined and converted to steroid mass by relating it to the plasma cholesterol specific activity:time curve. Recovery studies confirmed that extraction of both fractions by this technique was complete even in the presence of colestipol, and no modification was required to elute the bile acids from the resin.

The excretion within the neutral steroid fraction of radioactive $\beta$-sitosterol was determined simultaneously by double isotope counting. Recoveries averaged 94% (range: 81-103%) during the placebo period and 93% (range: 87-100%) during colestipol therapy (Table 5-2). Such figures indicate a virtual absence of degradative steroid losses during intestinal transit, since about 5% of $\beta$-sitosterol is absorbed (Ahrens, 1970). This confirms and extends other recent reports of almost complete steroid recovery during the consumption of solid diets (Kudchodkar et al, 1972a), and contrasts with the considerable losses which may occur during formula feeding (Ahrens, 1970).

Chromium oxide excretion was measured by the method of Davignon et al (1968). All faecal analyses were performed in duplicate.

The validity of both methods employed for measuring cholesterol turnover requires that cholesterol metabolism be in a steady state. Although it is impossible to establish with certainty that such conditions existed, the usual criteria of stable body weight, constant diet and stable plasma lipid levels suggested that a steady state was present at least during the studies carried out in the placebo period.
With colestipol, turnover studies were started only after plasma cholesterol levels had been constant for at least one month.

**Biliary cholesterol and cholic acid**

Aliquots of bile were refluxed for 1 hour with 1N NaOH in 90% ethanol and the cholesterol extracted with petroleum ether. Cholesterol specific activity was then determined, using the Auto Analyzer for measurement of mass. The lower phase was evaporated, dissolved in 2N NaOH and saponified for 2 hours at 15 psi. Bile acids were extracted with diethyl ether after acidification to pH2 with concentrated HCl, and methylated in 5% HCl-methanol overnight. Cholic acid methyl ester was isolated by 2-stage thin layer chromatography (Grundy et al, 1965; Kottke et al, 1966) and its specific activity determined, the mass of the trimethylsilyl derivative being quantified by gas chromatography using 5α-cholestane as internal standard (Grundy et al, 1965). (In the case of the two subjects who had received cholesterol-26-14C the specific activity of bile cholesterol only could be determined, since the 26-carbon is removed during bile acid synthesis).

**Calculations**

Plasma volumes were estimated as described by Edwards and Whyte (1960). All calculations for 2-pool analysis and double isotope counting were performed using the FOCAL system on a PDP-8 series computer (see Appendix II).

**RESULTS**

**Plasma Cholesterol and Triglyceride Concentrations**

Colestipol therapy reduced plasma concentrations of cholesterol by 20 to 80 mg/100ml (mean = 54mg/100ml) and increased those of triglyceride by 32 to 249mg/100ml (mean = 79mg/100ml). Both of these results were statistically significant (paired t-test analysis: P<0.01) and are presented in Table 5-1. They have been discussed in more detail in Chapter 4.
Cholesterol Turnover: 2-Pool Studies

The changes in plasma cholesterol specific activity are represented by Figure 5-2, which illustrates the changes observed in subjects LO, EA and RB, the three subjects in whom all investigations, including the biliary studies, were carried out. Equilibration of plasma free and esterified cholesterol specific activities became established within 3 to 5 days. Thereafter, each plasma total cholesterol specific activity-time curve appeared to be bi-exponential for the duration of the placebo period (68 to 179 days). Half-times for the rapid components of the curves ranged from 3.9 to 7.0 days (mean = 5.3 days). Those for the slow component ranged from 33.7 to 61.2 days and are presented in Table 5-3.

Colestipol therapy produced an immediate acceleration in specific activity decay in each of the four subjects studied (Figure 5-2). During this early period the fall in free cholesterol specific activity was initially more rapid than that in esterified cholesterol specific activity, although equilibration was restored before the second infusion. The implications of these observations, as well as those of associated changes in plasma lipoproteins and plasma cholesterol esterifying activity are discussed later (Chapters 6 and 7).

Following infusion of the second isotope the equilibration of free and esterified cholesterol tended to be more rapid than during the placebo period, being in all cases complete by the third day. This may have been a consequence of increased plasma LCAT activity (see Chapter 7). The subsequent decay in total cholesterol specific activity again appeared to be bi-exponential during the 82 to 149 days of measurement. Half-times for the rapid component were not significantly different (P>0.2) from those after the first infusion (range = 3.4 to 6.3 days; mean = 4.6 days). However, the half-times of the slow component were significantly faster with colestipol therapy (Table 5-3).

Double isotope counting permitted the continuation of the first curve to be followed simultaneously with the second exponential of the second curve (Figure 5-2).
The former had become flatter in every subject suggesting a possible third, very slow pool, since it was falling at a rate which was significantly slower ($P = 0.004$) than the second exponential of the placebo period. This slowest, final exponential derived from the first labelling was also significantly slower than the simultaneously calculated second exponential derived from the second labelling ($P = 0.0002$).

The results of kinetic analyses are presented in Table 5-4. Colestipol therapy produced an increase in cholesterol production rate ($P_{d}$) in every subject, which ranged from 0.40 to 1.53g/day. This was accompanied by a decrease of 1.7 to 6.8g in the size of pool $A$, the rapidly equilibrating pool of body cholesterol. When this pool is divided into the fraction contributed by plasma cholesterol ($M_{ap}$) and that contained within other tissues ($M_{ax}$), it is apparent that in 4 out of 6 subjects (AL, EA, RB, MW) the reduction in pool $A$ reflected decreases in both of these components. In 2 subjects, however, $M_{ax}$ was either unchanged (AT) or increased (DS) by colestipol therapy. Although the rate constant for the total removal of cholesterol from the slowly equilibrating pool $B$ ($k_{bb}$) was unaffected by resin therapy, that for total removal from pool $A$ ($k_{aa}$) was significantly increased. This latter change was apparently due to an increase in the rate constant for excretion from pool $A$ ($k_{a}$), that for transfer of cholesterol from pool $A$ to pool $B$ ($k_{ab}$) having been unaffected. The reduction in $M_{a}$ in the presence of an unchanged $k_{ab}$ produced a significant decrease in the rate of transfer of cholesterol from pool $A$ to pool $B$ ($r_{ab}$)‡.

Bile Analysis

The specific activities of plasma and biliary cholesterol were identical in the two subjects not receiving therapy (bile v. plasma, dpm/mg: subject RB, 536 v 548; subject KO, +

‡ As discussed in Chapter 3 the values derived for $k_{a}$, $k_{ab}$ and $r_{ab}$ by this technique are approximate only. An estimate of the size of pool $B$ (the slowly equilibrating pool of body cholesterol) has not been calculated for the reasons discussed in the same Chapter.
In the 5 subjects receiving colestipol therapy, however, the specific activity of biliary cholesterol was significantly lower than that of plasma cholesterol, although no significant differences were observed between the specific activities of plasma cholesterol and those of biliary cholic acid (Table 5-5).

**Cholesterol Turnover: Isotopic Steroid Balance**

These results are presented in Table 5-6.

Faecal bile acid excretion was increased in all subjects by a mean of 1236 mg/day (305%) during colestipol therapy. Endogenous neutral steroid excretion was increased in 2 subjects (AL, AT) and was decreased in one subject (RB) (P<0.01). Since the changes in the remaining 5 patients were small, the overall effect of colestipol on neutral steroid excretion (as assessed by paired t-test analysis) was not statistically significant (P = 0.3). Cholesterol turnover as estimated by total faecal endogenous steroid excretion was accordingly increased by a mean of 1329 mg/day (122%).

**DISCUSSION**

The results of two-pool analysis obtained during the control period are similar to those obtained by previous workers in subjects with hyperlipoproteinaemia, with the exception that the mean value of 1.40g/day for cholesterol

\[\text{The method of isotopic steroid balance requires that the specific activities of faecal endogenous steroids are similar to the plasma cholesterol specific activity which existed several days before the faecal collection, an established fact for subjects with average cholesterol turnover 3 or more weeks after the infusion of radioactive cholesterol (see Chapter 3). Since, however, the specific activity of bile cholesterol was consistently lower than that of plasma cholesterol during colestipol therapy, the estimates of faecal endogenous neutral steroid excretion during this time have been corrected by multiplying by the appropriate ratio of plasma cholesterol specific activity/bile cholesterol specific activity. In those patients in whom duodenal intubation was not performed the average ratio of 1.21 was used.}\]
production rate in patients with type II hyperlipoproteinemia, is slightly higher than those reported in previous studies (Nestel, et al, 1969; Goodman and Noble, 1968). There appear, however, to be adequate reasons for this difference. Hypertriglyceridaemia (Sodhi and Kudchodkar, 1971), excess body weight (Miettinen, 1971a) and ethinyl oestradiol therapy (Nestel et al, 1965) have all been shown to be associated with an increased rate of cholesterol turnover, and this may also be true for diets rich in polyunsaturated fatty acids (reviewed by Nestel, 1970a). One or more of these factors was present in each of the subjects included in the present study. The existence of factors influencing cholesterol turnover should not, however, invalidate the later assessment of colestipol therapy since body weight, relevant drug therapy and pre-existing diets were all maintained constant throughout the study.

One effect of colestipol therapy was to significantly reduce the size of pool A, the rapidly equilibrating pool of body cholesterol, from a mean of 29.0g to a mean of 25.4g. In 4 subjects this change reflected a reduction not only in the plasma pool, consequent upon the decrease in the plasma concentration of cholesterol, but also in the remaining component of pool A, $M_{ax}$. In the other subjects, however, there was either no change in $M_{ax}$ (AT) or an increase (DS), despite a fall in plasma cholesterol level. Accordingly there was no correlation between the changes in $M_{ap}$ and those in $M_{ax}$. The latter includes most of the cholesterol within the liver and possibly the intestine (Chobanian and Hollander, 1962), the two organs in which cholesterol synthesis is most active (Wilson, 1972b). Since cholesterogenesis within these organs is known to be inhibited by bile salts, either directly or indirectly (Wilson 1972b), and accordingly to be stimulated by the administration of the bile acid-sequestering resin cholestyramine (Grundy et al, 1971), the failure of the fall in $M_{ap}$ to be accompanied by a similar change in $M_{ax}$ in subjects AT and DS almost certainly reflected enhanced synthesis within these tissues. In a recent study in rats, colestipol did not influence the size of pool A (Phillips and Elfring, 1972). Previous
studies with other cholesterol-lowering drugs have demonstrated varying effects on body cholesterol pools in man. Goodman and Noble (1968), found cholestyramine therapy not to affect the size of pool A, while Kudchodkar et al (1972c) found the same drug to expand the tissue component of that pool. Neomycin (Samuel et al, 1968) and clofibrate (Grundy, Ahrens, Salen et al, 1972), however, were both reported to produce substantial reductions in body cholesterol pools, possibly because compensatory increases in cholesterol synthesis do not occur with these two drugs (Samuel et al, 1968; Grundy, Ahrens, Salen et al, 1972).

No attempt was made in the present study to calculate the size of pool B, where the bulk of body cholesterol is stored, for the reasons previously given (Chapter 3). The reduction in cholesterol transfer from pool A to pool B ($r_{ab}$) might well have reduced the size of pool B. However, this is dependent in part on the effect of colestipol on cholesterol synthesis within pool B, any stimulation of which would tend to maintain, and might even increase, its size. The observation that long-term treatment with cholestyramine may decrease the size of xanthomata (Khachadurian, 1968), the cholesterol of which is mainly a component of pool B (Samuel et al, 1972), suggests that other slowly equilibrating pools of cholesterol, including that of arterial wall (Chobanian and Hollander, 1962), may also diminish in size during resin therapy. This is supported by the report of diminished atherogenesis in experimental animals receiving colestipol (Kritchevsky et al, 1973). Although, carcass analyses have failed to show any change in the total body cholesterol of dogs after long-term biliary diversion (Kirchman et al, 1970), a procedure having similar metabolic effects to those of cholestyramine therapy (Boyd and Percy-Robb, 1971), the balance between bile acid loss and compensatory overproduction of cholesterol may be different in that species.

The fractional increase during colestipol therapy in the rate constant for clearance of cholesterol from pool A ($k_a$) was positively correlated with that in the faecal excretion
of bile acids \((r = +0.76, P<0.05)\). These changes, which are presented in Figure 5-3, were of similar magnitude to those reported in experimental animals (Phillips and Elfring, 1972; Parkinson et al, 1973), and confirm the mode of action of colestipol in lowering plasma cholesterol levels by enhancing its catabolism to bile acids within the liver. Although there was no correlation between the changes in either bile acid or neutral steroid excretion alone and the fall in plasma cholesterol level, the latter was significantly related to the increase in total endogenous steroid excretion in the 6 type II hyperlipoproteinaemic patients \((r = +0.89, P<0.05)\) (Figure 5-4). This suggests that the cholesterol-lowering effect of colestipol was influenced by changes not only in bile acid synthesis but also, to some extent, in neutral steroid excretion. In the present study 2 subjects (AL, AT) developed an increase in endogenous neutral steroid excretion \((P<0.01)\), 5 showed no change, and one subject (RB) demonstrated a decrease \((P<0.01)\) (Table 5-6). There is evidence that cholestyramine, which has a similarly variable effect in this respect (Moutafis and Myant, 1969a; Grundy et al, 1971; Nazir et al, 1972), may have independent and opposing effects on neutral steroid excretion, decreasing both the secretion of cholesterol into bile (Redinger and Passi, 1972) and, in some subjects, the absorption of cholesterol by the intestine (Grundy and Ahrens, 1969). The net effect on neutral steroid excretion may depend on a balance between these two factors, and may influence the cholesterol-lowering effect of bile acid-sequestrant therapy.

The absence of any significant increase in \(k_{ab}\) or decrease in \(k_{bb}\) makes it unlikely that the decrease in plasma cholesterol level involved a major transfer into tissue cholesterol stores.

The increased faecal excretion of total endogenous steroids confirms that cholesterol turnover was enhanced by colestipol therapy. To date there have been few comparisons of the estimates of cholesterol turnover obtained by the 2-pool and the isotopic steroid balance techniques. In Table 5-7 the values obtained by isotopic steroid balance have
been expressed as a per cent of those obtained simultaneously by two-pool analysis in the same subjects. It can be seen that during the placebo period faecal analysis gave values that were on average only 72% of those derived from the specific activity-time curves. Other workers have also found that steroid balance gives estimates consistently lower than those gained by two-pool analysis. Grundy and Ahrens (1969) reported that in man steroid balance averaged 85% of the value for cholesterol production rate obtained by isotope dilution, while others have observed values of 62-76% (Lofland et al, 1968) and 64-72% (Manning et al, 1971) in non-human primates. Similar values for steroid balance have been obtained by the isctopic and gas-liquid chromatographic methods (Grundy and Ahrens, 1966; Kudchodkar et al, 1972b), excluding an error in the estimation of faecal steroids by the isotopic method. There appear to be a number of possible reasons for the difference. Firstly the value obtained by steroid balance includes neither cholesterol which is excreted via the skin (Bhattacharyya et al, 1972) nor that which is metabolized by the adrenal glands (Borkowski et al, 1972), which may account for approximately 80 and 50 mg/day respectively. Secondly, it has been demonstrated that the two-pool model of cholesterol turnover, although the most convenient one, is an oversimplification. It omits a very rapid fall in plasma cholesterol specific activity during the first few hours after the infusion (Samuel et al, 1968), and ignores the existence of a third, very slowly equilibrating pool. Evidence for the latter was obtained in the present study by following both specific activity-time curves simultaneously during the period of resin therapy. Although there had been an early rapid decline in the first curve immediately after starting therapy, it had flattened considerably several months later. This finding, which is similar to that made by Moutafis and Myant (1969a) during cholestyramine therapy, could derive theoretically from a later decrease in cholesterol turnover, a gradual increase in cholesterol pool size, the presence of a third very slowly equilibrating pool of exchangeable cholesterol, or a combination of these factors. In the
present patients the first two possibilities would not be in accord with the finding that the half-time for the late part of the first curve was consistently longer than that for the simultaneous fall in the second curve, since they would be expected to influence both curves equally (Figure 5-2, Table 5-3). This observation thus confirms other recent evidence for a very slowly equilibrating pool of exchangeable cholesterol, obtained by following plasma cholesterol specific activity-time curves for a year or more (Samuel et al, 1972; Goodman et al, 1973; Samuel and Lieberman, 1973), and by determining the specific activity of cholesterol in various body tissues (Quintao et al, 1971b; Myant, 1971). Analysis of cholesterol metabolism in terms of a two-pool model accordingly overestimates turnover when compared with values obtained by more detailed analysis of the specific activity-time curves (Goodman et al, 1973; Samuel and Lieberman, 1973). Thus, under normal conditions cholesterol turnover is underestimated by steroid balance and overestimated by two-pool analysis.

