SOME DIETARY STUDIES ON THE METABOLISM
OF CHOLESTEROL AND BILE ACIDS

by

Juliet R. Gray, B.Sc., S.R.D.

A thesis submitted in accordance with
the requirements of the University of
Surrey for the degree of Doctor of
Philosophy.

Department of Biochemistry,
University of Surrey,
Guildford, Surrey. December 1978
Acknowledgements

I should like to extend my sincere thanks to my supervisors, Professor D.V. Parke and Dr. D.L. Williams, for their guidance and encouragement during these studies.

I should also like to thank Dr. R.K. Lowing and the staff of the Pathology Laboratory, St. Peters Hospital, Chertsey, who were responsible for the analyses of serum triglyceride concentrations in Chapter 3. I am also most grateful to Mr. M.G. Wright of the British Industrial Biological Research Association in preparation of tissues for histology and to Dr. P. Grasso for examining these preparations.

My thanks are also extended to my fellow students for discussion, particularly Mr. R. Rush who also gave me valuable assistance in the terminal experiment of Chapter 2. I am also grateful to Mr. P. Scobie-Trumper and the staff of the University Animal Unit for their assistance in caring for my animals. Thanks are also due to Mrs. M. Whatley and her secretarial staff and to the technical staff, notably Mrs. K. Cromwell, for continued assistance.

Finally, I am indebted to Mrs. Cleopatra Ioannides for typing this thesis and to the Science Research Council for financial support.
TO MY PARENTS AND
TO TIM
Abstract

Some dietary studies have been carried out on the metabolism of cholesterol and bile acids, in an attempt to elucidate the roles of three nutrients, namely, polyunsaturated fats, ascorbic acid and iron, which are known to influence the incidence of hyperlipidaemia and atherosclerosis. The rate-limiting enzyme of bile acid biosynthesis, cholesterol 7\alpha-hydroxylase, is a member of the mixed function oxidase group of enzymes, in which cytochrome P-450 acts as the terminal oxidase. Thus, the influence of these nutrients on some parameters of mixed function oxidase metabolism was also examined.

The hypercholesterolaemia and atheroma, induced by feeding a diet containing 20% saturated fat to rabbits, was prevented by giving a 20% polyunsaturated fat diet. However, the mechanism of the observed hypocholesterolaemia was shown not to be due to the influence of the unsaturated fat on the activity of cholesterol 7\alpha-hydroxylase.

Ascorbic acid deficiency was shown to impair the metabolism of both cholesterol and foreign compounds in the guinea pig. However, there is apparently a critical threshold level of dietary intake required to permit normal metabolism, which shows an inter-individual and inter-species variability. "Megadose" quantities of ascorbic acid did not appear to adversely affect cholesterol or foreign compound metabolism.

The administration of an iron-deficient diet to rats was shown to influence serum cholesterol concentrations, at least partly by an effect on cholesterol 7\alpha-hydroxylase activity;
however the direction of this effect was not consistent. It is possible that the influence of dietary iron deficiency on cholesterol and foreign compound metabolism may be dependent on the relative depletion of iron stores and/or on an interaction with other trace minerals such as copper.

Finally, a hypothesis has been proposed, which suggests that cytochrome P-450 may play a central role in the pathogenesis of atherosclerosis.
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General Introduction.</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>The Interaction Between the Hepatic Metabolism of Cholesterol and Foreign Compounds in the Rabbit.</td>
<td>37</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>The Effects of Ascorbic Acid Depletion and Supplementation on Cholesterol and Foreign Compound Metabolism in the Guinea Pig.</td>
<td>100</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>The Effects of Dietary Iron Deficiency on Cholesterol and Foreign Compound Metabolism in the Rat.</td>
<td>175</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>General Discussion.</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>219</td>
</tr>
</tbody>
</table>
Chapter 1

General Introduction
Introduction

I Diet, Atherosclerosis and Coronary Heart Disease

Cardiovascular diseases, notably coronary heart disease, are considered to be the major cause of death in the Western world today. Twenty five percent of all deaths in England and Wales (Lancet, 1975) and as many as 50% of deaths in the United States (Gotto et al., 1976) have been attributed to this cause.

Coronary heart disease results from atherosclerosis of the coronary arteries and may present clinically as angina pectoris, myocardial infarction or sudden death. The term atherosclerosis was introduced by Marchand in 1904. It has been defined by WHO (1958) as "a variable combination of changes of the intima of arteries, consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits and associated with medial changes". The arteries involved in human atherosclerosis are the aorta and its immediate large branches, together with the coronary and cerebral arteries; in animals the distribution of lesions is more variable (Roberts and Straus, 1965).

Various classifications of the disease process have been made, based on the morphology of these lesions (WHO, 1958; Haust and More 1972; Strong et al., 1972). It is thus possible to define four to six stages in the development of the atheromatous plaque, each progressing to the next, according to the degree of fatty infiltration and subsequent calcification and necrosis.
Whereas in experimental atherosclerosis, a two-stage classification of fatty streaks and fibrous plaques suffices (Armstrong, 1976), in man, the classification of the early lesion as a fatty streak has been questioned (McGill, 1968; Haust and More, 1972). The fatty streak may be considered as a collection of smooth muscle cells assuming a lipid storage function i.e. "foam cells". Fibrous plaques are a similar collection of smooth muscle cells which synthesise large amounts of collagen, elastin and various interstitial glycoproteins. There would appear to be a gradual transition from one type of lesion to the other, occurring in the first two to three decades of life. It has thus been suggested that, to be effective, any intervention studies should be established prior to this (Hermus, 1975).

Current theories of the pathogenesis of atherosclerosis have largely evolved from the original hypothesis of Virchow (1856), concerning the imbibition of lipid, and of Rokitansky (1852), who considered the initial lesion to result from the incorporation of formed elements from the blood. The former has undergone modification to become the lipid or infiltration hypothesis which suggests that lipids traverse the arterial wall from the plasma and subsequently interact with glycoproteins within the intima, thus promoting intimal proliferation. There is evidence that accumulation of lipid could result from an increased uptake of plasma lipids, their decreased elimination, or both (Portman, 1970; Bondjers and Bjorkerund, 1977; Niehaus et al., 1977).