In comparison with the findings during the placebo period, however, significantly better agreement was obtained when cholesterol turnover was stimulated by colestipol therapy (Table 5-7). The possibility that any difference between the two estimates was exaggerated during the control period by losses of $^3$H from cholesterol and bile acids to body water was excluded by the results of the double-labelling experiment (subject AH). This demonstrated that the decline in plasma cholesterol specific activity and the results of isotopic steroid balance were the same for both cholesterol-$4^{-13}$C and cholesterol-$1a,2a^-3$H (Figure 5-1). The likeliest explanation for the improved agreement is that when cholesterol turnover is increased, especially within pool A, the error incurred by omitting to consider the third pool will be reduced. Furthermore, the present bile studies confirm a previous report that, although the specific activities of bile cholesterol and plasma cholesterol are normally similar three or more weeks after labelling of plasma cholesterol, that of bile cholesterol
decreases relative to that of plasma cholesterol when turnover is stimulated (Grundy et al., 1971). Thus, under conditions of greatly enhanced cholesterol synthesis hepatic cholesterol fails to equilibrate completely with that of plasma before its secretion into the bile. This will result in an underestimation of cholesterol turnover by compartmental isotope dilution analysis, since a fraction of total turnover is now "by-passing" the plasma. Thus, factors will operate during enhanced cholesterol turnover which will tend to approximate the values obtained by two-pool analysis and steroid balance. This is supported by the improved agreement between these two techniques obtained in very obese subjects, who have high turnover rates comparable to those observed during colestipol therapy (Nestel, Shcreibman and Ahrens, 1973). The relationship between the present estimates of turnover is illustrated in Figure 5-5. The correlation coefficient for the pooled data was statistically highly significant \( r = +0.93, P<0.001 \).

Bile cholic acid specific activities were consistently greater than those of bile cholesterol during colestipol therapy \( P<0.002 \). However, they may also have been reduced relative to those of plasma cholesterol by treatment, since the ratio of cholic acid specific activity/plasma cholesterol specific activity is usually greater than the observed value of 1.00 ± 0.08 (mean ± SD) (Lindstedt, 1962). This may be due to the fact that the cholic acid was derived partly from newly synthesized hepatic cholesterol, as bile cholesterol appeared to be. However, an alternative explanation is provided by the increased fractional turnover of bile acids which is known to occur during resin therapy (Garbutt and Kenney, 1972).

The increase in cholesterol turnover was accompanied not only by a decrease in the levels of plasma cholesterol, but also by a significant increase in those of plasma triglyceride (Table 5-1). Similar changes in plasma lipids have also been noted during cholestyramine therapy (Grundy et al., 1971), and reflect reciprocal changes in the concentrations of VLDL and LDL (see Chapter 6). The association of
high VLDL levels, low LDL levels and increased bile acid production has also been noted in other circumstances, such as obesity (Wilson and Lees, 1972; Miettinen, 1971a), hyperlipoproteinaemia of types IV and V (Fredrickson et al, 1968; Kottke, 1969) and carbohydrate feeding (Wilson and Lees, 1972; Whyte et al, 1973). The results of the investigation described in Chapter 6 suggest that the turnover of plasma cholesterol involves influx of cholesterol predominantly within VLDL and its efflux mainly from LDL prior to catabolism to bile acids by the liver. Such a scheme provides an explanation for the association of changes described above.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex:Age (yrs)</th>
<th>Height (cm)</th>
<th>Standard*</th>
<th>Mean Placebo</th>
<th>Mean Treatment</th>
<th>Mean Plasma Cholesterol (mg/100 ml) Placebo: Treatment</th>
<th>Mean Plasma Triglyceride (mg/100 ml) Placebo: Treatment</th>
<th>Pathological Features</th>
<th>Other Drug Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO</td>
<td>M:52</td>
<td>187</td>
<td>80.0</td>
<td>93.5</td>
<td>91.1</td>
<td>388:361</td>
<td>484:733</td>
<td>IV</td>
<td>Hypertension; Cerebrovascular accident; Methyl Dopa; Chlorothiazide</td>
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<td>64.7</td>
<td>71.0</td>
<td>72.3</td>
<td>291:241</td>
<td>168:202</td>
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<td></td>
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<td>F:48</td>
<td>156</td>
<td>52.6</td>
<td>59.8</td>
<td>59.5</td>
<td>477:397</td>
<td>227:307</td>
<td>IIb</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td>M:35</td>
<td>169</td>
<td>66.7</td>
<td>62.0</td>
<td>62.9</td>
<td>292:272</td>
<td>103:143</td>
<td>IIa</td>
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<td>AH</td>
<td>F:47</td>
<td>159</td>
<td>54.0</td>
<td>64.5</td>
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<td>289:--</td>
<td>216:--</td>
<td>IIb</td>
<td>Amitryptiline</td>
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<td>80.0</td>
<td>79.7</td>
<td>357:278</td>
<td>127:159</td>
<td>IIa</td>
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</table>

* Average weight for height and sex at 20–24 years of age (Society of Actuaries, 1959).
† Type of hyperlipoproteinaemia (World Health Organization, 1970).
Table 5-2
Faecal recovery of orally administered radioactive β-sitosterol in eight patients with hyperlipoproteinaemia during the consumption of solid diets

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>PLACEBO (%</th>
<th>COLESTIPO (%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN ± SD</td>
<td></td>
</tr>
<tr>
<td>LO</td>
<td>81</td>
<td>94</td>
</tr>
<tr>
<td>AL</td>
<td>100</td>
<td>100</td>
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<td>95</td>
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<tr>
<td>MW</td>
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<td>93</td>
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</table>

**MEAN ± SD**

<table>
<thead>
<tr>
<th>PLACEBO</th>
<th>COLESTIPOL</th>
</tr>
</thead>
<tbody>
<tr>
<td>94 ± 7.3</td>
<td>93 ± 4.2</td>
</tr>
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</table>

**P**

0.75

*P value obtained by paired t-test analysis.
## Table 5-3
Half-times for slow decline of plasma cholesterol specific activity

<table>
<thead>
<tr>
<th>Patient</th>
<th>Placebo Period</th>
<th>Colestipol Period</th>
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</thead>
<tbody>
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<td></td>
<td>1st Isotope†</td>
<td>2nd Isotope</td>
</tr>
<tr>
<td></td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td>LO</td>
<td>50.5 (49-98)*</td>
<td>38.0 (55-97)</td>
</tr>
<tr>
<td>AL</td>
<td>52.6 (40-68)</td>
<td>37.0 (42-83)</td>
</tr>
<tr>
<td>AT</td>
<td>52.2 (42-84)</td>
<td>33.4 (40-82)</td>
</tr>
<tr>
<td>DS</td>
<td>33.7 (35-84)</td>
<td>27.7 (49-111)</td>
</tr>
<tr>
<td>AH</td>
<td>61.2 (130-179)</td>
<td>-</td>
</tr>
<tr>
<td>EA</td>
<td>49.1 (43-86)</td>
<td>30.2 (43-113)</td>
</tr>
<tr>
<td>RB</td>
<td>37.2 (48-91)</td>
<td>33.0 (48-149)</td>
</tr>
<tr>
<td>MW</td>
<td>43.6 (42-93)</td>
<td>23.1 (42-112)</td>
</tr>
</tbody>
</table>

| P † | 0.0001 | 0.0002 |
| Mean Difference | -13.8 | +21.9 |

* ‡ ³H and ¹⁴C were used as the first and second isotopes respectively in all patients except LO, in whom the reverse order was employed.

*(A-B): half-time was calculated from 6-10 specific activity determinations made between day A and day B after isotope infusion (during which time cholesterol SA appeared to decline mono-exponentially).

† P values were obtained by paired t-test analysis.
### Table 5-4
Parameters of cholesterol distribution and turnover in eight during colestipol therapy (CO).

<table>
<thead>
<tr>
<th>PR (_a) (g/day)</th>
<th>M (_a) (g)</th>
<th>M (_{ap}) (g)</th>
<th>M (_{ax}) (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PL CO</td>
<td>PL CO</td>
<td>PL CO PL CO</td>
</tr>
<tr>
<td>LO</td>
<td>- -</td>
<td>13.0 12.0</td>
<td>- -</td>
</tr>
<tr>
<td>AL</td>
<td>1.60 2.00</td>
<td>34.1 27.3</td>
<td>7.9 6.7</td>
</tr>
<tr>
<td>AT</td>
<td>1.16 2.28</td>
<td>23.6 21.9</td>
<td>11.6 9.7</td>
</tr>
<tr>
<td>DS</td>
<td>1.36 1.86</td>
<td>18.5 20.2</td>
<td>7.7 7.2</td>
</tr>
<tr>
<td>AH</td>
<td>1.37 -</td>
<td>30.4 -</td>
<td>7.4 6.5</td>
</tr>
<tr>
<td>EA</td>
<td>1.42 2.53</td>
<td>36.4 30.5</td>
<td>13.2 11.0</td>
</tr>
<tr>
<td>RB</td>
<td>1.32 2.23</td>
<td>25.5 23.2</td>
<td>8.6 7.4</td>
</tr>
<tr>
<td>MW</td>
<td>1.57 3.10</td>
<td>35.8 29.4</td>
<td>10.7 8.3</td>
</tr>
</tbody>
</table>

| P\(^+\) | 0.004 | 0.048 | <0.001 | 0.09 |
| Mean    | +0.98 | -3.6  | -1.4   | -    |

PR \(_a\): cholesterol production rate, defined as the rate of into pool A excluding recycled cholesterol from pool B; with all tissue cholesterol which exchanges rapidly with that

M \(_{ax}\): M \(_a\) minus M \(_{ap}\); k \(_{aa}\): rate constant for total transfer of total transfer of cholesterol from pool B; k \(_a\): rate transfer of cholesterol from pool A to pool B; r \(_{ab}\): mass transfer r \(_{ab}\) are calculated assuming that no cholesterol is excreted the size of pool B, the slowly equilibrating pool of cholesterol xanthomata, and arterial wall belongs (see text).

\(^+\) P values obtained by paired t-test analysis.
patients during the administration of a placebo (PL) and

<table>
<thead>
<tr>
<th>$-k_{aa}$ (days$^{-1}$)</th>
<th>$-k_{bb}$ (days$^{-1}$)</th>
<th>$-k_{a}$ (days$^{-1}$)</th>
<th>$-k_{ab}$ (days$^{-1}$)</th>
<th>$r_{ab}$ (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL CO</td>
<td>PL CO</td>
<td>PL CO</td>
<td>PL CO</td>
<td>PL CO</td>
</tr>
<tr>
<td>0.089 0.098</td>
<td>0.037 0.030</td>
<td>0.042 0.068</td>
<td>0.047 0.031</td>
<td>- -</td>
</tr>
<tr>
<td>0.119 0.149</td>
<td>0.041 0.045</td>
<td>0.047 0.073</td>
<td>0.072 0.076</td>
<td>2.46 2.07</td>
</tr>
<tr>
<td>0.101 0.154</td>
<td>0.032 0.033</td>
<td>0.049 0.104</td>
<td>0.051 0.050</td>
<td>1.21 1.08</td>
</tr>
<tr>
<td>0.148 0.124</td>
<td>0.050 0.037</td>
<td>0.074 0.092</td>
<td>0.075 0.032</td>
<td>1.38 0.64</td>
</tr>
<tr>
<td>0.085 -</td>
<td>0.025 -</td>
<td>0.045 -</td>
<td>0.040 -</td>
<td>1.22 -</td>
</tr>
<tr>
<td>0.106 0.123</td>
<td>0.052 0.038</td>
<td>0.039 0.083</td>
<td>0.067 0.040</td>
<td>2.43 1.21</td>
</tr>
<tr>
<td>0.098 0.141</td>
<td>0.045 0.034</td>
<td>0.052 0.096</td>
<td>0.046 0.045</td>
<td>1.18 1.05</td>
</tr>
<tr>
<td>0.116 0.177</td>
<td>0.057 0.059</td>
<td>0.044 0.105</td>
<td>0.072 0.072</td>
<td>2.59 2.11</td>
</tr>
<tr>
<td>0.05</td>
<td>0.09</td>
<td>0.001</td>
<td>0.11</td>
<td>0.029</td>
</tr>
<tr>
<td>+0.027</td>
<td>-</td>
<td>+0.039</td>
<td>-</td>
<td>-0.52</td>
</tr>
</tbody>
</table>

entry of newly synthesized and absorbed cholesterol

$M_a$: size of pool A, consisting of plasma cholesterol together of plasma; $M_{ap}$: size of the plasma cholesterol pool.

cholesterol from pool A ($=k_a+k_{ab}$); $k_{bb}$: rate constant for excretion from pool A; $k_{ab}$: rate constant for of cholesterol from pool A to pool B ($=M_a k_{ab}$); $k_a', k_{ab}$ and directly from pool B. No attempt has been made to estimate to which most of the cholesterol of adipose tissue, muscle,
Table 5-5: Specific activities* of plasma cholesterol, bile cholesterol and bile cholic acid, collected on the same day, during colestipol therapy.

<table>
<thead>
<tr>
<th>Patient of Study</th>
<th>Day of Study</th>
<th>Specific activity (dpm per mmol)</th>
<th>Bile cholesterol specific activity</th>
<th>Bile cholic acid specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma Cholesterol</td>
<td>Bile Cholesterol</td>
<td>Bile Cholic Acid</td>
</tr>
<tr>
<td>LO</td>
<td>76:136</td>
<td>92</td>
<td>74</td>
<td>86</td>
</tr>
<tr>
<td>AT</td>
<td>54:113</td>
<td>372</td>
<td>331</td>
<td>367</td>
</tr>
<tr>
<td>EA</td>
<td>64:119</td>
<td>428</td>
<td>323</td>
<td>393</td>
</tr>
<tr>
<td>RB</td>
<td>54:114</td>
<td>352</td>
<td>334</td>
<td>381</td>
</tr>
<tr>
<td>MW</td>
<td>63:120</td>
<td>338</td>
<td>245</td>
<td>360</td>
</tr>
</tbody>
</table>

\[ \text{p} = 0.02 \quad \text{0.46} \]

Mean ± SD

\[ 0.83 \pm 0.09 \quad 1.00 \pm 0.08 \]

* Specific activities derived from the second isotope.
† Numbers indicate respectively the time (in days) since the infusion of the second isotope and since commencing colestipol therapy.
‡ P values obtained by paired t-test analysis against plasma cholesterol SA.
Table 5-6  Faecal excretion of endogenous steroids during the administration of either a placebo or colestipol 15g daily

<table>
<thead>
<tr>
<th>Patient</th>
<th>Endogenous Neutral Steroids</th>
<th>Bile Acids</th>
<th>Total Endogenous Steroids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Colestipol</td>
<td>Placebo</td>
</tr>
<tr>
<td>LO</td>
<td>1057 ± 66</td>
<td>1031 ± 146</td>
<td>459 ± 47</td>
</tr>
<tr>
<td>AL</td>
<td>444 ± 20</td>
<td>719 ± 25</td>
<td>372 ± 15</td>
</tr>
<tr>
<td>AT</td>
<td>516 ± 56</td>
<td>938 ± 35</td>
<td>305 ± 47</td>
</tr>
<tr>
<td>DS</td>
<td>742 ± 63</td>
<td>738 ± 12</td>
<td>463 ± 53</td>
</tr>
<tr>
<td>AH</td>
<td>461 ± 43</td>
<td>-</td>
<td>359 ± 53</td>
</tr>
<tr>
<td>EA</td>
<td>562 ± 75</td>
<td>672 ± 52</td>
<td>338 ± 54</td>
</tr>
<tr>
<td>RB</td>
<td>841 ± 39</td>
<td>589 ± 34</td>
<td>419 ± 45</td>
</tr>
<tr>
<td>MW</td>
<td>622 ± 54</td>
<td>768 ± 71</td>
<td>478 ± 66</td>
</tr>
</tbody>
</table>

P<sup>+</sup> 0.3  0.0003  0.0002

Mean Difference  -  +1236  +1329

* Eight-day faecal collections analysed as four two-day pools.
† P values obtained by paired t-test analysis.
### Table 5-7

**Effect of colestipol therapy on the relationship between two independent estimates of cholesterol turnover**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Placebo</th>
<th>Colestipol</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>51</td>
<td>89</td>
</tr>
<tr>
<td>AT</td>
<td>71</td>
<td>102</td>
</tr>
<tr>
<td>DS</td>
<td>93</td>
<td>114</td>
</tr>
<tr>
<td>AH</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>EA</td>
<td>64</td>
<td>110</td>
</tr>
<tr>
<td>RB</td>
<td>96</td>
<td>106</td>
</tr>
<tr>
<td>MW</td>
<td>70</td>
<td>93</td>
</tr>
</tbody>
</table>

Mean ± S.D.  

<table>
<thead>
<tr>
<th>Production Rate (PR)</th>
<th>Placebo</th>
<th>Colestipol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72 ± 16.7</td>
<td>102 ± 9.7</td>
</tr>
</tbody>
</table>

*$^{TFES \times 100\%}$

* PR = production rate (cholesterol turnover as estimated by 2-pool analysis); TFES = total faecal endogenous steroids (cholesterol turnover as estimated by isotopic steroid balance).

*P value obtained by paired t-test analysis.
FAECAL STEROIDS

( mg/day, mean±s.e.m. )

<table>
<thead>
<tr>
<th></th>
<th>NEUTRAL</th>
<th>ACIDIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C</td>
<td>583±20</td>
<td>1288±117</td>
</tr>
<tr>
<td>$^{3}$H</td>
<td>576±22</td>
<td>1240±117</td>
</tr>
</tbody>
</table>

Figure 5-1

Decay of plasma cholesterol specific activity and estimates of faecal neutral and acidic steroid excretion after the simultaneous intravenous administration of cholesterol-$4-^{14}$C and cholesterol-$1\alpha,2\alpha-{^3}$H (subject AH).
Figure 5-2
Decay of plasma cholesterol specific activity before and during the administration of colestipol.