Both original theories have been united to form a second
important hypothesis, the "response-to-injury hypothesis" i.e. that atherosclerotic lesions are generated as a result of injury to the arterial endothelium (Ross and Glomset, 1976; Ross et al., 1977). The cause of injury may be hyperlipidaemia (Ross and Harker, 1976); other factors such as uraemia, infection or immunological injury (Minick and Murphy 1974); mechanical injury such as the increased shear stress in hypertension or arterial dilatation (Ross and Glomset, 1976; Helin et al., 1971) or a combination of these. Injury to the endothelium may alter both its nature as a barrier to blood constituents and also endothelial cell-cell or cell-connective tissue attachment, such that shear in the blood flow causes focal desquamation of the endothelium. Subsequent to this platelets may adhere and aggregate at the site of injury. Infiltration of platelet factors and other plasma constituents such lipoproteins and hormones may then lead to smooth muscle cell proliferation.

A further theory of atherogenesis is that proposed by Benditt and Benditt (1973). This is known as the "monoclonal hypothesis", which suggests that each lesion results from the proliferation of a single smooth muscle cell. Each lesion is considered to be a benign neoplasm, derived from a cell transformed by a mutagenic stimulus such as a virus, cigarette smoke or other environmental contaminant (Benditt, 1977).

In recent years, it has become apparent that to some extent, regression of these atherosclerotic lesions is possible. The reversal of atherosclerosis, which has been reviewed by Prichard (1974) and Armstrong (1976), has been demonstrated in rabbits, birds, swine and primates (Vesselinovitch et al.,
1974; Clarkson et al., 1973; Doaud et al., 1974; Friedman et al., 1976 and Vesselinovitch et al., 1976). Reports based on autopsy data and on angiographic evaluation of the coronary and peripheral circulation of human subjects indicate regression of atherosclerosis after suitable dietary or drug therapy (Barndt, 1977; Wissler and Vesselinovitch, 1977). This emphasizes the importance of elucidating risk factors associated with the disease, in the hope that treatment at an early stage may enable reversal or retardation of the disease process.

**Risk Factors For Coronary Heart Disease**

The epidemic increase in coronary heart disease amongst young and middle-aged men has led to an immense research effort to elucidate its pathogenesis. As a result, the concept of the disease as being multifactorial in origin and associated with certain risk factors has emerged (Epstein et al., 1965; Truett et al., 1967). The presence of such factors, or more precisely indicators, in an individual or his environment, may be used to predict the likelihood of development of clinical disease.

There has been much discussion in the literature about the relative importance of these risk indicators and their classification. Stamler et al. (1972) used the following classification:

(a) Those involving social environment and life style e.g. diet habitually high in saturated fat, cholesterol and calories; cigarette smoking; sedentary living.

(b) Those involving endogenous biochemical - physiological
regulatory mechanisms, but amenable to exogenous influences e.g. diet, pharmaceuticals, hyperlipoproteinaemia, hypertension, hyperglycaemia, hyperuricaemia and rapid resting heart rate.

(c) Those involving organ pathology e.g. ECG abnormalities, hypothyroidism and renal disease.

(d) Those involving fundamental biology and not generally amenable to exogenous influences e.g. age and sex.

The Framingham study, a prospective longitudinal cohort study, was established in 1948 with the aim of observing the evolution of cardiovascular disease in a general population sample (Kannel et al., 1971). It is from this study that the three primary risk indicators have emerged: elevated serum cholesterol, hypertension and cigarette smoking. Factors of secondary importance are heredity, sex, hypertriglyceridaemia, obesity, diabetes, physical activity, stress and personality type, there is also a considerable interrelationship between these factors (Gotto et al., 1976). However, the relevance of these risk indicators, especially hypercholesterolaemia, to the prophylaxis and treatment of cardiovascular disease is still widely debated.

The Relationship Between Atherosclerosis, Coronary Heart Disease and Lipid Metabolism

There is a well-established relationship between an elevation in plasma lipid, notably plasma cholesterol concentration and the development of atherosclerosis. As discussed by Kaunitz (1977) the number of scientific investigations presenting evidence supporting this association is legion, but the evidence may be
summarized in four main areas.

(i) The atherosclerotic lesion itself has a high cholesterol content. Windaus (1910) reported that human atheromatous aortas contained about six times as much free cholesterol and twenty times as much esterified cholesterol as normal aortas.

(ii) Numerous studies in experimental animals have demonstrated the development of atheroma as a consequence of hypercholesterolaemia induced by cholesterol feeding, although there is considerable inter- and intra-species variation in this response (Clarkson et al., 1976).

(iii) Epidemiological studies have demonstrated a definite statistical relationship between elevated plasma cholesterol concentration and an increased incidence of coronary heart disease (Keys et al., 1963; Dayton et al., 1970; Kannel et al., 1971; Carlson and Bottiger, 1972).

(iv) Patients suffering from familial hypercholesterolaemia have a high risk of developing atherosclerotic complications (Aldersbergh, 1951; Epstein et al., 1959; Fredrickson et al., 1967; Olsson, 1975).

The majority of lipids circulate in the plasma associated with specific proteins in the form of soluble lipoproteins. These complexes contain a core of non-polar lipid (triglycerides and cholesterol esters), a surface layer of more polar lipid (free cholesterol and phospholipids), together with the apoproteins (Morrisett et al., 1975; Jackson et al., 1976). Lipoproteins can be classified according to density, a property determined by the relative ratios of lipid to protein in the molecule. Two schemes of nomenclature which are used inter-
changeably have been derived from their principal methods of separation i.e. ultracentrifugation and electrophoresis on paper or cellulose acetate. In order to understand the role of these lipoproteins in lipid transport as related to atherosclerosis, their structure and function will be briefly described; the subject has been reviewed in detail by Jackson and his colleagues (1976).

The properties of these lipoproteins are summarized in Table 1.1. Their chemical composition has been extensively investigated (Lindgren et al., 1955; Ewing et al., 1965; Levy et al., 1967). Chylomicrons are the largest lipoproteins, composed mainly of exogenous triglyceride in transit from the small intestine to the liver and adipose tissue. Endogenous triglycerides comprise 50%-70% of the very low density lipoproteins together with 10-25% cholesterol. Their role is to transport this triglyceride from the liver and to some extent the intestine to other tissues such as adipose tissue and muscle. During their breakdown, there is an interchange of apoprotein with other lipoproteins. The remnant particles, known as intermediate density lipoproteins, deliver the bulk of their cholesterol to the liver and are subsequently degraded into low density lipoproteins. These are largely composed of cholesterol (40-45%) and are thus the major vehicle for cholesterol transport in plasma. High density lipoproteins comprise 50% protein, 35% phospholipid and only 15-20% cholesterol. Nevertheless, it has become apparent that these particles play an important role in transport of cholesterol to the liver for subsequent catabolism and excretion, as they
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Chylomicrons</th>
<th>Very Low Density</th>
<th>Low Density</th>
<th>High Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic nomenclature</td>
<td>Chylomicrons</td>
<td>Pre- $\beta$</td>
<td>$\beta$</td>
<td>$\lambda$</td>
</tr>
<tr>
<td>Ultracentrifugal Characteristics</td>
<td>1.006</td>
<td>0.94 - 1.006</td>
<td>1.006 - 1.063</td>
<td>1.063 - 1.250</td>
</tr>
<tr>
<td>Density Range g/ml</td>
<td>400 - 15,000</td>
<td>20 - 400</td>
<td>0 - 20</td>
<td>0 - 3, sediment</td>
</tr>
<tr>
<td>Lipid/Protein Ratio</td>
<td>Exogenous</td>
<td>Endogenous</td>
<td>Endogenous</td>
<td>Endogenous</td>
</tr>
<tr>
<td>Origin</td>
<td>After Meals</td>
<td>Usual</td>
<td>Usual</td>
<td>Usual</td>
</tr>
<tr>
<td>Presence in Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $S_F$ denotes the negative sedimentation coefficient in Svedberg Units at density 1.006 - 1.063 g/ml in NaCl solution at 26°C.
+ Adapted from Skipski (1972).
interact with lecithin: cholesterol acyl transferase in plasma, taking up cholesterol in the ester form (Glomset, 1968).

Early studies linking circulatory lipid to coronary heart disease, emphasised the importance of elevation of the low and very low density lipoprotein fractions. Thus, Fredrickson et al. (1967) in their classification of the hyperlipoproteinaemias noted that in both Type II (elevated low density) and Type IV (elevated very low density) there was an increased incidence of coronary heart disease. Similarly, an increased concentration of low density lipoproteins and their subsequent filtration to the arterial intima have been implicated as pathogenetic in the disease, possibly as a result of low density lipoprotein receptor deficiency (Brown and Goldstein, 1974; Brown and Goldstein, 1976).

In recent years however, the emphasis has changed to the association of high density lipoprotein with the incidence of coronary heart disease, as the two are apparently inversely correlated. This observation was made several years ago (Barr et al., 1951; Nikkila et al., 1953; Jencks et al., 1956; Brunner et al., 1962) but was not considered important until 1975, when Miller and Miller proposed that a reduction in high density lipoprotein was likely to accelerate the development of atherosclerosis by impairing the clearance of cholesterol from the arterial wall. Thus, a decrease in high density lipoprotein levels may be considered as an additional and independent risk factor in coronary heart disease (Castelli et al., 1977, Cooperative Lipoprotein Phenotyping Study; Gordon et al., 1977, Framingham Study). Similarly, the findings
of the Tromsø Heart Study indicate that high density lipoprotein concentrations may be a better predictor of coronary heart disease than serum cholesterol itself (Miller et al., 1977).

Diet in Relation to Hyperlipidaemia and Atherosclerosis

The observed association between atherosclerosis and serum lipids has made them the focus of attempts to limit the disease. Results from numerous animal experiments, epidemiological investigations and studies of populations subjected to changes in their habitual diets, have emphasised the role of diet as a major determinant of serum lipid concentration. The majority of these investigations have been concerned with the influence of dietary lipid. Thus, the "lipid theory" of coronary heart disease, whereby a reduction in serum cholesterol concentration in an individual by modification of dietary fat intake is thought to diminish the risk of a coronary event, has become widely accepted (Ahrens, 1976). The importance of dietary lipid will be further considered in Chapter 2. However, may it suffice to say that on the basis of existing experimental and epidemiological data, despite considerable scepticism and uncertainty on the part of some of the leading investigators (Ahrens, 1976; Mann, 1977), recommendations have been made both in this country and the United States for an overall modification in diet, particularly with respect to dietary lipid (D.H.S.S. 1974; Royal College of Physicians, London, 1976; Select Committee on Nutrition and Human Needs, U.S. Senate, 1977).

In addition to lipid, certain other dietary components such as carbohydrate, protein and fibre are known to influence
serum lipid concentration. Deficiencies of some vitamins, notably ascorbic acid, and minerals may affect the degree of lipidaemia; their importance will be discussed in Chapters 3 and 4 respectively.

Thus, as observed by Kritchevsky (1976), whilst the change in type and quantity of dietary fat has been implicated in the epidemic increase in cardiovascular disease, our diet has changed overall. He summarizes the data of Friend (1967), Rizek et al., (1974) and Gortner (1975), which demonstrate trends in nutrient intake in the United States between 1909 and 1974. From this he concludes that:

(a) Although protein availability has not changed, the ratio of animal to vegetable protein has doubled.
(b) Carbohydrate availability has fallen by 21%.
(c) Dietary fibre intake has fallen by 30%.
(d) Fat availability has increased by 20%, although the polyunsaturated: saturated fatty acid ratio has risen from 0.21 to 0.43.
(e) Cholesterol intakes have risen only slightly from 509 to 540mg per day.

These trends have also been observed in the U.K. and other Western countries and correlate with an increased death rate (Masironi, 1970).

Relationship Between Dietary Protein Intake and Hyperlipidaemia

Yudkin (1957) and Yerushalmy and Hilleboe (1957) demonstrated a strong positive correlation between the intake of animal protein and the incidence of heart disease. Vegetarians
are known to have lower plasma lipid levels than the average population, although this could also result from difference in fibre, vitamin or mineral intake (Sacks et al., 1975). Young women consuming a diet containing 50g of vegetable protein were shown to have lower serum cholesterol concentrations than those eating an equivalent amount of animal protein (Walker et al., 1960). Similarly, Hodges et al., (1967) substituted soya protein for mixed protein in the diet of human volunteers, with a consonant decrease in mean serum cholesterol level from 300mg to 200mg/100ml. More recently the substitution of soya for animal protein in the diets of normolipaemic (Carroll et al., 1978) and hyperlipidaemic (Sirtori et al., 1977) individuals, has been shown to lower serum cholesterol.