Each point represents the mean of values obtained from three subjects (LO, EA, RB). FC = free cholesterol; EC = esterified cholesterol; TC = total cholesterol.
Figure 5-3
Relationship between the increase in faecal bile and excretion (ΔFBA) and the increase in the rate constant for cholesterol elimination from pool A (ΔKa) in seven subjects treated with colestipol.
Figure 5-4

Relationship between the increase in cholesterol turnover (isotopic steroid balance) and the decrease in plasma cholesterol concentration in six patients with type II hyperlipoproteinaemia during treatment with colestipol 15g daily.
Figure 5-5
Relationship between estimates of cholesterol turnover obtained by two-pool analysis and those obtained by isotopic steroid balance during the administration of a placebo (■) or colestipol 15g daily (●).
ACUTE CHANGES IN LIPOPROTEIN CHOLESTEROL DURING COLESTIPOL THERAPY: DIFFERENCES IN THE HYPERLIPOPROTEINAEMIAS

Although cholesterol is present in all lipoproteins, it is likely that different classes of lipoproteins are concerned with specific functions in relation to cholesterol transport. The acute effects on lipoprotein cholesterol of rapidly altering cholesterol turnover have been examined to determine whether individual lipoproteins are involved in specific metabolic functions, and whether specific patterns of change characterize the hyperlipoproteinaemias.

Radioactively labelled cholesterol was infused into hypercholesterolaemic subjects. Several weeks later cholesterol metabolism was studied with the aid of colestipol or clofibrate, an inhibitor of cholesterol synthesis. The acute changes in the mass, radioactivity and specific activity of free and esterified cholesterol within VLDL, LDL, and HDL were studied. During the acute phase of colestipol treatment there was an increased uptake of cholesterol from plasma into the lipoprotein apo B pool. Changes in free cholesterol were more marked than those in esterified cholesterol and suggested that the influx of cholesterol into plasma was occurring mainly in the former. The changes in VLDL when long-term clofibrate therapy was stopped resembled those seen when colestipol was started: rises in VLDL cholesterol and radioactivity and a fall in specific activity. Since these studies suggested that the colestipol-induced rise in VLDL cholesterol reflected increased influx, and the fall in LDL cholesterol increasing efflux, the response to colestipol was compared in additional hyperlipidaemic subjects and 2 normal subjects before and during carbohydrate-rich diets. The rise in VLDL cholesterol was greatly enhanced in subjects with hyperlipoproteinaemia of types IV and V and in those eating carbohydrate.
ABSTRACT

Although cholesterol is present in all lipoproteins, it is likely that different classes of lipoproteins are concerned with specific functions in relation to cholesterol transport. The acute effects on lipoprotein cholesterol of rapidly altering cholesterol turnover have been examined to determine whether individual lipoproteins are involved in specific metabolic functions, and whether specific patterns of change characterize the hyperlipoproteinaemias. Radioactively labelled cholesterol was infused into hypercholesterolaemic subjects. Several weeks later cholesterol metabolism was altered with the aid of colestipol or clofibrate, an inhibitor of cholesterol synthesis. The acute changes in the mass, radioactivity and specific activity of free and esterified cholesterol within VLDL, LDL and HDL were measured serially during the first week of treatment. With colestipol therapy the mass and, to a lesser extent, the radioactivity of VLDL cholesterol rose, while the specific activity fell, suggesting an increased influx into plasma of newly synthesized cholesterol within VLDL. By contrast, the mass, radioactivity and specific activity of LDL cholesterol fell, being consistent with an increased efflux of cholesterol from plasma within this lipoprotein. Changes in free cholesterol were more marked than those in esterified cholesterol and suggested that the efflux of cholesterol into plasma was occurring mainly as the former. The changes in VLDL when long-term clofibrate therapy was stopped resembled those seen when colestipol was started: rises in VLDL cholesterol mass and radioactivity and a fall in specific activity. Since these studies suggested that the colestipol induced rise in VLDL cholesterol reflected increased influx, and the fall in LDL cholesterol increasing efflux, the response to colestipol was compared in additional hyperlipidaemic subjects and 2 normal subjects before and during carbohydrate-rich diets. The rise in VLDL cholesterol was greatly enhanced in subjects with hyperlipoproteinaemia of types IV and V and in those eating carbohydrate,
and was least in subjects with type IIa hyperlipoprotein-aemia and in normal subjects. LDL cholesterol fell in normal and type II subjects, but tended to rise in type IV and type V subjects and during high carbohydrate diets. It is suggested that cholesterol influx was stimulated more in type IV and type V subjects than in type II or normal subjects and that this was reflected in a greater transfer of cholesterol from VLDL to LDL.

that LDL may be derived from the metabolism of VLDL and represent a cholesterol-rich lipoprotein remnant that has been diverted most of its triglyceride (Gustafson, 1966). This concept has been supported by the precursor-product relationship of both the protein and triglyceride fatty acid moieties of VLDL and LDL (Navel, 1961; Gitlin et al, 1958; Bilheimer et al, 1972; Quaifordt et al, 1970), and by the difficulty in demonstrating LDL clearly elsewhere than in plasma (Baroec et al, 1971). The rapid isotopic equilibration of free cholesterol which occurs among the lipoproteins (Shapiro et al, 1966) makes it difficult to determine whether cholesterol first appears preferentially in a specific lipoprotein after the injection of a radiolabelled cholesterol precursor (Goodman, 1964). However, at least in the cholesterol-fed rat this appears to occur largely within VLDL (uchems et al, 1965).

Some differences in the handling of cholesterol in the various lipoproteins have been observed. When free cholesterol enters plasma it becomes esterified at different rates in HDL, VLDL and LDL, being fastest in HDL and slowest in LDL. This has been demonstrated in man by both in vitro (Gleseet et al, 1966; Akhunn and Gleeveet, 1968) and in vivo (Goodman, 1964) techniques.

In the present study an attempt has been made to define the specific roles of the various lipoproteins by examining the acute changes in lipoprotein cholesterol which occurred when cholesterol turnover was stimulated by the administration of colestipol. Further studies were subsequently carried out with clofibrate, a known inhibitor of cholesterol synthesis (Grundy, Ahrens, Salan et al, 1972; Fageau and Fini, 1972; Steinberg, 1970). By inducing rapid changes in cholesterol transport, it was possible to show early changes in cholesterol metabolism in at least VLDL.
INTRODUCTION

It seems certain that the differences in the composition of the different plasma lipoproteins reflect specific functions. Thus, the triglyceride-rich VLDL transport newly formed triglyceride (Havel, 1961). On the other hand, although cholesterol is carried predominantly in LDL, the specific role of LDL in cholesterol transport has not been defined. It has been postulated that LDL may be derived from the metabolism of VLDL and represent a cholesterol-rich lipoprotein remnant that has been divested of most of its triglyceride (Gustafson, 1966). This concept has been supported by the precursor-product relationship of both the protein and triglyceride fatty acid moieties of VLDL and LDL (Havel, 1961; Gitlin et al, 1958; Bilheimer et al, 1972; Quarfordt et al, 1970), and by the difficulty in demonstrating LDL clearly elsewhere than in plasma (Bersot et al, 1971). The rapid isotopic equilibration of free cholesterol which occurs among the lipoproteins (Shapiro et al, 1966) makes it difficult to determine whether cholesterol first appears preferentially in a specific lipoprotein after the injection of a radiolabelled cholesterol precursor (Goodman, 1964). However, at least in the cholesterol-fed rat this appears to occur largely within VLDL (Roheim et al, 1963).

Some differences in the handling of cholesterol in the various lipoproteins have been observed. When free cholesterol enters plasma it becomes esterified at different rates in HDL, VLDL and LDL, being fastest in HDL and slowest in LDL. This has been demonstrated in man by both in vitro (Glomset et al, 1966; Akanuma and Glomset, 1968) and in vivo (Goodman, 1964) techniques.

In the present study an attempt has been made to define the specific roles of the various lipoproteins by examining the acute changes in lipoprotein cholesterol which occurred when cholesterol turnover was stimulated by the administration of colestipol. Further studies were subsequently carried out with clofibrate, a known inhibitor of cholesterol synthesis (Grundy, Ahrens, Salen et al, 1972; Fulton and Hsia, 1972; Steinberg, 1970). By inducing rapid changes in cholesterol transport, it was possible to show early changes in cholesterol metabolism in at least VLDL...
and LDL. The studies were, therefore, extended to subjects with various types of hyperlipoproteinaemia to determine whether these were characterized by specific patterns in the redistribution of lipoprotein cholesterol during colestipol therapy.

METHODS

Experimental Subjects

Twenty one subjects were studied (Table 6-1). Typing of the lipoprotein pattern was based on the relative proportions of cholesterol and triglyceride in individual lipoproteins (World Health Organization, 1970). Two patients were taking drugs known to influence cholesterol metabolism, thyroxine sodium (Kurland et al, 1961) (NT, for marginally lowered thyroid function tests) and ethinyl oestradiol (Nestel et al, 1965) (AT, for menopausal symptoms), at the respective daily doses of 100 μg and 0.02 mg. Both drugs were continued at constant dosage throughout the study. Two subjects (EA, DS) had been eating a diet low in cholesterol and high in polyunsaturated fatty acids for one year, with resultant lowering of plasma cholesterol levels by 80 and 100 mg/100 ml respectively. The nature of the investigation was fully explained and each subject gave informed consent.

Clinical Procedures

Generally the test subjects had been eating their usual diets for several months before entering the study and continued to do so until its completion. The exceptions were 2 normal subjects who were studied in hospital during high carbohydrate diets. Body weights remained essentially constant. All blood samples were drawn after a 12-14 hour overnight fast and were collected into chilled tubes containing disodium EDTA (final plasma concentration 2.5 mM).

Study 1: Acute effects of colestipol

Body cholesterol was isotopically labelled in 3 subjects with type II hyperlipoproteinaemia (AH, EA, RB) by an intravenous infusion of 50-120 μc of cholesterol-1α,2α-3H
(Radiochemical Centre, Amersham), either dissolved in 200 ml 0.9% saline or complexed with autologous plasma lipoproteins. Each patient was then seen weekly for the determination of the plasma concentrations of free cholesterol, esterified cholesterol and triglyceride, and the specific activities of free and esterified cholesterol. Twelve to 26 weeks after the infusions colestipol was given at a daily dose of 15 g to produce rapid increases in cholesterol synthesis and catabolism. The acute changes produced in the concentrations of free cholesterol, esterified cholesterol and triglyceride and in the specific activities of free and esterified cholesterol within VLDL, LDL and HDL were examined in serial blood samples collected immediately before treatment and during the first 5 to 14 days after its commencement.

Study 2: Long-term effects of colestipol

These studies were carried out in the 3 preceding subjects, in an additional 4 patients with type II hyperlipoproteinaemia (AL, AT, DS, MW) and in one patient with type IV hyperlipoproteinaemia (LO). Analyses of free cholesterol, esterified cholesterol and triglyceride and in the specific activities of free and esterified cholesterol within VLDL, LDL and HDL were performed on 2 to 5 different days before therapy and again on a similar number of blood samples 8 to 12 weeks after its commencement.

Study 3: Acute effects of starting and stopping clofibrate

The acute response of plasma lipoproteins to therapy with clofibrate (2 g daily) was investigated in 2 subjects (MT, MM), each of whom had received an intravenous infusion of 50-80 μc cholesterol-1α,2α-3H 3 weeks previously.

The effects of stopping long-term clofibrate therapy (2g daily for 5 months), thereby stimulating the synthesis of cholesterol without directly stimulating catabolism, were examined in a single subject (DL), who had been given an infusion of 50 μc of cholesterol-1α,2α-3H 4 weeks previously.

Study 4: Acute effects of colestipol in relation to lipoprotein type

The acute effects of colestipol therapy on total cholesterol and triglyceride within VLDL and LDL were compared
in 3 normal subjects (NM, DY, RF) and in a total of 11 patients with 4 types of hyperlipoproteinaemia, including the 3 who had been studied in greater detail with radio-labelled cholesterol (AH, EA, RB) plus 8 others (MD, EB, AC, ES, LO, JF, MH, CG). Blood samples were collected on 2 days immediately before treatment and on 3-5 occasions during the first 8 days after its commencement.

Experiment 5: Acute effects of colestipol during high-carbohydrate diets

The effects of carbohydrate feeding on the acute response of lipoprotein lipids to colestipol therapy was examined in 2 normal subjects (DY and RF). Studies were performed first during a diet providing 45%, 40% and 15% of total calories as carbohydrate, fat and protein, and then during a carbohydrate-rich diet (75%: 15%: 15%). Both diets were solid and cholesterol intake was kept constant at 500 mg/day.

LABORATORY PROCEDURES

Studies with radiolabelled cholesterol

In vitro esterification of plasma cholesterol was inhibited by the addition of p-chloromercuriphenylsulphonate (PCMPS) to a final concentration of 2mM (Glomset et al, 1970). Plasma was separated by centrifugation immediately after venesection in order to minimise in vitro exchange of free cholesterol between erythrocytes and plasma lipoproteins.

Plasma lipids were extracted in the solution described by Dole (1956) and separated by thin-layer silicic acid chromatography (Kieselgel G; hexane:diethyl ether:methanol: acetic acid, 180:40:6:4, solvent). Free cholesterol, esterified cholesterol and triglyceride were eluted separately with diethyl ether, after visualization with Rhodamine G under ultraviolet light, and were redissolved in a known volume of 95% redistilled isopropanol. Cholesterol and triglyceride mass were then measured by a semi-automated procedure (Auto Analyzer II, Technicon Instruments Corporation).
Duplicate analyses gave consistent agreement within ± 2%. Cholesterol radioactivity was measured in a liquid scintillation counter using PPO -dimethyl POPOP in toluene as scintillator solvent. Counting efficiency was established by automatic external standardization.

Plasma lipoproteins were isolated within 24 hours of venepuncture. Aliquots of plasma were layered under 0.15 M NaCl (d = 1.006 g/ml) and the VLDL separated by centrifugation at 100,000 g for 16 hours at 14°C in the 40.3 rotor of a Beckman preparative ultracentrifuge (Havel et al, 1955). After isolating the VLDL by a tube-slicing technique, the LDL were precipitated from the infranate by the addition of manganese chloride and heparin (Burstein et al, 1970), separated by centrifugation (3,000 r.p.m. for 10 minutes) and resuspended in 0.9% saline. The free cholesterol, esterified cholesterol and triglyceride of each of the 3 major lipoprotein classes were then isolated and assayed for mass and radioactivity as described for whole plasma. Total recovery of cholesterol and triglyceride averaged 95.3±6.0% and 93.2±7.8% respectively (mean ± SD). It was established that the separation of lipoproteins by this procedure was unaffected by the presence of PCMPS at the concentration employed, and that it gave values for lipoprotein lipids similar to those obtained by sequential ultracentrifugation.

Studies without radiolabelled cholesterol

Collection of blood, separation of plasma lipoproteins into VLDL, LDL and HDL, and the extraction of lipids were carried out as described above. However, only total cholesterol and triglyceride concentrations were measured, and therefore preliminary chromatographic separation was not performed. Furthermore, PCMPS was not added to the samples of plasma.

In subjects AL, DS, AH, RB and MW more detailed separation of lipoproteins into subclasses Sf 20-400, Sf 12-20, Sf 0-12 and d>1.063 was carried out on selected days by sequential ultracentrifugation at adjusted densities of 1.006, 1.019 and 1.063 g/ml as described by Havel et al (1955). Chylomicrons (Sf>400) were separated from the plasma
of subjects JF and MH by centrifugation for 30 minutes at 26,000 g and 20°C after overlaying with 0.15 M NaCl (Hatch and Lees, 1968).

RESULTS

Study 1: Acute effects of colestipol

Within 24 to 48 hours of commencing resin therapy changes had occurred in all 3 lipoprotein fractions, especially in VLDL and LDL. These are presented in Table 6-2 and illustrated in Figures 6-1 and 6-2. Within each lipoprotein there was an acceleration in the decay of the cholesterol specific activity-time curve, with that of free cholesterol initially falling more rapidly than that of esterified cholesterol (the specific activity-time curve of cholesterol before colestipol therapy is shown in Figures 6-1 and 6-2). In VLDL these changes were associated with a clear and persistent increase in cholesterol mass in all 3 subjects and with an increase in cholesterol radioactivity in 2 subjects (EA and AH); in the remaining subject there was a transient though marked rise in radioactivity on the second day. Of the rise in cholesterol mass, the fraction transported as free cholesterol appeared to increase slightly during the first week in subjects EA and RB, but this was not as clearcut as the greater initial fall in free cholesterol specific activity compared to esterified cholesterol specific activity. The triglyceride concentration within VLDL rose together with that of cholesterol, especially that of free cholesterol.