Many early feeding studies in animals, particularly the rabbit, provided evidence for a hypercholesterolaemic effect of casein and beef protein (reviewed by Kritchevsky, 1976 and Carroll, 1978). Other workers (Lofland et al., 1961; Clarkson et al., 1962; Middleton et al., 1967) varied the quantity of both cholesterol and protein in diets fed to pigeons or squirrel monkeys and found that the high protein diets were most atherogenic. Howard et al. (1965) observed that the hypercholesterolaemia and atherosclerosis induced in rabbits by feeding a cholesterol-free, high fat, purified diet could be reduced by substituting soya flour or soya bean meal for casein. These results have been confirmed by other workers (Carroll and Hamilton, 1975; Hermus, 1975; Hamilton and Carroll, 1976). In general, proteins derived from animal sources gave a hypercholesterolaemic response, although pork and albumin were
exceptions to this (Carroll and Hamilton, 1975). It has also been shown in rabbits that the serum cholesterol concentration may be influenced by the amino acid content of the diet (Huff et al., 1977).

**Relationship Between Dietary Carbohydrate Intake and Hyperlipidaemia**

As a result of finding correlations between coronary heart disease mortality and sucrose intake, Yudkin has proposed that sucrose consumption is of prime importance in the aetiology of coronary heart disease (Yudkin, 1957; Yudkin, 1963; Yudkin and Roddy, 1964; Yudkin, 1966). He has postulated that sucrose may act by influencing serum lipid levels, platelet adhesiveness or hormonal balance (Yudkin, 1972). In general, sucrose intake does not appear to affect serum cholesterol levels, although it does elevate serum triglycerides (Anderson et al., 1963; Denbesten et al., 1973). Yudkin's hypothesis has been criticised (Grande, 1967; St Hamler et al., 1972) and there is apparently considerable interaction between the influences of sucrose and saturated fat (Macdonald, 1967; Macdonald, 1972). There is a definite variation in response to sucrose feeding in experimental animals and man. Yudkin has thus suggested that it may only be atherogenic in some individuals (Worcester et al., 1975).

**Relationship Between Dietary Fibre Intake and Hyperlipidaemia**

Recently attention has been focussed on the role of complex carbohydrates and non-nutritive fibre in coronary heart disease.
Many experiments, carried out in rabbits fed semipurified diets, showed some interaction between the fibre and protein components of these diets with respect to hypercholesterolaemia (Kritchevsky et al., 1977b). It has been proposed (Trowell, 1972; Burkitt et al., 1974) that a deficiency of dietary fibre may be a causative factor in coronary heart disease.

Human studies designed to test this hypothesis give conflicting results. Some workers have observed an hypocholesterolaemic effect on feeding rolled oats (deGroot et al., 1963), bengal gram (Mathur et al., 1968) or lignin (Thiffault et al., 1975), whilst others using wheat bran, have been unsuccessful (reviewed by Truswell and Kay, 1976), Jenkins et al. (1975) showed that dietary pectin and guar gum significantly reduced serum cholesterol concentrations in healthy volunteers whilst wheat fibre was without effect. The hypocholesterolaemic action of pectin has also been demonstrated by Judd et al. (1977). Thus, in humans the response would seem to depend on the nature of the dietary fibre.

Similar conclusions can be drawn from experimental studies in animals. In the rat, both pectin and lignin were hypocholesterolaemic (Judd et al., 1975; Chang and Johnson, 1976), whereas wheat fibre had a minimal effect (Kay and Truswell, 1974). In the rabbit, wheat straw and alfalfa were more hypocholesterolaemic than pure cellulose (Story et al., 1976). These experiments will be discussed in greater detail in Chapter 2.

II Cholesterol Metabolism and its Control

The pathways of cholesterol metabolism which influence
tissue and plasma cholesterol concentration are summarized in Fig 1.1.

Methods used to investigate these pathways have been whole body sterol balance studies and analyses of plasma cholesterol kinetics (Sabine, 1977). Data derived from these studies has facilitated the understanding of the three major aspects of cholesterol metabolism i.e. absorption, synthesis and catabolism and their interrelationships.

**Cholesterol Absorption**

Dietary cholesterol mixes in the small intestinal lumen with cholesterol derived from the bile and desquamated mucosal cells and with sterol newly synthesised in the intestine itself (Dietchsky and Wilson, 1970b), although a more recent study in rabbits (Rudel et al., 1972) showed that within the mucosal cell, absorbed and newly synthesised cholesterol remained separated. Cholesterol is incompletely absorbed and transported in the splenic circulation to the liver via the thoracic duct as very low density lipoprotein and chylomicrons (Borgstrom, 1969; Quintao et al., 1971; Kudchodkar et al., 1973).

Absorbed cholesterol is used in the synthesis of plasma lipoproteins (8-12 mmol/day) and bile acids (1 mmol/day) or is re-excreted in the bile (2.5 mmol/day) (Angelin, 1977). The percentage of cholesterol absorbed is dependent on various factors: species, age and sex (Lutton and Chevallier, 1972), the nature of dietary fatty acids and rate of bile flow (Dietchsky and Wilson, 1970b). The size of the bile acid pool also influences cholesterol absorption. Decreased bile acid
Fig 1.1 Summary of Cholesterol Metabolism (Lewis, 1976)

Cholesterol is derived from dietary cholesterol absorption (1), reabsorption of endogenous cholesterol (2), and from synthesis in small intestine (3), liver (4) and extrahepatic tissues (5). Cholesterol enters plasma from the small intestine (6), the liver (7), (8) and peripheral tissues (9). Uptake of cholesterol from plasma occurs peripherally (10) and by the liver (11), (12). Hepatic cholesterol is derived by processes (4) (synthesis), (6) (absorption) and (11) (uptake from plasma); the liver disposes of cholesterol by lipoprotein synthesis (7), (8), by biliary excretion of cholesterol (13) and by synthesis and secretion of bile acids (14). Bile acids are largely reabsorbed and recycle in an enterohepatic circulation (15), ultimately being excreted in the faeces (16). Neutral sterol excreted in the faeces (17) is derived from biliary excretion (13), unabsorbed dietary cholesterol (18) and from desquamated intestinal mucosal cells (19).
reabsorption, induced by cholestyramine treatment or bile duct ligation, inhibits cholesterol absorption, whilst expansion of the bile acid pool by the administration of taurocholate enhances it (Siperstein et al., 1952; Lutton et al., 1973).