In contrast, LDL demonstrated similar percentage decreases in both free and esterified cholesterol mass, accompanied by a rapid fall in cholesterol radioactivity (Figure 6-2). The early changes in the specific activities of free and esterified cholesterol were similar to those occurring within VLDL. There was no consistent effect on lipid mass within HDL during the first week of colestipol therapy. However, the changes in specific activity were similar to those described for VLDL and LDL.
Study 2: Long-term effects of colestipol

The long-term effects of resin therapy on lipoprotein lipid mass are presented in Table 6-3 and summarised in Figure 6-3. Although the specific activity of plasma cholesterol continued to fall, those of free and esterified cholesterol were again equal after several weeks of therapy (Table 6-2). The concentration of total cholesterol stabilized at values that were significantly lower than in the control period. In all patients the cholesterol and triglyceride concentrations during long-term therapy remained significantly higher in VLDL and significantly lower in LDL, though within LDL the reduction in triglyceride was much less than that in cholesterol, giving rise to a significant increase in the triglyceride/cholesterol ratio. No significant changes were seen in HDL lipid mass.

Because the triglyceride/cholesterol ratio increased significantly in LDL with long-term colestipol therapy, the possibility was considered that this reflected an abnormal accumulation of Sf 12-20 lipoproteins. Accordingly both Sf 12-20 and Sf 0-12 lipoproteins were examined in 5 subjects (Table 6-4). The relative amounts of cholesterol in the two lipoprotein fractions were, however, within the range reported for healthy subjects (Nichols, 1967).

Study 3: Acute effects of starting and stopping clofibrate

Administration of clofibrate produced rapid and substantial falls in VLDL cholesterol mass and radioactivity and in VLDL triglyceride mass (Table 6-5, Figure 6-4). The changes in LDL lipids were smaller than those in VLDL and occurred more slowly (e.g. in both subjects on the second day, triglyceride and cholesterol mass had fallen at least 50% in VLDL, but had changed little in LDL). On the other hand, cholesterol specific activity declined slowly in both VLDL and LDL, although an adequate pre-treatment specific activity-time curve was not available for comparison. The triglyceride but not the cholesterol concentration also fell in HDL. The changes in the one subject in whom clofibrate was stopped resembled those seen after starting colestipol, of 32±5.1 mg/100 ml and 44±16.4 mg/100 ml respectively (mean ±SD, n = 7).
but were slower and did not disturb the normal equilibrium between free and esterified cholesterol (Table 6-5, Figure 6-5). In VLDL the cholesterol mass and radioactivity rose while the specific activity fell. The triglyceride concentration also rose. In LDL and HDL cholesterol mass did not change. The triglyceride mass increased in both LDL and HDL, raising the triglyceride/cholesterol ratio.

Study 4: Acute effects of colestipol in relation to lipoprotein type

These results are presented in Table 6-6.

Although the changes produced in patients with type IIa hyperlipoproteinaemia were similar to those observed in normal subjects, they differed considerably from those observed in patients who were already hypertriglyceridaemic. Thus, the increment in VLDL cholesterol and triglyceride was greatest in the subjects with type IV or type V hyperlipoproteinaemia, least in the normal and type IIa subjects and intermediate in type IIb subjects (Table 6-6). Accordingly, in the pooled data the mean increment in VLDL lipid mass was positively correlated with the corresponding pretreatment level (VLDL cholesterol: \( r = +0.73, P<0.005, n = 14 \); VLDL triglyceride: \( r = +0.55, P<0.05, n = 13 \) (Figure 6-6). Furthermore, in the patients with type IV or type V hyperlipoproteinaemia LDL lipids were either unchanged or increased during the first 8 days of colestipol therapy, in contrast to the rapid fall which was consistently observed in the other subjects (Table 6-6). In subject LO (in whom the long-term effects of colestipol were also examined) LDL cholesterol eventually decreased to 18 mg/100 ml below the pre-treatment level. This was, however, a small reduction relative to that observed in the type II patients (average fall = 67 mg/100 ml). Within the group with type II hyperlipoproteinaemia there was no significant correlation between the fall in LDL cholesterol and the pre-treatment level \( (r = -0.57, P>0.1, n = 8) \). In type V hyerlipoproteinaemia these changes were associated with increases in chylomicron cholesterol and triglyceride of 82±16.1 mg/100 ml and 442±104 mg/100 ml respectively (mean ±SD, \( n = 7 \)).
Study 5: Acute effects of colestipol during high-carbohydrate diets

The acute changes in VLDL and LDL lipids which were seen with colestipol therapy in type IV and type V patients could be partially reproduced in normal subjects during high-carbohydrate diets. These results are summarised in Figures 6-7 and 6-8. Whereas during the control diet colestipol produced only a small increase in VLDL cholesterol and a decrease in that of LDL, when treatment was commenced after 6 or 7 days on a high-carbohydrate diet the increment in VLDL cholesterol was increased 2 to 5-fold and there was an associated transient rise in LDL cholesterol.

DISCUSSION

Observations on the effects of bile salts in experimental animals indicate that impairment of their intestinal absorption by resin therapy will stimulate both the synthesis of cholesterol in the liver (Hamprecht et al, 1971) and intestine (Dietschy, 1968a), and the catabolism of cholesterol to bile acids in the liver (Boyd and Percy-Robb, 1971). Such responses to cholestyramine therapy have been demonstrated in man (Grundy et al, 1971) and it was established in the preceding study (Chapter 5) that colestipol has similar effects. The detection of altered bile composition by other workers within 24 to 48 hours of commencing resin therapy (Juul and van der Linden, 1969), and of increased faecal bile acid excretion within several days (Nazir et al, 1972) indicate that cholesterol metabolism is affected rapidly. It is, therefore, likely that the changes in lipoprotein cholesterol during colestipol therapy occurred in a setting of greatly enhanced cholesterol synthesis and catabolism.

Colestipol therapy produced an immediate increase in the rate of decay of plasma cholesterol specific activity in all 3 subjects in Study 1. This was associated with a temporary but clear disturbance in the equilibrium between free and esterified cholesterol, the specific activity of the former initially falling much more rapidly than that of the latter. These findings provide evidence that the colestipol-induced increase in cholesterol synthesis was
associated with a rapid influx of newly synthesised cholesterol into plasma and that this occurred predominantly in the unesterified form. Other observations have also suggested that esterification of cholesterol occurs after the entry of free cholesterol into plasma: 1. A precursor-product relationship between plasma free and esterified cholesterol is a consistent finding after infusions of radio-labelled cholesterol precursors such as mevalonic acid (Nestel and Monger, 1967) and acetate (Moutafis and Myant, 1969b); 2. The specific activity of esterified cholesterol is higher in plasma than in the liver after injecting radiocholesterol into man (Nestel and Couzens, 1966b); and 3. Comparative rates of cholesterol esterification in vivo and in vitro suggest that esterification occurs mostly in the plasma (Nestel and Monger, 1967). The similarity between the specific activity changes within VLDL, LDL and HDL is consistent with the rapid transfer of free cholesterol which is known to occur between the different lipoproteins (Shapiro et al, 1966; Roheim et al, 1963; Goodman, 1964). The restoration in the equilibrium between free and esterified cholesterol during long-term colestipol therapy, despite a continued enhancement of cholesterol turnover (Chapter 5), may reflect an increase in plasma LCAT activity (Chapter 7).

The acute increment in VLDL cholesterol mass suggests that it was predominantly within this lipoprotein that the influx of new cholesterol was occurring. Similar conclusions have been drawn from rat liver perfusion studies (Roheim et al, 1963; Windmueller and Spaeth, 1967), while transport of intestinal cholesterol within VLDL has been established in the same species (Windmueller et al, 1970). The acute rise which occurred in VLDL cholesterol radioactivity may indicate the additional transport of some preformed stored cholesterol; alternatively it may merely reflect isotopic exchange between the expanded VLDL cholesterol pool and the cholesterol of body tissues. The parallel increase in VLDL triglyceride is in accord with other evidence that the various lipid and protein components participate in the turnover of VLDL. Thus, the synthesis of both the protein...
(Eaton and Kipnis, 1969) and triglyceride (Quarfordt et al., 1970) moieties of VLDL is stimulated, and the plasma concentration of VLDL cholesterol increased (Schonfeld, 1970) by high carbohydrate diets, when the main stimulus is presumably on triglyceride fatty acid synthesis (Barter et al., 1972), while perfusion studies have shown positive correlations between the synthesis and secretion of VLDL cholesterol, triglyceride and phospholipid (Windmueller and Spaeth, 1967; Heimberg et al., 1965).

The effects of colestipol on the metabolism of LDL cholesterol in the first study differed considerably from those on VLDL metabolism. Colestipol produced a rapid fall not only in the specific activity of LDL cholesterol but also in its mass and radioactivity. These changes suggest that the efflux of plasma cholesterol occurred predominantly from LDL when the catabolism of cholesterol to bile acids was enhanced. The simultaneous decline in the three measured LDL lipids suggests in addition that the clearance of cholesterol was occurring mostly within intact LDL, a concept which is supported by the report that cholestyramine increases the fractional turnover of LDL protein (Langer et al., 1969).

An alternative explanation for the reciprocal changes which occurred in VLDL and LDL cholesterol pools during Study 1 would be that colestipol inhibited VLDL conversion to LDL. Such an explanation would appear to be unlikely, however, for the following reasons:

1. The changes observed in the cholesterol specific activity-time curves indicated that colestipol increased the fractional turnover of cholesterol.

2. Reduced VLDL conversion to LDL might be expected to result in the accumulation of lipoprotein "intermediates" of Sf 12-20 (Bilheimer et al., 1971), an effect which was excluded by the subfractionation studies (Table 6-4).

3. As mentioned previously, resin therapy has been shown to increase the fractional turnover of LDL protein (Langer et al., 1969).
The clofibrate studies (Study 3) were consistent with the interpretation of the colestipol-induced changes in VLDL metabolism. Although the effects of clofibrate in some areas of lipid metabolism are uncertain, there is general agreement that the major effect on cholesterol metabolism is that of inhibition of synthesis (Steinberg, 1970; Fulton and Hsia, 1972; Nestel et al, 1965; Grundy, Ahrens, Salen et al, 1972). In the single subject (DL) in whom clofibrate therapy was stopped, the presumed increase in cholesterol synthesis was associated with rapid increments in VLDL cholesterol mass and radioactivity. While such changes could also reflect decreased VLDL clearance, this is rendered unlikely by the rapid fall in cholesterol specific activity. By contrast, suppression of cholesterol synthesis in the two clofibrate treated subjects (MT, MM) was associated with immediate and substantial falls in VLDL cholesterol mass and radioactivity.

In the clinical trial of colestipol (Chapter 4) it was noted that the rise in plasma triglyceride was most marked in subjects who were already hypertriglyceridaemic. This was examined more systematically in the present study, and clear differences were found in the acute response of plasma lipoproteins to colestipol in subjects with various types of hyperlipoproteinaemia (Study 4). Although all subjects showed a rise in VLDL lipids, the increase was positively correlated with the pre-treatment level. Thus, the increments in the markedly hypertriglyceridaemic subjects (types IV and V) were significantly greater than in those with type II hyperlipoproteinaemia, and even amongst the latter those with type IIb (having elevated VLDL as well as LDL) showed greater increments in VLDL than those with type IIa (Table 6-6). Furthermore, the increment in VLDL lipids was "normal" only in subjects with type IIa hyperlipoproteinaemia. If colestipol merely exaggerates the basal rates of VLDL synthesis and secretion, then these findings may indicate that the turnover of VLDL lipids is generally increased in types IIb, IV and V hyperlipoproteinemia. There is, furthermore, other evidence that cholesterol transport is increased in subjects with type IV hyperlipoproteinaemia: the \textit{in vivo} turnover of esterified cholesterol
(Nestel, 1970b), the excretion of endogenous faecal steroids (Sodhi and Kudchodkar, 1973) and the production of bile acids (Kottke, 1969) have all been reported to be enhanced. The present findings are in accord with these observations, although they by no means exclude the additional possibility that the removal of VLDL lipids is abnormal in such patients. The studies with carbohydrate-rich diets (Study 5) provide further evidence that the colestipol induced rise in VLDL cholesterol is enhanced in a setting of increased VLDL turnover (Figures 6-7, 6-8). Such diets have been shown to stimulate VLDL secretion by the liver (Wolfe and Ahuja, 1972) and the turnover of esterified cholesterol (Nestel, 1970b). Subjects with type IV hyperlipoproteinemia may respond to carbohydrate diets with an exaggerated increase in VLDL lipids (Quarfordt et al, 1970). The findings in Study 5 therefore support the proposition that the enhanced rise in VLDL lipids in types IIb, IV and V hyperlipoproteinemia reflects increased input.

The findings with LDL lipids also demonstrated differences among the hyperlipoproteinemias (Table 6-6). In all subjects with type II hyperlipoproteinemia (both type IIa and type IIb) there was an immediate fall in LDL cholesterol, which in absolute terms did not differ significantly from that seen in normal subjects. By contrast, LDL cholesterol rose or remained unchanged in patients with type IV or type V hyperlipoproteinemia, and increased in the two subjects eating carbohydrate-rich diets (Figures 6-7, 6-8). The smaller fractional reduction in LDL cholesterol in the type II subjects compared with that in the normal subjects is in accord with previous findings that in the metabolic steady state the clearance rate of cholesterol from plasma is diminished in type II hyperlipoproteinemia (Nestel et al, 1969; Myant, 1971). On the other hand, total exchangeable cholesterol turnover is not reduced in type II hyperlipoproteinemia (Nestel et al, 1969; Myant, 1971), though bile acid production may be low in some individuals (Miettinen, 1970a). The subjects who showed an increase in LDL cholesterol concentration during colestipol also demonstrated an enhanced increment in VLDL
cholesterol. These findings with LDL may, therefore, be explained in terms of increased conversion of VLDL to LDL secondary to the relatively large rise in VLDL. Alternatively, there may have been a simultaneous influx of cholesterol into plasma within both lipoproteins. A third possibility of diminished cholesterol removal from both lipoproteins is unlikely in the presence of a drug that greatly enhances cholesterol turnover.

The variable response of LDL cholesterol raises the question of the interrelationship of VLDL and LDL under normal circumstances. There are several lines of evidence that the protein (Gitlin et al, 1958; Bilheimer et al, 1972; Gulbrandsen et al, 1971) and triglyceride fatty acid moieties (Havel, 1961; Quarfordt et al, 1970; Barter and Nestel, 1972) of VLDL are transferred to smaller lipoproteins, including LDL, as the result of VLDL metabolism, while the conversion of whole VLDL to LDL has been demonstrated both in vitro (Shore and Shore, 1962) and in vivo (Gulbrandsen et al, 1972). Such conversion cannot be shown for cholesterol because of the rapid isotopic equilibration of free cholesterol which occurs between lipoproteins, but the reciprocal fall in VLDL cholesterol and the rise in LDL cholesterol that occur during weight loss (Wilson and Lees, 1972), after heparin infusions (Nichols et al, 1968) and during long-term clofibrate therapy (Wilson and Lees, 1972) have been put forward in support of such interconversion. There are also circumstances in which the cholesterol rises in VLDL but falls in LDL. This has been described in some subjects with hypertriglyceridaemia (Fredrickson et al, 1968), during therapy with bile acid sequestering resins such as cholestyramine (Jones and Dobrilovic, 1970) and colestipol (Tables 6-2 and 6-3) and with long-term carbohydrate-rich diets (Wilson and Lees, 1972). It is unlikely that this necessarily reflects diminished transfer of VLDL cholesterol to LDL, since cholesterol turnover appears to be enhanced in at least the first two of these situations (Sodhi and Kudchodkar, 1973; Goodman and Noble, 1968; Chapter 5). The possibility that VLDL and LDL may compete for common synthetic machinery or that one lipoprotein
may exert an inhibitory effect on the synthesis of the other also need consideration. However, while such mechanisms would explain the divergence between VLDL and LDL concentrations during colestipol therapy in normal and type II subjects, they are rendered unlikely by the simultaneous rise in both lipoproteins which occurred in the subjects with type IV and type V hyperlipoproteinaemia and in those on carbohydrate-rich diets. The likeliest explanation for the present findings would appear to be that in normal subjects and in those with type II hyperlipoproteinaemia the loss of cholesterol within LDL in response to colestipol exceeds the influx within VLDL; a new steady state is eventually reached with an expanded pool of VLDL cholesterol and a diminished pool of LDL cholesterol. However, in patients with type IV and type V hyperlipoproteinaemia and during carbohydrate-rich diets, the greater influx of VLDL (and hence greater conversion to LDL) compensates for the loss of cholesterol from LDL. Finally, it is noteworthy that the colestipol induced rise in LDL lipids in the carbohydrate-fed subjects occurred at a lower pre-existing level of VLDL lipids and in association with a relatively smaller increment in VLDL lipids, than in the subjects with type IV or type V hyperlipoproteinaemia: this may indicate a less efficient conversion of VLDL to LDL in the latter.

The increase in the Sf>400 fraction during colestipol in the patients with type V hyperlipoproteinaemia may indicate that the hyperchylomicronaemia in this condition is of endogenous origin, since it is unlikely that colestipol increased cholesterol absorption. Alternatively it may mean that VLDL and chylomicrons compete for the same clearance mechanism.

The present discussion has so far been confined to VLDL and LDL, since changes in HDL were on the whole unremarkable. No consistent effects were observed on HDL cholesterol mass in any study, and the changes in HDL cholesterol specific activity and radioactivity (Studies 1 and 3) can be explained by the exchange of free cholesterol between lipoproteins.
However, it is noteworthy that the present data concerning HDL demonstrate a positive correlation between VLDL triglyceride concentration and the triglyceride/cholesterol ratio in HDL ($r_s = +0.90$, $P<0.001$). This is illustrated in Figure 6-9, and supports a previous report that the mass of triglyceride carried \emph{in vivo} within HDL is related to the existing concentration of VLDL (Lindgren et al, 1965). This may be related to the transfer of triglyceride from VLDL to HDL which has been noted \emph{in vitro} (Nichols and Smith, 1965). These findings with triglyceride are in accord with other evidence that HDL may be associated with VLDL metabolism. Thus, HDL and VLDL share common polypeptides which move from HDL to VLDL during alimentary lipaemia (Havel et al, 1973) and which activate lipoprotein lipase (La Rosa et al, 1970; Bier and Havel, 1970), while heparin induced lipolysis is associated with reciprocal changes in VLDL and HDL concentrations (Nichols et al, 1968).
<table>
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<th>Subject</th>
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<th>Age (years)</th>
<th>Body Weight (kg)</th>
<th>Mean Plasma Concentration (mg/100 ml)</th>
<th>Lipoprotein* pattern</th>
<th>Experiment†</th>
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<td>444</td>
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<td>69</td>
<td>304</td>
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</tr>
<tr>
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<td>63</td>
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* World Health Organization (1970)
† See text for experimental details
Table 6-2
Acute effects of colestipol on cholesterol and triglyceride of whole plasma, VLDL, LDL and HDL in 3 patients with type II hyperlipoproteinaemia

<table>
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<th>B</th>
<th>H</th>
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<td>TC</td>
<td>316</td>
<td>320</td>
<td>285</td>
<td>298</td>
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</table>

† Colestipol was begun on day 0
* TC : total cholesterol, mg/100 ml; % FC : per cent of cholesterol unesterified; TG : triglyceride, mg/100 ml; FCSA : free cholesterol specific activity, dpm/mg; ECSA : esterified cholesterol specific activity, dpm/mg; TCR : total cholesterol radioactivity, dpm/ml.
Table 6-3

Long-term effects of colestipol therapy on cholesterol and triglyceride of VLDL, LDL and HDL in 8 patients with hyperlipoproteinaemia.

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<th>HDL</th>
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<td>%PC</td>
<td>TG*</td>
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</table>

Each value is the mean of 2 to 5 determinations made on separate days during the control period (C) and after 8 or more weeks of therapy (T). Mean coefficients of variation: VLDL TC, 21%; LDL TC, 10%; HDL TC, 13%; VLDL TG, 26%; LDL TG, 10%; HDL TG, 14%.

* mg/100 ml plasma.

+ P values obtained by paired t-test analysis.
Table 6-4

Distribution of cholesterol and triglyceride among different subfractions of d<1063 lipoproteins after several months of colestipol therapy

<table>
<thead>
<tr>
<th>PATIENT</th>
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<th>Sf 20-400 TC</th>
<th>Sf 12-20 TC</th>
<th>Sf 0-12 TC</th>
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<td>Sf&gt;400 TG</td>
<td>Sf 20-400 TG</td>
<td>Sf 12-20 TG</td>
<td>Sf 0-12 TG</td>
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<tr>
<td>AL</td>
<td>&lt;5</td>
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<tr>
<td>DS</td>
<td>&lt;5</td>
<td>19</td>
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<td>RB</td>
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<td>30</td>
<td>124</td>
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<tr>
<td>MW</td>
<td>&lt;5</td>
<td>55</td>
<td>220</td>
<td>31</td>
</tr>
</tbody>
</table>

* TC = total cholesterol (mg/100 ml plasma)
† TG = triglyceride (mg/100 ml plasma)
Table 6-5

Acute effects of commencing (M.M., M.T.) and stopping (D.L.)† clofibrate therapy on cholesterol and triglyceride of whole plasma, VLDL, LDL and HDL in 3 patients with hyperlipoproteinaemia.

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>M.T.</th>
<th>M.M.</th>
<th>D.L.</th>
</tr>
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<tbody>
<tr>
<td>DAY ‡</td>
<td>-4</td>
<td>-2</td>
<td>+2</td>
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<tr>
<td>WP</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TC</td>
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</tr>
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<td>TG</td>
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<td>213</td>
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</tr>
<tr>
<td>VLDL</td>
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<tr>
<td>ECSA</td>
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<td>1182</td>
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</tr>
<tr>
<td>TCR</td>
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<td>365</td>
<td>140</td>
</tr>
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<td>LDL</td>
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<td></td>
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<tr>
<td>TC</td>
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<td>255</td>
<td>251</td>
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<td>26.1</td>
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<tr>
<td>TCR</td>
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</tbody>
</table>

† Clofibrate was stopped after 5 months' therapy.
‡ Clofibrate therapy was started or stopped on day 0.
* Units used: TC, mg/100 ml; TG, mg/100 ml; FCSA and ECSA, dpm/mq; TCR, dpm/ml.
Table 6-6  Acute changes in lipoprotein lipids produced by colestipol therapy in normal subjects and in patients with various types of hyperlipoproteinaemia.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Lipoprotein</th>
<th>Lipoprotein Pattern</th>
<th>Normal</th>
<th>Type IIa</th>
<th>Type IIb</th>
<th>Types IV and V</th>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>VLDL</td>
<td>+3.4±0.7</td>
<td>+6.8±1.4</td>
<td>+30.3±6.7</td>
<td>+74.2±15.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(4:12)</td>
<td>(4:12)</td>
<td>(3:10)</td>
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<tr>
<td></td>
<td>Cholesterol</td>
<td>P†</td>
<td>NS</td>
<td>&lt;0.005</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LDL</td>
<td>-24.0±3.7</td>
<td>-42.6±8.1</td>
<td>-55.4±11.1</td>
<td>+13.8±7.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3:8)</td>
<td>(4:12)</td>
<td>(4:12)</td>
<td>(3:10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Triglyceride</td>
<td>P†</td>
<td>NS</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
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<td></td>
<td>VLDL</td>
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<td>+98.4±24.3</td>
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<td>(4:12)</td>
<td>(4:12)</td>
<td>(2:6)</td>
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<tr>
<td></td>
<td>LDL</td>
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<td>-2.5±3.2</td>
<td>-8.7±4.4</td>
<td>+8.9±4.9</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(3:8)</td>
<td>(4:8)</td>
<td>(4:12)</td>
<td>(2:7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.005</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

† mean ± SEM mg/100ml.

*Numbers in parenthesis indicate respectively the number of subjects studied and the total number of observations made.

‡ P values obtained by t-test comparison of adjacent columns. NS indicates P>0.05.
Figure 6-1

The acute effects of colestipol administration on the free cholesterol (●), esterified cholesterol (Θ) and triglyceride (■) of VLDL in patients with type II hyperlipoproteinaemia. Each point represents the mean value for 3 subjects. The broken line represents an extension of the control specific activity-time curve for both free and esterified cholesterol of whole plasma. Colestipol therapy was begun on day 0.
The acute effects of colestipol administration on the free cholesterol (●), esterified cholesterol (●) and triglyceride (■) of LDL in patients with type II hyperlipoproteinaemia. Each point represents the mean value for 3 subjects, with the exception that triglyceride concentrations were followed in 2 subjects only. The broken line represents an extension of the control specific activity - time curve for both free and esterified cholesterol of whole plasma. Colestipol therapy was begun on day 0.
Figure 6-3

Long-term effects of colestipol therapy on plasma concentrations of free cholesterol, esterified cholesterol and triglyceride within VLDL, LDL and HDL in 8 patients with hyperlipoproteinaemia. The concentrations shown are the overall means of all values obtained before colestipol and after eight or more weeks of continuous therapy. P values were obtained by paired t-test analysis of individual subject means.
Figure 6-4

The acute effects of starting clofibrate therapy on the free cholesterol (●), esterified cholesterol (Θ) and triglyceride (■) of VLDL and LDL in two patients with type II hyperlipoproteinaemia (mean values). Clofibrate therapy was commenced on day 0.
Figure 6-5
The acute effects of stopping long-term clofibrate therapy (day 0) on the free cholesterol (●), esterified cholesterol (●) and triglyceride (■) of VLDL and LDL in a patient with type IIb hyperlipoproteinaemia.
Figure 6-6

Relationship between the initial VLDL cholesterol concentration and the acute increase produced by colestipol in normal subjects (Θ) and in patients with hyperlipoproteinaemia of types IIa (●), IIb (●), IV (■) and V (◆).

Pretreatment values are the means of 2 determinations performed on different days; values for acute increase are the means of 3 to 5 determinations performed on days 2 to 8 of treatment.
Figure 6-7
Effects of carbohydrate feeding on the acute response of plasma lipoprotein lipids to colestipol therapy (▪▪▪▪▪▪). Subject DY. ▪ = triglyceride; ▪ = total cholesterol.
Figure 6-8
Effects of carbohydrate feeding on the acute response of plasma lipoprotein lipids to colestipol therapy (---). Subject RF. ■ = triglyceride; ○ = total cholesterol.
Figure 6-9
Relationship between VLDL triglyceride concentration (VLDL TG) and the triglyceride to cholesterol ratio within HDL (HDL TG/TC) in 10 subjects with hyperlipoproteinaemia. Each point represents the values obtained from a single subject during a control period (■), or after one or more weeks of therapy with colestipol (●) or clofibrate (○). Where more than one determination was made the mean value is given. $r_s = \text{Spearman rank correlation coefficient.}$
CHAPTER 7

INCREASED PLASMA CHOLESTEROL ESTERIFYING ACTIVITY DURING COLESTIPOL THERAPY IN MAN

...
ABSTRACT

In vitro plasma cholesterol esterifying activity was measured before and during colestipol therapy in the seven subjects with type II hyperlipoproteinaemia who were described in Chapters 4 and 5. Long-term therapy was associated with a 53 ± 17.1% increase in LCAT activity expressed as % free cholesterol esterified per hour, and a 37 ± 23.8% increase in terms of μg free cholesterol per ml plasma esterified per hour (mean ± SD). This increase occurred despite the fall in the plasma concentration of cholesterol. Total plasma LCAT activity was positively correlated with the rate of cholesterol turnover (r = +0.87, P<0.001). Before colestipol therapy values for total LCAT activity exceeded those for cholesterol turnover (1.69 ± 0.22 v 1.22 ± 0.16 g/day, mean ± SD, n = 7), but they did not differ significantly during colestipol therapy (2.17 ± 0.19 v 2.34 ± 0.40 g/day, n = 6). The results suggest that most exchangeable cholesterol molecules are at some stage esterified within plasma on at least one occasion during the normal turnover of body cholesterol.
INTRODUCTION

The plasma enzyme, lecithin:cholesterol acyltransferase (LCAT), catalyzes the transfer of an acyl group from lecithin to cholesterol (Glimset, 1968). Cholesteryl esters normally comprise 65-75% of total plasma cholesterol, and although esterifying enzymes exist also in the liver (Monger and Nestel, 1967a) and intestine (Blomstrand and Ahrens, 1958), it is highly probable that in man plasma cholesterol is esterified mostly by LCAT. This is based largely on the similarity between the values for cholesterol esterification obtained by *in vitro* (Glimset, 1962) and *in vivo* (Nestel and Monger, 1967) techniques and on the virtual absence of plasma cholesteryl esters in the plasma of subjects with familial LCAT deficiency (Norum and Gjone, 1967). However, neither the physiological significance of plasma cholesteryl esters nor the factors regulating cholesterol esterification are fully understood. In particular, the relationship of plasma cholesterol esterifying activity to the *in vivo* turnover of total body exchangeable cholesterol has not been defined. In the present study the rate of total plasma cholesterol esterification has been compared with that of cholesterol turnover in 7 subjects with type II hyperlipoproteinaemia, both during the administration of a placebo and after cholesterol metabolism had been stimulated by therapy with colestipol.

MATERIALS AND METHODS

**Experimental Subjects**

Eight subjects were studied, 7 patients with type II hyperlipoproteinaemia and one normal control. Typing of the lipoprotein pattern (World Health Organization, 1970) was made according to individual lipoprotein cholesterol and triglyceride concentrations. All subjects had been eating their usual diets for several months before entering the study and continued to do so until its completion. Body weights remained essentially constant. Two subjects (EA, DS) had been eating a diet low in cholesterol and high in polyunsaturated fatty acids for one year, with resultant lowering
of plasma cholesterol levels by 80 and 100 mg/100ml respectively. One patient (AT) was taking ethinyl oestradiol, a drug known to influence cholesterol metabolism (Nestel et al, 1965) at the daily dose of 0.02 mg. This was continued throughout the study.

Experimental Design and Clinical Procedures

The study was performed on an out-patient basis with each subject eating his habitual diet. Comparisons of plasma LCAT activity were made during a control period, when a placebo of microcrystalline cellulose was taken, and after several months of therapy with colestipol (15 g daily).

Plasma free and esterified cholesterol concentrations were measured frequently, in vitro esterification of cholesterol having been inhibited by the addition of p-chloromercuriphenylsulphonate to a final plasma concentration of 2 mM (Glomset et al, 1970). Plasma LCAT activity was measured in triplicate on two separate days during the placebo period and again after 2-3 months of resin therapy. Blood was collected for this purpose into chilled tubes containing disodium EDTA to a final concentration of 2.5 mM (Stokke and Ncram, 1971). In one patient (AH) LCAT assays were also performed serially during the first week of colestipol therapy.

Freshly drawn plasma from the single normolipidaemic control subject (NM; plasma cholesterol = 155 mg/100ml) was assayed for LCAT activity in parallel with each test assay, to assess both the reproducibility of the test system and the biological variability of LCAT activity over a period of several months.

All blood samples were drawn after a 12-14 hour overnight fast.

Laboratory Procedures

Plasma cholesterol

The concentrations of plasma total, free and esterified cholesterol (separated by thin-layer chromatography) were measured by a semi-automated procedure based on the
colorimetric method of Liebermann and Burchard, as previously described (Auto Analyzer II, Technicon Instruments Corporation).

**Plasma LCAT activity**

Assay of *in vitro* lecithin:cholesterol acyltransferase activity was performed essentially as described by Stokke and Norum (1971). Plasma was separated immediately after collection by centrifugation at $4^\circ$C, it having been previously established that plasma LCAT was inhibited at this temperature. Radiolabelled cholesterol was incorporated into plasma lipoproteins by incubating 100 $\mu$l plasma for 4 hours at $37^\circ$C and pH 7.1 with 30 $\mu$l of an albumin-stabilized emulsion of purified cholesterol-4-$^{14}$C or cholesterol-1a,2a-$^{3}$H in the presence of a reversible LCAT inhibitor (20 $\mu$l of a 10 mM solution of Ellman reagent in 0.2 M phosphate buffer). The reaction was activated by adding 20 $\mu$l of a 0.1 M solution of mercaptoethanol and allowed to proceed for one hour when 20 volumes of chloroform:methanol (2:1) was added. Lipids were extracted with additional chloroform:methanol (x3). Free and esterified cholesterol were separated by thin-layer silicic acid chromatography (Kieselgel G; hexane:diethyl ether:methanol:acetic acid, 180:40:6:4, solvent), eluted with diethyl ether and assayed for radioactivity and mass (Zlatkis et al, 1953). Plasma LCAT activity was calculated as % free cholesterol (FC) esterified per hour and as $\mu$g FC per ml of plasma esterified per hour.

This assay provides a measure of the *in vivo* rate of plasma cholesterol esterification, since it is dependent on the concentrations of plasma substrates as well as on enzyme activity. A value for total plasma cholesterol esterifying activity was calculated for each subject as the product of the mass esterification rate *in vitro* and the estimated plasma volume (Edwards and Whyte, 1960).

**RESULTS**

**Reproducibility of Plasma LCAT Assay**

The coefficients of variation for triplicate assays performed on the same plasma samples averaged $5.6 \pm 4.7\%$
(n=41) for percent esterification and 8.1 ± 4.7% (n=41) for mass esterification (mean ± SD).

Day to Day and Seasonal Variations in LCAT activity

The results of 14 pairs of assays performed on two different days during a single study period differed from their means by an average of 6.3 ± 4.3% for percent esterification and 7.0 ± 4.7% for mass esterification (mean ± SD). There was no evidence of any seasonal variation in LCAT activity in the control subject, mean values of 4.8, 4.7, 4.9, 4.6 and 4.6%FC/hr and of 23, 19, 21, 20 and 18 µgFC/ml/hr being obtained respectively during the months of March, May, July, October and January. This agrees with the findings of Monger and Nestel (1967b).

LCAT activity following colestipol therapy

No change in plasma LCAT activity was observed during the first week of resin therapy in subject AH. Rates of 3.5, 3.9, 3.5, 4.1 and 3.9 percent FC esterified per hour and of 30, 35, 26, 30 and 26 µg FC/ml plasma esterified per hour were observed on days 0, 1, 2, 3 and 7 of treatment.