Some investigators (Kaplan et al., 1963; Wilson and Lindsay, 1965) consider that in man there is a maximum rate of absorption of approximately 500mg per 24 hours. Conversely, Borgstrom (1969) considers the rate to be unlimited, although the percentage absorbed is reduced at higher intakes. This was confirmed by Grundy and Ahrens (1969). In some individuals, the effects of increased absorption are compensated for by two mechanisms: enhanced excretion of faecal neutral sterols and suppression of endogenous cholesterol synthesis (Quintao et al., 1971).

In contrast to other species, such as the rat, dog or squirrel monkey, there is no increase in cholesterol degradation to bile acids in man.

Cholesterol Synthesis

Most tissues are capable of cholesterogenesis, although the liver and intestine predominate (Dietchsky and Wilson, 1970a) and together with skin are considered to contribute in excess of 90% of endogenous cholesterol (Lewis, 1976). Cholesterol is synthesised from acetate via a series of steps, of which the formation of mevalonate from β-hydroxy β-methylglutaric CoA (HMGCoA), catalysed by the microsomal enzyme HMGCoA reductase, is rate-limiting (Siperstein and Fagan, 1966). This enzyme is subject to a negative feedback control; thus hepatic synthesis is inhibited by cholesterol itself. In rat liver, exogenous
cholesterol (derived from chylomicrons) is more effective than endogenous cholesterol in this respect (Weis and Dietchsky, 1969). Intestinal cholesterogenesis is apparently largely regulated by bile acids (Dietchsky, 1968), although McIntyre and Isselbacher (1973) have shown that intestinal cholesterol may also play some part. In some tissues, cholesterol synthesis may be depressed due to feedback inhibition by low density lipoproteins, mediated by receptors on the cell surface (Balasubramaniam et al., 1976). It has been proposed that a major function of these receptors is to permit the transport of low density lipoprotein into the cell, where they suppress the activity of HMGCoA reductase and thereby inhibit cholesterol synthesis (Brown et al., 1975).

**Cholesterol Excretion**

Under steady-state conditions, the cholesterol eliminated in the faeces and urine should equal that derived from synthesis and absorption. A small quantity of cholesterol is excreted through the skin (Bhattacharyya et al., 1972). In man, the principal route of excretion is via the faeces, which contain unabsorbed cholesterol from the diet, biliary excretion and desquamated epithelial cells (Dietchsky and Wilson, 1970c; Lewis, 1976). In the rat, faecal excretion of cholesterol itself is relatively low compared with that of bile acids (Lutton, 1976). In the large bowel, cholesterol undergoes bacterial metabolism, largely through ring saturation, to yield coprostanol and cholestanol (Snog-Kjaer et al., 1956; Rosenfeld et al., 1967).

The second major route of cholesterol excretion is via its'
catabolism to bile acids. Bloch et al. (1943) reported the conversion of isotopically labelled cholesterol to bile acids. More than 80% of cholesterol is excreted in this form in the rat (Bergstrom, 1952; Siperstein et al., 1952) and in man (Siperstein and Morray, 1955); however, Lewis and Myant (1967) state that bile acids account for only 28-59% of the net sterol loss in man.

The transformation of cholesterol to bile acids occurs via a series of oxidative steps, involving the introduction of hydroxyl groups into the steroid nucleus and shortening of the side chain (Dietchsky and Wilson, 1970c). This is summarized in Fig 1.2. The initial reaction is the introduction of a hydroxyl group in an α-configuration at the 7-position of cholesterol, to form cholest-5-ene-3β,7α-diol. In subsequent steps, the 3β-hydroxy group is inverted to the 3α-position via a 3-keto intermediate, the Δ5 double bond is saturated and the orientation of the A and B rings is shifted from a trans- to a cis-relation i.e. from a 5α-H to a 5β-H sterol.

In the pathway leading to cholic acid, 12α-hydroxylation also occurs. Subsequent steps involve the loss of the three terminal carbon atoms from the side chain and oxidation of the C24 carbon to a carboxylic acid group. This process is achieved by ω-oxidation to the corresponding 26-oic acid, followed by β-oxidation and cleavage of the three-carbon unit in the form of propionyl-CoA (Suld et al., 1962). In this manner, 5β-cholestone-3α, 7α, 12α-triol and 5β-cholestone-3α, 7α-diol are converted to choly-CoA and chenodeoxycholy-CoA respectively. These compounds may then be conjugated through a peptide linkage to
Fig. 1.2  Biosynthetic Pathways of Primary Bile Acids

Cholesterol

7α-Hydroxycholesterol

Cholest-4-ene 3-one-7α-ol

Cholic acid

Cholest-4-ene 3-one-7α,12α-diol
either glycine or taurine and secreted into the bile as taurocholic, glycocholic, taurochenodeoxycholic or glycochenodeoxycholic acids, which are known as primary bile acids.

Within the intestine, further chemical transformations may be effected by bacteria. Deconjugation to the free bile acids and \( \alpha \)-dehydroxylation to form deoxycholic from cholic acid or lithocholic from chenodeoxycholic acid may occur (Norman and Sjovall, 1958). These are known as secondary bile acids.

Bile acids are efficiently reabsorbed by active transport from the distal ileum, although lithocholic acid is mostly lost in the faeces. Unconjugated bile acids which are reabsorbed are conjugated with taurine or glycine prior to re-excretion in the bile, which thus contains a mixture of conjugated primary and secondary bile acids. There is considerable species variation in the pattern of bile acids present in bile (Haslewood, 1967), but in man, cholic, chenodeoxycholic and deoxycholic acids predominate in the proportions 1.1:1.0:0.9 (Einarsson et al., 1974). The bile salt pool, which is largely in the biliary tract, liver, small intestine and portal circulation, also varies in size (Sabine, 1977) but in man is of the order of 3g (Lewis, 1976). It is known to recycle several times each day (Hofman, 1967; Shefer et al., 1969), but it is apparent that different bile acids may have different rates of enterohepatic circulation (Sabine, 1977). Approximately 10% of the pool is lost each day.

In man and other species bile acid synthesis is under complex control. The major rate-limiting event is the initial step in the biosynthetic pathway i.e. \( \alpha \)-hydroxylation and is
apparently under negative feedback control from the intestinal bile acid pool (Shefer et al., 1970). Evidence supporting this emanates from studies in cholestyramine-treated or bile-fistula rats, where the activity of cholesterol 7α-hydroxylase was induced (Shefer et al., 1968; Johansson, 1970), whilst that of other enzymes in the pathway was unaffected. However, Johansson (1970) has suggested that the 12α-hydroxylase enzyme may have a secondary regulatory function. Further studies (Shefer et al., 1970; Mosbach et al., 1971) have shown that the incorporation of 14C from cholesterol into bile acids was inhibited in the presence of the circulating bile acid pool, but that incorporation of 14C from cholest-5-ene 3α, 7α-diol (the product of 7α-hydroxylation) was unaltered in the presence or absence of the bile acid pool. The characteristics of the 7α-hydroxylase enzyme system and the factors influencing its activity will be discussed in the next section.