After eight or more weeks of therapy, however, cholesterol esterifying activity showed a significant increase in the 7 patients, particularly in subject AH. This was shown for both %FC esterified/hour and µgFC/ml plasma esterified/hour (or g FC/total plasma volume esterified per day) (Table 7-1). Results obtained with plasma samples from the single control subject (NH) averaged 4.7 ± 0.56 %FC/hr and 20.1 ± 3.43 µgFC/ml/hr for assays performed in parallel with placebo assays, and 4.7 ± 0.27 %FC/hr and 20.1 ± 1.00 µgFC/ml/hr for assays performed in parallel with colestipol assays (mean ± SD).

Resin therapy also produced a significant fall in the plasma cholesterol concentration and a small but significant increase in the fraction unesterified (Table 7-2). Neither of these, however, was correlated with plasma LCAT activity.

Each patient also participated in a simultaneous study of the effects of colestipol on cholesterol pool sizes and turnover, the results of which were discussed in Chapter 5.
Cholesterol turnover was estimated both by isotope dilution analysis and isotopic steroid balance during each study period. In Table 7-2 the mean values obtained by these techniques are given for each subject. It can be seen that resin therapy produced a significant increase in body cholesterol turnover.

During the placebo period values for total plasma LCAT activity averaged 0.47 g/day (39%) more than the corresponding values for cholesterol turnover (paired t-test: \( t = 5.073, P<0.0001 \)), while during colestipol the values did not differ significantly (total LCAT activity: 2.17 ± 0.19 g/day; cholesterol turnover: 2.34 ± 0.40 g/day, mean ± SD, \( n = 6 \); \( t = 1.745, P>0.05 \)). Total plasma LCAT activity was significantly correlated with cholesterol turnover both in the pooled data (\( r = +0.87, P<0.001 \)) and in the colestipol data (\( r = +0.94, P<0.01 \)). However, no correlation was apparent within the narrow range of values observed during the placebo period (\( r = +0.22, P>0.05 \)). These relationships are illustrated in Figure 7-1.

**DISCUSSION**

The function of cholesterol esterification within plasma has not been established. In subjects with familial LCAT deficiency an almost absence of plasma cholesteryl esters, hypertriglyceridaemia, abnormal lipoprotein composition and increased erythrocyte cholesterol have been reported (Norum and Gjone, 1967; Glomset et al, 1970; Gjone et al, 1968). Although the proportion of cholesterol that is esterified in whole plasma is relatively constant in healthy subjects, there is considerable variability in the rate of esterification (Akanuma and Glomset, 1968; Goodman, 1964) and in the proportion of esterified cholesterol within different lipoproteins. Within the triglyceride-rich very low density lipoproteins the ratio of esterified to free cholesterol rises as the size and triglyceride content of the lipoprotein falls (Gustafson et al, 1965). This may be related to the experimental observation that cholesteryl esters can provide the non-polar constituent required for
lipoprotein stability (Lux et al, 1972). Such reports suggest that plasma cholesterol esterification is related to lipid transport, and are consistent with the present finding of an increase in LCAT activity accompanying the colestipol-induced rise in cholesterol turnover. This occurred despite an average fall of 11% in the plasma concentration of free cholesterol and a probable fall in that of phospholipid (Jones and Dobrilovic, 1970), changes which might be expected to retard in vitro cholesterol esterification under other circumstances (Glomset, 1968; Monger and Nestel, 1967b). Increased plasma cholesterol esterification has been reported in other conditions characterized by enhanced lipid transport. This has been demonstrated in obesity by both in vivo (Nestel and Monger, 1967) and in vitro (Akanuma et al, 1973) techniques, while cholesteryl ester turnover is also enhanced in type IV hyperlipoproteinaemia (Nestel, 1970b), the nephrotic syndrome (McKenzie and Nestel, 1968) and carbohydrate feeding (Nestel, 1970b). By contrast, clofibrate therapy is associated with a reduction in cholesteryl ester turnover (Sodhi et al, 1971). It cannot be determined from these studies, however, whether the changes in cholesterol esterification were related primarily to alterations in cholesterol transport, triglyceride transport or lipoprotein metabolism in general. Although triglyceride turnover was not estimated in the present study, the strength of the correlations between total exchangeable cholesterol turnover and total plasma LCAT activity suggests that the rate of the reaction may be regulated according to changes in the metabolism of cholesterol. It was demonstrated in Chapter 6 that the increased cholesterol turnover during resin therapy is associated with a greatly increased influx into plasma of newly synthesized free cholesterol. The extremely small increase in the plasma free/esterified cholesterol ratio during long-term therapy thus attests to the efficiency of the LCAT-regulating system in maintaining a stable lipoprotein composition under such circumstances. However, it must be conceded that esterification cannot be an essential requirement for plasma cholesterol transport since subjects with
LCAT deficiency, either familial (Norum and Gjone, 1967) or secondary to liver disease (Calandra et al, 1971), do metabolize cholesterol, albeit abnormally in several respects.

The similar order of magnitude of the values for total LCAT activity and those for total cholesterol turnover suggests that most cholesterol molecules are esterified within plasma at some stage during their passage from sites of synthesis to those of excretion and catabolism. However, it is interesting to note that during the control period the rates for total cholesterol esterification were significantly greater than those for the turnover of total body cholesterol. This must indicate a recycling of cholesterol through plasma after the hydrolysis of cholesteryl esters within body tissues. Such hydrolytic activity has been detected in a variety of animal tissues, including the liver, intestine, adipose tissue, kidney and muscle (Goodman, 1965). Thus, plasma cholesteryl ester turnover may represent the balance between the continuous processes of cholesteryl ester formation within plasma, their subsequent hydrolysis within body tissues, and the partial re-incorporation of the resulting free cholesterol into plasma lipoproteins, together with other cholesterol derived from de novo synthesis and absorption.

The approximation of the values for total LCAT activity and cholesterol turnover which occurred during colestipol therapy probably reflects the excretion and catabolism of a greater proportion of newly synthesized cholesterol directly from the liver, a process which has been demonstrated to occur under conditions of greatly enhanced cholesterol turnover (Chapter 5).

The present data provide no information concerning the mechanism by which LCAT activity is regulated. The cholesterol esterifying activity of whole plasma is dependent not only on the LCAT enzyme itself but also on other plasma components including the plasma lipoproteins. In parallel studies of plasma lipoprotein lipids (Chapter 6) colestipol had no effect on the concentration of HDL cholesterol, the preferred substrate for LCAT activity (Akanuma and Glomset,
Nor are alterations in the plasma bile salt concentration during resin therapy (Carey and Williams, 1961) likely to influence LCAT activity (Calandra et al, 1971). The possibility that the LCAT assays may have been affected by a plasma cholesteryl ester hydrolase is excluded by the absence of liver disease (Jones et al, 1971). Whatever the regulating mechanism may be, the failure of esterifying activity to increase in subject AH during the first week of therapy suggests that the response to altered lipid metabolism is relatively slow. Such a mechanism may involve increased synthesis and secretion of the enzyme by the liver (Osuga and Portman, 1971).
Table 7-1  Plasma cholesterol esterifying activity* in seven patients with type II hyperlipoproteinaemia during the administration of either a placebo or colestipol 15g daily

<table>
<thead>
<tr>
<th>Patient</th>
<th>%FC† esterified/hour</th>
<th>ug FC/ml plasma esterified/hour</th>
<th>g FC/total plasma volume esterified/day</th>
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<td>Placebo</td>
<td>Colestipol</td>
<td>Placebo</td>
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<td>AL</td>
<td>3.5</td>
<td>4.8</td>
<td>29</td>
</tr>
<tr>
<td>AT</td>
<td>2.6</td>
<td>4.1</td>
<td>28</td>
</tr>
<tr>
<td>DS</td>
<td>3.3</td>
<td>4.7</td>
<td>22</td>
</tr>
<tr>
<td>AH</td>
<td>3.6</td>
<td>6.7</td>
<td>27</td>
</tr>
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</tr>
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<td>RB</td>
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<td>25</td>
</tr>
<tr>
<td>MW</td>
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<td>28</td>
</tr>
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</tr>
<tr>
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<td>1.14</td>
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</tr>
<tr>
<td>t**</td>
<td>6.158</td>
<td></td>
<td>3.956</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td></td>
<td>&lt;0.0002</td>
</tr>
</tbody>
</table>

* Each value is the mean of triplicate determinations performed on each of two days (6 values)
** Paired t-test analysis.
† FC = free cholesterol
Table 7-2

Plasma cholesterol concentration, degree of esterification of plasma cholesterol and rate of cholesterol turnover in seven patients before (P) and during (C) colestipol therapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma cholesterol concentration (mg/100 ml)</th>
<th>% Plasma cholesterol unesterified</th>
<th>Cholesterol turnover (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>C</td>
<td>P</td>
</tr>
<tr>
<td>AL</td>
<td>291</td>
<td>241</td>
<td>31</td>
</tr>
<tr>
<td>AT</td>
<td>477</td>
<td>397</td>
<td>29</td>
</tr>
<tr>
<td>DS</td>
<td>292</td>
<td>272</td>
<td>27</td>
</tr>
<tr>
<td>AH</td>
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<td>256</td>
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</tr>
<tr>
<td>EA</td>
<td>444</td>
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</tr>
<tr>
<td>RB</td>
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<td>26</td>
</tr>
<tr>
<td>MW</td>
<td>357</td>
<td>278</td>
<td>29</td>
</tr>
</tbody>
</table>

\[ t^* = 6.002 \quad 2.970 \quad 6.456 \]

\[ P < 0.0001 \quad < 0.003 \quad < 0.0001 \]

**MEAN DIFFERENCE**

\[ -54 \quad +1.4 \quad +1.09 \]

* Paired t-test analysis.

† Means of values obtained by 2-pool analysis and isotopic steroid balance.

---

*Figure 7-1*

The relationship between total plasma cholesterol esterifying activity and cholesterol turnover in 7 patients with type II hyperlipoproteinemia before (■) and during (●) long-term colestipol therapy.
Figure 7-1
The relationship between total plasma cholesterol esterifying activity and cholesterol turnover in 7 patients with type II hyperlipoproteinaemia before (■) and during (○) long-term colestipol therapy.
CHAPTER 8

ALtered Bile Acid Metabolism During Treatment with Phenobarbitone
ABSTRACT

The effects of phenobarbitone on cholesterol and bile acid metabolism have been examined in healthy humans, using gas chromatographic quantification of faecal steroid excretion and isotope dilution to measure the pool size and turnover of bile acids.

In 3 of 4 subjects phenobarbitone stimulated the synthesis of bile acids and this was associated with an increased pool size of cholic acid. Faecal neutral steroid excretion was not clearly affected. The fourth subject who did not respond was also exceptional in not showing an increase in the plasma clearance of antipyrine.

The 3 responsive subjects also developed significant increases in plasma cholesterol and triglyceride concentrations. These findings were associated in one subject with an early rise in very low density lipoprotein and a fall in plasma cholesterol specific activity, changes compatible with increased cholesterol synthesis.

METHODS

Experimental Design

Four healthy male volunteers were studied under metabolic ward conditions. Clinical details are given in Table 8-1. Each subject consumed a eucaloric solid food diet of constant composition (protein : fat : carbohydrate, 1:2:3; ratio of polyunsaturated to saturated fatty acids = 0.18). Cholesterol intake, quantified by gas chromatography (Schiller et al., 1955), ranged from 512 to 578 mg/day (mean = 550 mg/day). After 4-5 days on this diet measurements

Phenobarbitone is known to induce enzymes of the endo-

philosis of neonatal jaundice 

esothermic of drug and steroid

Hormone hydroxylations (Nade et al., 1961; Nade et al., 1967b; Nade et al., 1968; Kuntzman, 1969). It is not surprising, therefore, that cholesterol metabolism has been shown to be influenced by phenobarbitone in experimental animals (Jones and Armstrong, 1955; Schefer, Kassner and Nosbach, 1972). The effects of phenobarbitone on cholesterol and bile acid metabolism in normal human subjects have, however, not been studied to date. The following account records the results of such an investigation.
INTRODUCTION

Phenobarbitone is known to induce enzymes of the endoplasmic reticulum within the liver (Kuntzman, 1969). The stimulation of these enzymes appears to have important clinical connotations. These include the use of phenobarbitone in the treatment of Gilbert's disease (Hunter et al, 1971b) and in the prophylaxis of neonatal jaundice (Trolle, 1968), the development of osteomalacia during long-term anticonvulsant therapy (Hahn et al, 1972), the antagonism of other drugs by phenobarbitone (Cucinell et al, 1965), the development of steroid resistance in asthmatics (Brooks et al, 1972) and possibly the development of folate deficiency in epileptics (Maxwell et al, 1972).

The rate-limiting enzymes that regulate both the synthesis of cholesterol (β-hydroxy-β-methyl-glutaryl CoA reductase) and its catabolism (7α-hydroxylase) reside within the hepatic endoplasmic reticulum (Shefer, Hauser, Lapar et al, 1972; Boyd and Percy-Robb, 1971), and appear to have some co-factor requirements similar to those of drug and steroid hormone hydroxylations (Wada et al, 1967a; Wada et al, 1967b; Wada et al, 1968; Kuntzman, 1969). It is not surprising, therefore, that cholesterol metabolism has been shown to be influenced by phenobarbitone in experimental animals (Jones and Armstrong, 1965; Shefer, Hauser and Mosbach, 1972). The effects of phenobarbitone on cholesterol and bile acid metabolism in normal human subjects have, however, not been studied to date. The following account records the results of such an investigation.

METHODS

Experimental Design

Four healthy male volunteers were studied under metabolic ward conditions. Clinical details are given in Table 8-1. Each subject consumed a eucaloric solid food diet of constant composition (protein:fat:carbohydrate, 15:39:46%; ratio of polyunsaturated to saturated fatty acids = 0.16). Cholesterol intake, quantified by gas chromatography (Miettinen et al, 1965), ranged from 512 to 979 mg/day (mean = 682 mg/day). After 4-8 days on this diet measurements...
of cholesterol and bile acid metabolism were made. This was followed by 13-18 days of phenobarbitone therapy (240 mg daily), during the latter part of which the measurements were repeated. The investigation was fully explained and each subject gave informed consent.

Investigations
Faecal steroid excretion

All stools were collected for the duration of the study and stored at -25°C. The faecal excretion of cholesterol and bile acids and their products of bacterial degradation was quantified by gas chromatography essentially as described by Miettinen et al (1965) and Grundy et al (1965). Pools of faeces were homogenized and aliquots subjected to mild saponification in IN ethanolic NaOH for 60 minutes. Neutral steroids were then extracted with petroleum ether and an aliquot separated by thin-layer chromatography (Kieselgel G; heptane:diethyl ether, 45:55) into cholesterol, coprostanol and coprostanone, together with the corresponding plant sterols. After visualization under ultraviolet light with rhodamine G, the 3 fractions were eluted with diethyl ether and individually quantified by gas chromatography as the trimethylsilyl derivatives, using 5α-cholestane as internal standard (4 mm i.d. coiled glass column, length: 6 feet; carrier gas:nitrogen, 50 ml/min; liquid phase: 1% DC-560; column support: gas-chrom Q, 100-120 mesh; column temperature: 250°C; hydrogen flame ionization detector, temperature: 260°C). After the addition of 2 ml ION NaOH, the saponified fraction of the homogenate was further saponified under pressure (3 hrs; 15 psi) to ensure bile acid deconjugation. Free bile acids were extracted at pH2 with chloroform:methanol (2:1) and chloroform. Bile acids were methylated overnight in 5% HCl-methanol and separated from fatty acids (solvent:-benzene) and acidic pigments (solvent:-isoctane:isopropanol:acetic acid, 120:40:1) by 2-stage thin-layer chromatography. After visualization with I₂ vapour they were eluted with methanol, redissolved in ethyl acetate, and quantified as the trimethylsilyl derivatives in the same way as were the neutral
steroids. Results were corrected from the recovery of radiolabelled cholic acid \((^{14}\text{C})\) and cholesterol \((^{3}\text{H})\). This was consistently in excess of 85% for both fractions. The results were further corrected for variations in faecal flow by measuring the excretion of chromium oxide, 300 mg of which was consumed daily by each subject (Davignon et al, 1968). In view of the previous demonstration (Chapter 5), and that of others, that degradative neutral steroid losses are negligible during the consumption of solid diets, it was considered unnecessary to monitor possible losses of dietary \(\beta\)-sitosterol in all subjects. In the one subject (TK) in whom this was performed the recovery was essentially 100%. Phenobarbitone produced no detectable alteration in bowel habit in any subject.

**Bile acid turnover**

Cholic acid turnover was measured by isotope dilution analysis in all subjects, and that of chenodeoxycholic acid also in two subjects (RB, KO), during the control period and again during the latter part of the treatment period. Cholic-(carboxyl-\(^{14}\text{C}\)) acid* (control: 5-12 \(\mu\text{C}\); phenobarbitone: 10-23 \(\mu\text{C}\)) and chenodeoxycholic acid-\(^{3}\text{H}\)* (control: 11 \(\mu\text{C}\); phenobarbitone: 22 \(\mu\text{C}\)) were given intravenously in 20 ml 5% human albumin in 0.9% saline, after incubation at \(37^\circ\text{C}\) for 30 minutes. Four 5 ml duodenal aspirates were collected before breakfast over the following 5-8 days and stored at \(-25^\circ\text{C}\). An intubation on the morning of the second injection established that more than 97% of the first dose had been excreted by this time.

After the addition of the bile to 4 volumes of methanol: acetone (1:1), protein was removed by centrifugation after heating to \(37^\circ\text{C}\) for 2 minutes. An aliquot of the supernatant was saponified under pressure (3hrs; 15 psi) in 2N \(\text{NaOH}\), and the free bile acids extracted with diethyl ether at \(\text{pH} 2\) (Kottke et al, 1966). Bile acids were subsequently separated with endogenous bile acids, either by thin layer chromatography. Specific activities: cholic acid -\(^{14}\text{C}\), 46 \(\text{mC/mmol}\); chenodeoxycholic acid-\(^{3}\text{H}\), 0.7C/mmol.

* New England Nuclear, Boston. Radiochemical purity >98% by thin layer chromatography.
by thin-layer chromatography on Adsorbisil† No. 5 using iso-octane: ethyl acetate: acetic acid: n-butanol (20:10:3:3) as solvent system (Sundaram et al., 1971). Cholic acid and chenodeoxycholic acid were eluted separately with methanol, using the technique described by Goldrick and Hirsch (1963), after visualization with iodine vapour. Aliquots were assayed for mass by spectrophotometry in 65% sulphuric acid (Kottke et al., 1966; Wollenweber et al., 1966) and for radioactivity by liquid scintillation spectrometry, using 1 ml methanol plus 15 ml PPO-dimethyl POPOP in toluene as scintillator solvent. Bile acid pool size and production rate were calculated from the specific activity–time curve as originally described by Lindstedt (1957).

Although the values for total bile acid synthesis obtained by this technique were positively correlated with those obtained by faecal analysis (r = +0.96; P < 0.05), the former averaged 31 ± 7.4% (mean ± SD, n = 4) higher than the latter (P < 0.001). There is an increasing awareness that estimates of bile acid turnover tend to be higher by isotope dilution than by steroid balance (Einarsson and Hellström, 1972), but no controlled comparisons in man have been published to date. Hellström and Sjövall (1962) compared the production rate of deoxycholic acid obtained by isotope dilution with that obtained by faecal analysis in rabbits, and noted a discrepancy similar to that encountered in the present study. The possibility that faecal analysis might underestimate bile acid turnover (due to the existence of other important routes of elimination, tissue metabolism of bile acids or intestinal bacterial degradation) is unlikely in view of the virtually complete recovery of orally administered radioactive bile acids within human faeces reported by Grundy et al. (1965). Thus, isotope dilution would appear to overestimate bile acid synthesis. This could theoretically be due to an underestimation of the number of bile acid pools, to the excretion of a fraction of the administered dose before complete mixing with endogenous bile acids has occurred, or to a combination of these factors. Further work is indicated to answer this question.

† Applied Science Laboratories, State College, Pa., U.S.A.
In 2 subjects (RB, KO) bile acid synthesis was also estimated from the production rate of $^{14}\text{CO}_2$ after the intravenous administration of cholesterol-26-$^{14}$C, using a modification of the method described by Myant and Lewis (1966). During bile acid synthesis the 3 end-carbon atoms of the cholesterol side-chain are removed as propionyl-CoA, which is subsequently oxidized to CO$_2$ via succinyl-CoA. Hence, when body cholesterol is labelled with cholesterol-26-$^{14}$C, $^{14}$CO$_2$ is expired at a rate which is related to the rate of bile acid synthesis.

Each subject was given an intravenous infusion of 50 µC of cholesterol-26-$^{14}$C* in 200 ml 0.9% saline 3 to 7 weeks before entering the study. From the beginning of the treatment period, serial 70-100 litre collections of expired air were made over a timed period of 10-15 minutes essentially as described by Myant and Lewis (1966). The CO$_2$ from 20 litre aliquots was trapped by pumping air at 800 ml/min serially through two absorption tubes containing successively 5 ml and 3 ml of ethanolamine plus 10 ml toluene:2-methoxyethanol (1:1, v:v) containing 0.5% PPO and 0.03% POPOP. These tubes were cooled in iced water to minimize evaporation. A third tube containing 10 ml saturated aqueous barium hydroxide solution was used as a monitor to confirm the completeness of CO$_2$ absorption. The contents of the first two tubes were subsequently pooled, made up to 40 ml with toluene:2-methoxyethanol counting fluid, and a 20 ml aliquot assayed for radioactivity in a liquid scintillation counter. Counting efficiency was monitored by automatic external standardization and ranged from 40 to 45%. Air collections were made routinely between 11.30 a.m. and 12 midday, and on some days again 12 hours later. Plasma cholesterol specific activity was determined as described previously (see Chapter 5), and bile acid synthesis calculated

Specific activity = 24.2 mC/mmol.
from the formula:

\[
\text{Bile acid synthesis} = \frac{\text{dpm expired/day as } ^{14}\text{CO}_2}{\text{Plasma cholesterol specific activity (dpm/mg)}}
\]

(Myant and Lewis, 1966).

Duplicate assays from the same collection gave consistent agreement within 5%. There was no significant difference between production rates estimated from morning and evening air samples (paired t-test: P > 0.1, n = 11).

The values obtained by this technique were compared with those obtained simultaneously by chemical analysis of faeces. The former averaged 60 ± 16.9% (mean ± SD, n = 6) of the latter, a difference which was statistically highly significant (paired t-test, P < 0.002). This result is in accord with the findings of Myant and Lewis (1966) and Lewis and Myant (1967), who used lithium hydroxide to absorb \(^{14}\text{CO}_2\) and isotopic balance to measure faecal bile acid excretion.

Plasma lipids

Plasma cholesterol and triglyceride concentrations were determined every 2 or 3 days (Technicon Auto Analyzer II). Additional plasma from subject KO was collected on three days during both the control and treatment periods for the isolation of plasma lipoproteins by ultracentrifugation and precipitation, as described in Chapter 6. To assist the interpretation of changes in cholesterol metabolism, the plasma cholesterol specific activity-time curve was followed during both study periods in subjects RB and KO.

Plasma clearance of antipyrine

Hepatic enzyme induction was monitored by measuring the plasma antipyrine clearance (dose = 18 mg/kg by mouth) on the final day of both study periods (Brodie et al., 1949).

RESULTS

Subject RB failed to show any change in the metabolism of cholesterol or antipyrine (control v. treatment: plasma cholesterol (mg/100ml, mean±SEM), 233 ± 4 v. 229 ± 5; plasma triglyceride, 119 ± 6 v. 102 ± 4; plasma cholesterol specific activity-time curve unchanged; faecal neutral steroids
(mg/day), 1532 ± 125 v. 1378 ± 25; faecal bile acids, 171 ± 29 v. 202 ± 53; cholic acid pool (mg), 377 v. 365; cholic acid turnover (mg/day), 149 v. 140; chenodeoxycholic acid pool, 914 v. 845; chenodeoxycholic acid turnover, 127 v. 135; plasma antipyrine clearance (ml/min), 39 v. 42).

Each of the remaining 3 subjects, however, demonstrated both an increased antipyrine clearance (mean increase ± SD: 55 ± 11%) and a consistent response in plasma lipids and bile acid metabolism. Plasma cholesterol and triglyceride levels increased during treatment by an average of 29 mg/100 ml (16%, t = 6.089, p<0.001) and 25 mg/100 ml (25%, t = 5.368, p<0.001) respectively. In subject KO these changes were confined to very low density lipoproteins (VLDL) initially, but later occurred in low density lipoproteins (LDL). These findings were associated in the same subject with an early transient steepening of the plasma cholesterol specific activity-time curve. These results are summarised in Figure 8-1. Body weights remained constant.

The daily faecal excretion of neutral and acidic steroids decreased during the control period, which was probably due to a change in dietary habit (Figure 8-2). Phenobarbitone reversed this trend in bile acid excretion, the excretion rates during the last 6 days of treatment (442 ± 57 mg/day, mean ± SEM) being significantly greater (t = 2.755, P = 0.020) than those during the 5 days preceding treatment (233 ± 38 mg/day). Although neutral steroid excretion became constant, this probably reflected the attainment of a new steady state after the dietary change, rather than an effect of phenobarbitone.

The enhanced faecal bile acid excretion in these three subjects was accompanied by an increased pool size and production rate of the primary bile acids, as measured by isotope dilution. The results of the bile acid turnover studies are presented in Table 8-2 and summarised in Figure 8-2.

In subject KO the increased cholesterol catabolism produced by phenobarbitone was also detected from the estimations based on $^{13}$CO$_2$ production (Figure 8-3).
Although there was a considerable day-to-day variation with this technique, bile acid synthesis was positively correlated with the duration of treatment ($r = +0.72$, $P < 0.01$).

**DISCUSSION**

Other studies have indicated that microsomal enzyme induction by phenobarbitone is genetically controlled (Vesell and Page, 1969), and this may explain the failure of subject RB to respond. The present results thus suggest that when microsomal enzymes are induced, as indicated by increased plasma antipyrine clearance, steroid metabolism is affected. The increased bile acid production, demonstrated by three independent techniques, was the most marked effect of phenobarbitone and agrees with the results of similar studies with monkeys (Redinger and Small, 1973). The question arises whether this was a primary effect, or whether it was secondary to enhanced cholesterogenesis. The first possibility is supported by reports of increased 7α-hydroxylation of cholesterol by liver preparations from phenobarbitone-treated rats (Wada et al, 1968; Mosbach, 1969; Shefer, Hauser and Mosbach, 1972). However, the response of cholesterol 7α-hydroxylase is variable (Shefer, Hauser and Mosbach, 1972) and cholesterol 12α-hydroxylation is inhibited in the same species (Suzuki et al, 1968; Einarsson and Johansson, 1968). The alternative possibility, that enhanced bile acid production was secondary to increased cholesterogenesis, would be in accord with the observed changes in plasma lipids. That the increase in plasma cholesterol level reflected enhanced cholesterogenesis, rather than transfer of stored cholesterol from body tissues, seems likely in view of the early rises in VLDL and plasma triglyceride and the fall in plasma cholesterol specific activity. These changes were observed in other situations of enhanced cholesterogenesis in the study described in Chapter 6, namely the early phase of colestipol therapy and during the first few days after stopping treatment with clofibrate. The later rise in LDL may reflect increased conversion from VLDL (Gulbrandsen et al, 1971). Consistent with this interpretation is the finding of increased cholesterogenesis from acetate and mevalonate by liver preparations.
from rats (Wada et al, 1967a; Kato et al, 1968; Middleton and Isselbacher, 1969) and hamsters (Jones and Armstrong, 1965) treated with phenobarbitone, while enhanced synthesis from acetate has been noted in the rat intestine (Middleton and Isselbacher, 1969). Increased microsomal sterol demethylase activity has also been observed in rats (Moir et al, 1970). While enhanced cholesterogenesis may be secondary to altered bile acid metabolism, the phenobarbitone induced expansion of the bile acid pool in the present subjects would have tended rather to inhibit cholesterogenesis, (Boyd and Percy-Robb, 1971). The increased bile acid production with phenobarbitone may, therefore, reflect both primary stimulation of cholesterogenesis, and the induction of enzymes of cholesterol catabolism.

The expansion of the bile acid pool is a natural consequence of increased bile acid synthesis, in the absence of impaired intestinal reabsorption. This was also observed in phenobarbitone-treated monkeys (Redinger and Small, 1973). This result in normal subjects contrasts with the decrease in bile salt pool produced in some patients with primary biliary cirrhosis or intrahepatic cholestasis of other cause (Earnest et al, 1972; Stiehl et al, 1972; Sharp and Mirkin, 1970; Admirand and Bauer, 1971). This latter effect may be secondary to the increased biliary flow which is produced by phenobarbitone (Klaassen, 1971; Sharp and Mirkin, 1970), and which appears to be independent of microsomal enzyme induction (Klaassen, 1969). It may also be related to an increased hepatic transport of bile acids, which has been recently demonstrated in experimental animals (Schiff et al, 1971).

The effects of phenobarbitone on lipid metabolism appear to resemble those of ethanol. Although the effects of alcohol on bile acid turnover have not been clearly defined (Boyer, 1972; LeFèvre et al, 1972), increases in cholesterogenesis (LeFèvre et al, 1972) and lipoprotein secretion (Mistilis and Ockner, 1972; Baraona et al, 1973) have been demonstrated during its consumption. Since alcohol also produces a proliferation of the hepatic endoplasmic reticulum and induces drug-metabolizing enzymes (Rubin et al, 1968), the mechanism of alcohol-induced hyperlipidaemia (Losowsky et al, 1963) may be related to microsomal enzyme induction.
### Table 8-1

**Clinical Data**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yrs)</th>
<th>Weight (kg)</th>
<th>mg/100ml (mean ± SD)</th>
<th>Experiment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK</td>
<td>23</td>
<td>60.3</td>
<td>244 ± 6.5</td>
<td>1,2</td>
</tr>
<tr>
<td>GR</td>
<td>17</td>
<td>73.2</td>
<td>151 ± 12.3</td>
<td>1,2</td>
</tr>
<tr>
<td>KO</td>
<td>21</td>
<td>67.9</td>
<td>163 ± 6.5</td>
<td>1,2,3,4,5</td>
</tr>
<tr>
<td>RB</td>
<td>20</td>
<td>57.9</td>
<td>233 ± 10.6</td>
<td>1,2,3,4</td>
</tr>
</tbody>
</table>

* 1 Faecal neutral and acidic steroid excretion (GLC)
2 Cholic acid turnover
3 Chenodeoxycholic acid turnover
4 Plasma cholesterol specific activity
5 Plasma lipoprotein lipid analysis
Table 8-2

Effects of phenobarbitone (240 mg daily) on bile acid turnover in four normal subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Cholic Acid</th>
<th>Chenodeoxycholic Acid</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pool size (mg)</td>
<td>Production Rate (mg/day)</td>
<td>Pool size (mg)</td>
</tr>
<tr>
<td></td>
<td>C*</td>
<td>PB</td>
<td>C</td>
</tr>
<tr>
<td>TK</td>
<td>358</td>
<td>652</td>
<td>114</td>
</tr>
<tr>
<td>GR</td>
<td>636</td>
<td>1147</td>
<td>186</td>
</tr>
<tr>
<td>KO</td>
<td>827</td>
<td>1207</td>
<td>220</td>
</tr>
<tr>
<td>RB</td>
<td>377</td>
<td>365</td>
<td>149</td>
</tr>
</tbody>
</table>

* C = control period; PB = phenobarbitone period.
Figure 8-1
Effects of phenobarbitone on plasma cholesterol and triglyceride concentrations, plasma lipoproteins, and plasma cholesterol specific activity in three normal subjects. Chol = cholesterol; TG = triglyceride; VLDL = very low density lipoprotein; LDL = low density lipoprotein. Each vertical bar represents the standard error of the mean. Plasma lipoprotein lipids and cholesterol specific activity were measured in one subject only.
Figure 8-2

Effects of phenobarbitone on faecal steroid excretion, bile acid turnover and plasma antipyrine clearance in three normal subjects. CA = cholic acid; CDCA = chenodeoxycholic acid. Each vertical bar represents the standard error of the mean. The vertical arrow indicates the day of the second infusion of labelled bile acids.
Figure 8-3
Effect of phenobarbitone on bile acid synthesis as estimated from the rate of expiration of $^{14}$CO$_2$ derived from cholesterol-26-$^{14}$C (subject KO). Phenobarbitone (60 mg four times daily) was commenced on day 1.

○ = values estimated from morning collections;
■ = values estimated from evening collections.
In this thesis several studies have been described concerning pharmacological effects on cholesterol metabolism. The first study was a clinical trial of a new bile-acid-sequestering resin, colestipol, in the treatment of hyperlipoproteinemia (Chapter 4). The effects of colestipol on the body pools and metabolism of cholesterol were examined in parallel studies in the same patients, using the techniques of compartmental isotope dilution analysis, sterol balance, and bile analysis (Chapter 5). When the mode of action of colestipol on cholesterol metabolism had been established, it was then employed to investigate the relationship of the various plasma lipoproteins to cholesterol transport, and subsequently to examine the effect of different types of hyperlipoproteinemia (Chapter 6). Colesalpinol also was used to study the relationship between the rate of plasma cholesterol esterification and the turn-over of total body exchangeable cholesterol (Chapter 7).

In the final study the effects on cholesterol metabolism of phenobarbital, a known enzyme inducer, were examined, and the associated changes in plasma lipoproteins compared with those produced by colestipol (Chapter 6).