It has thus been assumed that alterations in total body cholesterol will be reflected by corresponding changes in the concentration of plasma cholesterol. Consequently an obvious area of research has been the relationship between bile acid turnover and hypercholesterolaemia. As a result of the known association between diet and hyperlipidaemia, relationships between diet and bile acid metabolism have been sought, these will be considered in greater detail in the relevant experimental chapters.

III Role of Cholesterol 7α-Hydroxylase and Its Relationship to Foreign Compound Metabolism

Cholesterol 7α-hydroxylase (EC 1.14) belongs to the class
of enzymes known as mixed function oxidases. This term was introduced by Mason (1957) and is specific for enzyme-catalysed hydroxylations in which one atom of a molecule of oxygen is introduced into the substrate, the other being reduced to water by a reduced coenzyme. Two electrons are consumed for each molecule of oxygen. The reaction may be represented thus:

\[ \text{RH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{ROH} + \text{NADP}^+ + \text{H}_2\text{O} \]

where RH is the substrate.

These enzymes, which are located in the hepatic endoplasmic reticulum are membrane-bound and are responsible for the metabolism of a wide range of exogenous substrates such as drugs, carcinogens and other foreign compounds, together with endogenous substrates such as steroids and fatty acids (Conney, 1967; Kuntzman, 1969; Gillette et al., 1972). Originally, this microsomal enzyme system was thought to consist of at least two functional protein components: a haemoprotein called cytochrome P-450 and a flavoprotein known as NADPH-cytochrome c reductase or NADPH-cytochrome P-450 reductase.

Cytochrome P-450 acts as the substrate- and oxygen-binding site of the system, whilst the reductase acts as an electron carrier, transferring electrons from NADPH to cytochrome P-450. Lu and Coon (1968) solubilised the system using detergent, and as a result identified 3 components: cytochrome P-450, NADPH-cytochrome c reductase and a lipid, shown to be phosphatidylcholine. More recently, both protein components have been purified (Imai and Sato, 1974; Levin et al., 1974; Van der Hoeven and Coon, 1974; Ryan et al., 1975). Using these preparations,
an absolute dependence on the 3 components for the metabolism of foreign compounds has been demonstrated (Levin et al., 1974; Lu and Levin, 1974; Van der Hoeven and Coon, 1974).

A proposed scheme for the series of reactions occurring during hydroxylation is shown in Fig 1.3. The sequence of events has been extensively investigated by studying changes in both optical and EPR spectra of the cytochrome during the reaction (reviewed by Estabrook et al., 1975). The mechanisms of subsequent reactions concerned with the transfer of the second electron and the introduction of the activated oxygen atom into the substrate are still unclear. The nature of the acceptor for the second electron remains undetermined, although Hildebrandt and Estabrook (1971) have postulated that cytochrome b5 may be important in this reaction. However, if this were so, it is difficult to understand why this cytochrome is not an essential component of the solubilised hydroxylating system.

**Cytochrome P-450 and Its Specificity**

Cytochrome P-450 is considered to be the most essential component of the enzyme system, as a consequence of its oxygen-activating role. The designation "P-450" to the cytochrome is derived from the observation that when complexed with carbon monoxide, which it binds avidly (Omura and Sato, 1962) it shows a maximal absorption in the Soret region at 450nm.

An induction of mixed function oxidase activity, concomitant with an induction of cytochrome P-450, has been observed following administration of, or accidental exposure to, various chemical agents (Conney, 1967; Remmer, 1972). The classical inducers
Fig. 1.3 A Schematic Diagram of Cytochrome P-450 Mediated Hydroxylations

From Fry and Bridges, 1977
of cytochrome P-450-catalysed reactions are phenobarbitone and 20-methylcholanthrene, the cytochromes induced by these agents being known as P-450 and P-448 respectively (Comai and Gaylor, 1973). Phenobarbitone induces the metabolism of a wide range of substrates, whereas methylcholanthrene is more specific in its induction (Conney, 1967).

Studies carried out over the last decade suggest that cytochrome P-450 is heterogeneous and that both between different animals and within each species there exist multiple forms of the cytochrome, specific for various substrates (Lu et al., 1972; Comai and Gaylor, 1973; Ryan et al., 1975; Thomas et al., 1976). Variation in the proportions of P-450 types in a particular microsomal preparation, could explain the species, strain, sex and age differences observed in microsomal metabolism (Lu, 1977). Likewise, it might be anticipated that just as pretreatment with a particular drug may affect the relative proportions of P-450 present, variations in the nutritional composition of the diet may also have some effect.

**Characteristics of Cholesterol 7α-Hydroxylase**

Cholesterol 7α-hydroxylase has been shown to require NADPH and molecular oxygen (Shefer et al., 1968; Mitton et al., 1971; Mitropoulos and Balasubramaniam, 1972). The reaction can be inhibited by carbon monoxide (Boyd et al., 1969; Wada et al., 1969) and the inhibition can be reversed by monochromatic light at 450nm (Boyd et al., 1971; Boyd et al., 1973). Bjorkhem et al. (1974), using partially purified preparations of cytochrome P-450, were able to restore the cholesterol 7α-hydroxylase
activity of inactive subfractions of liver microsomes. These findings suggest that cytochrome P-450 acts as the terminal oxidase in the \( \alpha \)-hydroxylase system.

Investigation of the factors controlling the activity of this enzyme have yielded equivocal results. As stated earlier, the rate of hydroxylation of foreign compounds by hepatic microsomes is largely determined by cytochrome P-450 concentration. For example, the rate of oxidative demethylation of ethylmorphine parallels changes in P-450 concentration induced by various experimental modifications (Balasubramaniam and Mitropoulos, 1975); similar findings were reported for aminopyrine (Ernster and Orrhenius, 1965). However, changes in the activity of cholesterol \( \alpha \)-hydroxylase do not consistently correlate with concentrations of either the cytochrome or its reductase. Thus, whereas \( \alpha \)-hydroxylase activity is induced in cholestyramine-treated or bile-fistula rats, cytochrome P-450 and ethylmorphine activities are unaffected (Balasubramaniam and Mitropoulos, 1975). Pretreatment of rats with phenobarbitone, whilst markedly inducing cytochrome P-450 concentration and ethylmorphine demethylation, has variable effects on cholesterol \( \alpha \)-hydroxylase. In general, its activity is unaltered, but certainly in the rat there is a notable strain variation (Einarsson and Johansson, 1968; Shefer et al., 1972; Balasubramaniam and Mitropoulos, 1975). Recently, Mellon and his colleagues (1978) have presented data which confirms this earlier work and also shows a lack of correlation of \( \alpha \)-hydroxylase activity with that of cytochrome c reductase or \( b_5 \).

These findings have led to speculation as to the mechanism
of induction of cholesterol \( \alpha \)-hydroxylase, bearing in mind this apparent dissociation from the components of the enzyme system. The existence of multiple forms of cytochrome P-450 may offer some explanation for these observations. It may be possible that there is a form of the cytochrome specific for the \( \alpha \)-hydroxylation of cholesterol (Wada et al., 1969; Atkin et al., 1972). If this specific form comprised only a small part of the bulk of cytochrome P-450, no observable change in total microsomal P-450 would be expected. Alternatively, Brown and Boyd (1974) have postulated that the in vivo regulatory mechanisms of cytochrome P-450 and cholesterol \( \alpha \)-hydroxylase are different. They propose the existence of an additional component to the enzyme system, such as a carrier protein specific for cholesterol, the concentration of which would control the amount of cholesterol accessible to the oxygen-activating site on the cytochrome.

In order to clarify these mechanisms, solubilisation of the microsomal P-450 required for \( \alpha \)-hydroxylation, as carried out for the drug hydroxylation reactions (Lu and Coon, 1968), has been attempted (Bjorkhem et al., 1974; Hattersley and Boyd, 1975). However, as there is considerable loss of activity of the enzyme during contact with the concentrated detergents used in the purification procedure, this approach has been unsuccessful.

A further area of uncertainty, of prime importance in establishing a suitable assay system, is that of the origin of intracellular cholesterol acting as preferential substrate for the enzyme in vivo. On incubating \([^{14}C]\) cholesterol with
hepatic microsomes, under conditions suitable for 7α-hydroxylation, the specific activity of the 7α-hydroxycholesterol generated is lower than that of the added cholesterol. This indicates that a proportion of endogenous cholesterol equilibrates with the added substrate and is thus accessible to the enzyme (Mitropoulos and Balasubramaniam, 1972; Balasubramaniam et al., 1973). These workers have proposed the term "substrate pool" for this fraction of microsomal cholesterol and have attempted to estimate its size, assuming immediate equilibration of the pool with added radioactive cholesterol. However, the form in which the cholesterol is added to the incubation system has been shown to influence the accessibility of the substrate to the enzyme (Balasubramaniam et al., 1973; Bjorkhem and Danielsson, 1975). The concept of "substrate pool" has consequently been criticised (Bjorkhem and Danielsson, 1975). Concern as to the interpretation of experiments in which cholesterol 7α-hydroxylation was determined by the addition of exogenous cholesterol, led to the development of an assay for 7α-hydroxylation of endogenous cholesterol using a mass fragmentographic technique (Bjorkhem and Danielsson, 1974). However, comparison of both assays in this and subsequent studies (Bjorkhem and Danielsson, 1975; Bjorkhem and Kallner, 1976; Bjorkhem et al., 1978) have shown them to give similar results.

The origin of the microsomal cholesterol per se used preferentially by the enzyme has been questioned. By the isolation of the liver microsomal fraction from rats administered intravenous [14C]cholesterol immediately prior to killing, it was shown that the specific activity of the products formed
in vitro was lower than that of the total microsomal cholesterol (Balasubramaniam et al., 1973). They concluded that plasma cholesterol does not rapidly equilibrate with the pool from which \( \Delta^\alpha \)-hydroxycholesterol is synthesised and consequently that this pool is derived from cholesterol synthesis \textit{de novo} in the liver. Evidence supporting this has been provided by Bjorkhem and Danielsson (1975), who showed that the specific activity of \( \Delta^\alpha \)-hydroxycholesterol formed from \( [5-^{3}\text{H}] \) mevalonic acid incubated with rat liver microsomes \textit{in vitro} was greater than that of the total microsomal cholesterol.

**Factors Affecting Cholesterol \( \Delta^\alpha \)-Hydroxylase Activity**

In addition to the regulatory action of the enterohepatic circulation of bile acids on cholesterol \( \Delta^\alpha \)-hydroxylase, factors known to influence this enzyme can be divided into hormonal and dietary.

The thyroid hormones are known to modify bile acid metabolism. Triiodothyronine has been shown to increase the rate of synthesis of total bile acids (Strand, 1963). In agreement with this, Wada \textit{et al.} (1969) and Balasubramaniam \textit{et al.} (1975) have shown a marked induction of cholesterol \( \Delta^\alpha \)-hydroxylase activity after administration of thyroxine; thyroidectomy decreases the activity (Balasubramaniam \textit{et al.}, 1975; Mayer, 1975). However, the work of Story \textit{et al.} (1974) does not confirm this. It has been suggested that thyroid status may also influence other enzymes in the synthetic pathway (Mitropoulos \textit{et al.}, 1968; Bjorkhem \textit{et al.}, 1973).

Cholesterol \( \Delta^\alpha \)-hydroxylase activity shows a pronounced
diurnal variation, with a maximum during the dark period (Gielen et al., 1969; Danielsson, 1972; Mayer, 1976). Thus it has been suggested that experiments investigating the effect of environmental changes on the enzyme should take this into account (Mayer, 1976). However, Bjorkhem et al. (1978) showed that the effects of modified fat diets on \( \Delta^\alpha \)-hydroxylation were the same, irrespective of the lighting conditions. The exact mechanism of the diurnal variation is uncertain. From studies in which the rhythm is maintained under conditions of both fasting and feeding (reviewed by Myant and Mitropoulos, 1977), it is apparent that there is an inherent periodicity in cholesterol \( \Delta^\alpha \)-hydroxylase activity which may be secondarily influenced by external factors such as diet or alteration in light cycles. This contention is supported by the findings of Van Cantfort (1973; 1974), who showed that in rats kept in continued darkness and in genetically blind mice, the diurnal rhythm is maintained. Plasma corticosterone concentration, which itself displays a pronounced diurnal response, is known to affect cholesterol \( \Delta^\alpha \)-hydroxylase activity. Gielen and coworkers (1975) reported abolition of the diurnal rhythm after hypophysectomy or adrenalectomy. This was confirmed by Balasubramaniam et al. (1975). Administration of glucocorticoids to rats increased the activity of cholesterol \( \Delta^\alpha \)-hydroxylase, an effect which could be counteracted by the injection of Actinomycin D (Gielen et al., 1976). These studies implicate a regulatory function for corticosterone in \( \Delta^\alpha \)-hydroxylase activity which may be at the level of RNA synthesis. Additional evidence was provided by Van Cantfort (1973) who showed that the circadian rhythm in
enzyme activity was not established until the 20th day after birth, which corresponds exactly with the appearance of a diurnal rhythm in adrenocorticotropic hormone secretion and plasma corticosterone concentration (Myant and Mitropoulos, 1977).