The clinical trial established that colestipol is an effective cholesterol-lowering drug in type II hyperlipoproteinemia. The fall in cholesterol level was subsequently shown to reflect a decrease in the plasma concentration of LDL and to be associated in most patients with a reduction in the rapidly equilibrating pool of tissue cholesterol. The metabolic studies further demonstrated that these changes derived predominantly from a four-fold increase in the conversion of cholesterol to bile acids. Whether the depletion of tissue cholesterol stores in this manner can reverse the process of atherosclerosis is not known, but is pertinent to the usefulness of cholesterol-lowering therapy. Since bile acid-sequesterants are being increasingly used in the treatment of hypercholesterolaemia, long-term trials of their efficacy in the prevention of clinical coronary heart disease are indicated.
In this thesis several studies have been described concerning pharmacological effects on cholesterol metabolism. The first study was a clinical trial of a new bile acid-sequestering resin, colestipol, in the treatment of hyperlipoproteinaemia (Chapter 4). The effects of colestipol on the body pools and metabolism of cholesterol were examined in parallel studies in the same patients, using the techniques of compartmental isotope dilution analysis, steroid balance and bile analysis (Chapter 5). When the mode of action of colestipol on cholesterol metabolism had been established, it was then employed to investigate the relationship of the various plasma lipoproteins to cholesterol transport, and subsequently to examine lipoprotein cholesterol metabolism in different types of hyperlipoproteinaemia (Chapter 6). Colestipol was also used to study the relationship between the rate of plasma cholesterol esterification and the turnover of total body exchangeable cholesterol (Chapter 7).

In the final study the effects on cholesterol metabolism of phenobarbitone, a known enzyme inducer, were examined and the associated changes in plasma lipoproteins compared with those produced by colestipol (Chapter 8).

The clinical trial established that colestipol is an effective cholesterol-lowering drug in type II hyperlipoproteinaemia. The fall in cholesterol level was subsequently shown to reflect a decrease in the plasma concentration of LDL and to be associated in most patients with a reduction in the rapidly equilibrating pool of tissue cholesterol. The metabolic studies further demonstrated that these changes derived predominantly from a four-fold increase in the conversion of cholesterol to bile acids. Whether the depletion of tissue cholesterol stores in this manner can reverse the process of atherosclerosis is not known, but is pertinent to the usefulness of cholesterol-lowering therapy. Since bile acid-sequestrants are being increasingly used in the treatment of hypercholesterolaemia, long-term trials of their efficacy in the prevention of clinical coronary heart disease are indicated.
Equally interesting was the finding of significant increases in the plasma concentrations of VLDL and triglyceride. This was associated with a two-fold increase in cholesterol production rate, due at least in part to enhanced hepatic cholesterogenesis. Close examination of the acute response of lipoprotein cholesterol to the colestipol-induced rise in cholesterol turnover suggested that cholesterol influx into plasma occurs predominantly as free cholesterol within VLDL, while efflux of cholesterol occurs mainly within LDL prior to its catabolism to bile acids within the liver. The long-term effects of colestipol thus reflected the attainment of a new steady state in which the plasma pool of triglyceride-rich VLDL was expanded due to increased input and that of the cholesterol-rich LDL was diminished due to increased clearance.

The conclusion that VLDL is the major vehicle for incoming cholesterol transport was supported by further studies. Thus, the acute response of VLDL to colestipol therapy was greatly enhanced in patients with hyperlipoproteinaemia of types IV and V and during carbohydrate-rich diets, conditions which appear to be associated with enhanced cholesterol transport. The effects of disturbing cholesterol metabolism with clofibrate, a known inhibitor of cholesterol synthesis, were also consistent with this hypothesis (Chapter 6). Other evidence was provided by the response to phenobarbitone therapy; changes in faecal steroid excretion and in plasma cholesterol specific activity suggesting enhanced cholesterol synthesis were again associated with elevated plasma levels of VLDL and triglyceride. These observations were in accord with previous reports of elevated VLDL (and triglyceride) levels in other conditions of enhanced cholesterol turnover, namely obesity (Miettinen, 1971a) and the nephrotic syndrome (Nestel, 1973).*

Conclusions drawn from such indirect evidence must, however, remain tentative until confirmed by a more direct approach. The study of plasma cholesterol transport is

* Personal communication.
complicated by two factors. The rapid exchange of free cholesterol between lipoproteins makes it difficult to determine by isotopic techniques whether cholesterol enters plasma preferentially in a particular lipoprotein, while the relatively low rate of cholesterol turnover precludes the possibility of comparing lipoprotein cholesterol gradients across the liver. However, if cholesterol does enter plasma predominantly in one lipoprotein, differences in lipoprotein free cholesterol specific activity might be detected by rapidly processing hepatic venous plasma by lipoprotein precipitation techniques. Additional indirect information could be obtained by examining the response of plasma lipoproteins to therapy with bile acids, which reduces cholesterol turnover, or with specific inhibitors of cholesterol synthesis, when non-toxic forms of these are available.

Since it has been established that VLDL transports newly synthesized triglyceride from the liver, these findings suggest a link between the turnover of cholesterol and triglyceride at the point of VLDL synthesis. This would be in accord with previous reports that the turnover of both lipids is increased in a variety of conditions, notably obesity, carbohydrate feeding, type IV hyperlipoproteinaemia and the nephrotic syndrome, while both are decreased during clofibrate therapy. Further information concerning the relationship between cholesterol and triglyceride metabolism might be obtained by combining recently developed techniques for studying splanchnic triglyceride production (Wolfe and Ahuja, 1972) and biliary lipid secretion (Grundy and Metzger, 1972). It is interesting in this context that a correlation has recently been reported between the secretion of triglyceride and the production of bile by the perfused rat liver (Marsh et al, 1972).

The conclusion that cholesterol subsequently leaves plasma mostly within LDL is consistent with the conversion of VLDL to LDL that has been suggested by studies of their triglyceride fatty acid and protein moieties. However, the positive identification of LDL cholesterol is the major precursor for bile acid synthesis will require further basic research. This might be achieved \textit{in vivo} by comparing the
rates of conversion to biliary bile acids of different plasma lipoproteins labelled in the cholesteryl ester moiety. The conclusion that cholesterol enters plasma mainly as free cholesterol in VLDL but leaves predominantly within LDL is in accord with the evidence that the esterification of cholesterol is related to its plasma transport, since the majority of LDL cholesterol is in the esterified form. This was subsequently substantiated by the demonstration of increased plasma cholesterol esterifying activity during the colestipol-induced rise in cholesterol transport (Chapter 7). Percent esterification was increased by an average of 53% during long-term therapy, while mass esterification was increased by an average of 37%. Total plasma LCAT activity was accordingly strongly correlated with the rate of cholesterol turnover. This possibility had been suggested earlier by the restoration during long-term treatment of the normal equilibrium between free and esterified cholesterol after this had been disturbed during the early period of therapy. Such enhanced esterification might be required to maintain lipoprotein stability or to provide a suitable substrate for bile acid synthesis.

The study of plasma LCAT activity also indicated that the turnover of plasma cholesteryl esters normally exceeds that of total body exchangeable cholesterol, suggesting that considerable recycling of cholesterol through plasma occurs after the hydrolysis of cholesteryl esters by body tissues. This could be tested by examining the fate of radiolabelled cholesteryl ester after its intravenous infusion as a component of plasma lipoproteins. Although there was no significant difference between total LCAT activity and cholesterol turnover during colestipol therapy, this finding was consistent with changes in bile cholesterol specific activity which indicated that during treatment a greater fraction of newly synthesized cholesterol was secreted by the liver directly into bile rather than into plasma.

In addition to supporting the interpretation of the colestipol-induced changes in lipoprotein cholesterol, the
studies with phenobarbitone provided other information of pharmacological interest. The production of increased plasma concentrations of both cholesterol and triglyceride during short-term studies indicated a need for long-term assessment of the effects of phenobarbitone on plasma lipids, as well as of those of other enzyme-inducing drugs. In addition, an increase in bile acid production was demonstrated during phenobarbitone therapy by 3 independent techniques, namely faecal analysis by gas-liquid chromatography, isotope dilution kinetics and the measurement of $^{14}$CO$_2$ production derived from infused cholesterol-26-$^{14}$C. This finding suggests that phenobarbitone might be of value in the management of patients with cholelithiasis or malabsorption, when this is due to inadequate bile acid synthesis.
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APPENDIX I

THE ETHICS OF CLINICAL RESEARCH

The benefits of modern medicine derive wholly from experimentation, and some risk, however slight, is inseparable from medical advance. It is the duty of clinical investigators, however, to ensure that in their desire to help patients, in general they do not cause the individual to suffer. It was in this context that the World Medical Association prepared a series of recommendations as a guide to persons involved in clinical research (Declaration of Helsinki, 1964). Those relevant to the present investigation are quoted below:

"Basic Principles."

1. Clinical research must conform to the moral and scientific principles that justify medical research and should be based on laboratory and animal experiments or other scientifically established facts.

2. Clinical research should be conducted only by scientifically qualified persons and under the supervision of a qualified medical man.

3. Clinical research cannot legitimately be carried out unless the importance of the objective is in proportion to the inherent risk to the subject.

4. Every clinical research project should be preceded by careful assessment of inherent risks in comparison to foreseeable benefits to the subject or to others."

"Non-Therapeutic Clinical Research."

1. In the purely scientific application of clinical research carried out on a human being, it is the duty of the doctor to remain the protector of the life and health of that person on whom clinical research is being carried out.

2. The nature, the purpose, and the risk of clinical research must be explained to the subject by the doctor.

3. Clinical research on a human being cannot be undertaken without his free consent after he has been informed: ...
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2. The nature, the purpose, and the risk of clinical research must be explained to the subject by the doctor.

3a. Clinical research on a human being cannot be undertaken without his free consent after he has been informed; ...
3b. The subject of clinical research should be in such a mental, physical, and legal state as to be able to exercise fully his power of choice.

4a. The investigator must respect the right of each individual to safeguard his personal integrity, especially if the subject is in a dependent relationship to the investigator.

4b. At any time during the course of clinical research the subject ... should be free to withdraw permission for research to be continued.

The investigator ... should discontinue the research if in his ... judgement, it may, if continued, be harmful to the individual."

The present investigations conformed to these recommendations in all respects.
1. Double isotope counting: standardization

01.10 I (SW)4.2
01.11 E
01.13 T !"DOUBLE ISOTOPE COUNTING - STANDARDISATION
01.14 T !"DEPRESS 'RETURN' AFTER ENTERING EACH NUMBER
01.16 S NC=4
01.21 T !"FOR C-14 STD, LIST -"!
01.22 T !"T. GR. COUNTS IN TIME T"!
01.23 F J=1,NCIA GC(J)
01.24 T !"COUNTING TIMES"!
01.25 F J=1,NCIA XX;S CE(J)=GC(J)*0.001/XX
01.26 T !"BLUE AES COUNTS/MIN"!
01.27 F J=1,NCIA BC(J)

02.05 S SE=0;S SB=0;S SP=0;S RS=0
02.10 F J=1,NCIA SE=SE+CE(J);S SB=SB+BC(J)
02.20 F J=1,NCIA SP=SP+CE(J)*BC(J);S RS=RS+BC(J)*RC(J)
02.30 S R1=(SP-SE*SB/NC)/(RS-SB*SB/NC);S B0=(SE-R1*SB)/NC

03.11 S S(1)=R1;S S(2)=R0
03.12 T !"T. RED COUNTS IN TIME T"!
03.13 F J=1,NCIA XX;S CE(J)=FLOG(XX/GC(J));S BC(J)=FLOG(BC(J))
03.20 D 2.0;S S(3)=R1;S S(4)=R0
03.30 T !"FOR TRITIUM STD, LIST -"!
03.40 F J=1,NCIA CE(J)
03.50 D 1.24;F J=1,NCIA XX;S CE(J)=CE(J)*0.00125/XX
03.60 D 1.26;F J=1,NCIA BC(J)
03.70 D 2.0;S S(5)=R1;S S(6)=R0
04.10 S Sw=-1;S I=1
04.20 F J=0,4;S XX=FCOM(J,S(I));S I=I+1
2. Double isotope counting: samples

03.10 I (Sw) 3.2/S Sw= -1; S I=1
03.20 F J=0,4/S S(I)=FCOM(J); S I=I+1
03.30 I (1-7) 3,4/S Sw= JD 3,4; 0 4.3
03.40 L D SA2CIL $ S A2C
03.50 L C IC2C
03.60 6

04.30 T !"For SAMPLES, LIST-"!
04.40 D S16 4.3

05.10 A "TOTAL NO. OF SAMPLES"NC;I (NC) 9.9,9.9
05.15 T !"Gr."
05.20 T "BLANK COUNTS";A RA
05.25 T !"Gr. COUNTS"; F J=1,NC;A CE(J)
05.29 A !"COUNTING TIME?"T
05.30 F J=1,NC;S CC(J)=(CE(J)-BA)/T
05.34 T !"BLUE AES COUNTS/MIN"!
05.35 F J=1,NC;A BC(J); S CE(J)=S(2)+S(1)*BC(J)
05.40 F J=1,NC;S CC(D)=CC(J)*100/CE(J)
05.42 F J=1,NC;D 9.43
05.45 T !"Red" ;D 5.20; T !"Red COUNTS"!
05.50 F J=1,NC;A XX;S CC(J)=XX-B)/T-CC(J)
05.51 F J=1,NC;S BC(J)=S(6)+S(5)*BC(J); S CC(J)=CC(J)*100/BC(J)
05.60 T !"" DPM COUNTING EFFICIENCY"!
05.65 F J=1,2;T " C-14 THITIUM"
05.70 F J=1,NC;I %10.01,ICD(J),CC(J),CE(J),BC(J)

09.43 S NR=FXPS(S(4)+S(3)*FLOG(BC(J))); S CC(J)=CC(J)*NR
09.90 T !:Q
*

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3. Analysis of double exponential curve

01.26 E
01.30 A "AFTEH WHAT TIME (IN UNITS USED) DOES CURVE FLATTEN OUT?" "TA
01.35 T !A W(I), S(I):I (W(I)-1000)*1.4, 2.1, 2.1
01.40 S I=I+1:G 1.35

02.10 S NT=W(I-1):S I=-1
02.25 S I=I+1:J (W(I)-TA) 2.25, 2.35, 2.33
02.35 I (NS) 0.35, 2.35, 2.4
02.35 S NS=I+G 2.50
02.40 I (W(I)-NT) 2.50, 2.45, 2.6
02.45 S NF=I+D 2.51G 2.6
02.55 S I=I=FLOG(S(I))
03.00 G 2.25
03.05 S NO=NF-NS+1

03.05 S SX=0;S SY=0;S SP=0;S XX=0
03.10 F I=NS,NF;S SX=SX+W(I):S SY=SY+S(I)
03.20 F I=NS,NF;S SP=SP+W(I):S XX=XX+W(I)*W(I)
03.30 S A0=(SP-SY*NO)/(XX-SX*NO):S NO=(SY-A0*SX)/NO

04.15 F I=0,NS-1:S S(I)=FLOG(S(I)-FEXP(A0+A1*W(I)))
04.20 S B0=FEXP(A0):S B1=A1
04.25 S NO=NS+1:S NF=NS-1:S NS=0:D 3.0
04.30 S A0=FEXP(A0):S SP=0.4343:AL=A1*SP:BE=B1*SP
04.45 S SP=0.69315:TS TA=SP/A1:S TB=SP/B1
04.55 T %!,"A0=","A0=","A0=","A0=","A0=","A0=",TA
04.60 T %!"B0=",B0=","B0=",BE=","BE=",B1=",TB
04.65 T %!"Q
4. Calculation of parameters of a 2-compartment model

01.02 T !!! "2 COMPARTMENT MODEL", !!
01.04 A ! "DOSE", R
01.06 A "AO", AO, "BO", BO, "AL", AL, "BE", BE
01.08 S MA=R/(AO+BO)*1000
01.09 T ! "MA=" MA
01.10 S Pr=0.001*AL*BE/(AL*BO+(BE*AO))
01.11 T ! "Pr=" Pr
01.12 S KA=((AO*AL)+(BO*BE))/(AO+BO)
01.14 T ! "KA=" KA
01.16 T ! "KBB =", AL+BE-KA
01.18 T !!, "ASSUMING KB=0"

02.02 S KK=(AO*BO)*AL*BE/(AL*BO+BE*AO)
02.03 T ! "KK=" KK
02.04 S KT=AO*BO*(AL-BE)/(AO+BO)*((AL+BO)+(BE*AO)))
02.05 T ! "KT=" KT
02.06 T ! "KA=" ((BO*AL)+(AO*BE))/(AO+BO)
02.08 T ! "HAA=" MA*KK
02.10 T ! "HAB=" MA*KK
02.12 T !!, "ASSUMING KB=0 AND SB=0"

03.02 T ! "MB=" MA*KT*KK/AL*BE
03.07 A !!! "GO AGAIN? (YES/NO)" BA; I (BA-0 YES) 3.09, 1.02, 3.09
03.09 Q
APPENDIX III
THE USE OF STATISTICAL ANALYSIS IN THIS THESIS

Two kinds of statistical test have been used:

1. **Parametric**

   The Student t-test has been used to determine whether two samples come from populations with the same mean. This is based on two assumptions, however, that the two populations are normally distributed and that the two distributions have the same variance (Goldstein 1964), and its use has accordingly been restricted to such situations.

2. **Non-Parametric**

   Non-parametric tests make no assumptions about the nature of the population distributions (Siegel, 1956). The Mann-Whitney U-test has, therefore, been employed in situations where the conditions necessary for a t-test were not fulfilled.

   Variance estimates have been compared by means of the variance ratio, or F-test.

   All regression analyses have been performed by the method of least squares. Correlations are expressed as the Bravais-Pearson coefficient of linear correlation \((r)\) or the Spearman rank correlation coefficient \((r_s)\), as considered appropriate.