With respect to the influence of diet on cholesterol 7α-hydroxylase, investigations to date have been limited, largely focussing on the effect of cholesterol feeding on its activity. This will be discussed in Chapter 2. However, it is likely that the nutritional status of the animal would influence this enzyme as the activities of the microsomal mixed function oxidase enzymes and their components are markedly influenced by alterations in diet.

Influence of Nutrition on Mixed Function Oxidase Activity

The influence of nutritional status on the metabolism of foreign compounds by the microsomal mixed function oxidase enzymes is a subject which has been frequently reviewed (Campbell and Hayes, 1974; Campbell and Hayes, 1976; Becking, 1976; Wade and Norred, 1976; Zannoni and Sato, 1976; Campbell, 1977; Conney et al., 1977; Kato, 1977). Variations in the quantity or quality of the major nutrients protein, carbohydrate or fat and of minerals or vitamins may influence foreign compound metabolism. The effects of dietary lipid, vitamins and minerals will be considered in Chapters 2, 3 and 4, but the role of carbohydrate, protein and the total energy content of the diet will briefly be discussed here.

Manipulation of dietary carbohydrate results in alterations in mixed function oxidase activity, although it is thought
that the influence of carbohydrate is not specific to the enzyme system itself but acts by modifying intermediary metabolism (Campbell and Hayes, 1974). Rats fed sucrose or a mixture of glucose and fructose were shown to display decreased biphenyl 4-hydroxylase activities associated with decreased levels of cytochrome P-450 (Dickerson et al., 1971). Similarly, Boyd et al. (1970) reported an increased toxicity of benzylpenicillin as a result of its decreased metabolism in rats fed high sucrose diets. Likewise, increased consumption of various monosaccharides prolonged the barbiturate-induced sleeping time of mice (Strother et al., 1971).

The effects of protein deficiency on mixed function oxidase activity have been thoroughly investigated; a reduction in either its quantity or quality results in diminished metabolism. Drill (1952) first reported the increased toxicity of drugs under conditions of protein deficiency. The in vitro oxidative microsomal metabolism of various substrates such as phenobarbital, strychnine, aminopyrine, zoxazolamine, aniline and ethylmorphine is reduced by protein deficiency (Kato et al., 1968; Mgbodile and Campbell, 1972), as is their capacity for induction (Mgbodile et al., 1973; Campbell, 1977). It is also evident that reduced quality of the protein influences metabolism. Rats fed different types of protein exhibited varying susceptibility to the lethal effects of heptachlor (Webb and Miranda, 1973). In agreement with this, in vivo and in vitro drug metabolism, cytochrome P-450 and microsomal protein concentrations were low in rats fed gluten compared to those fed casein as the protein source (Miranda and Webb, 1973).
The consequences of alteration in dietary carbohydrate and protein have also been studied in man (Kappas et al., 1976; Conney et al., 1977). These workers determined the antipyrine and theophylline half-lives in volunteers fed a "normal" home diet, a high protein/low carbohydrate diet and a high carbohydrate/low protein diet. Whereas the high protein diet resulted in a pronounced diminution in plasma half-life of both drugs, the high carbohydrate regime tended to increase them, thus giving support to the animal experiments.

Finally, it should be noted that foreign compound metabolism is measurably, although variably, influenced by starvation. Dixon et al. (1960) reported a decrease in both in vivo and in vitro hepatic drug metabolism in starved mice. Conversely, Kato et al. (1962) demonstrated a slight increase in meprobamate metabolism in fasted female rats. Later, Kato and Gillette (1965) concluded that there was a marked sex difference in response to starvation. Sex-dependent enzymes such as hexobarbitone and pentobarbitone hydroxylase and aminopyrine N-demethylase were impaired, whilst p-nitroanisole O-demethylation and zoxazolamine hydroxylation, which are less sex-dependent, were little affected. This sex difference was also observed by Gram et al. (1970); males tended to be more responsive to fasting.

Aim of Present Study

In view of the established association between dietary composition, hyperlipidaemia and bile acid metabolism, the present study was designed to investigate this association, emphasising the rate-limiting enzyme in bile acid biosynthesis,
cholesterol \( \alpha \)-hydroxylase. In this way, it was hoped to elucidate the role of diet in the pathogenesis of hypercholesterolaemia and atherosclerosis. As the enzyme is known to belong to the mixed function oxidase system, which is also responsible for foreign compound metabolism, studies on drug metabolism were carried out in parallel.
Chapter 2

The Interaction Between Dietary Lipid and the Hepatic Metabolism of Cholesterol and Foreign Compounds in the Rabbit
Introduction

A relationship between the lipid content of the diet and atherosclerosis in man, was noted as early as the 1st world war, when Aschoff in Germany observed a decline in frequency of severe atherosclerotic lesions at autopsy, which he attributed to a decrease in total fat intake in the population (Grande, 1974). Similarly, Malmros (1950) noted a reduction in mortality from atherosclerosis during the 2nd world war, a period when rationing enforced decreased intakes of dietary fat. Groen et al. (1952) observed that, in man, diets rich in animal fats increased serum concentrations of cholesterol compared with vegetarian diets. Numerous experiments have confirmed these findings; this earlier literature has been reviewed by Jollife (1961) and Page et al. (1957).

Much data is derived from epidemiological studies (reviewed by Stamler, 1967), notably those of Keys and his colleagues. In an early study (Bronte-Stewart et al., 1955), showed that a Capetown population consuming foods rich in fat had higher serum cholesterol levels than a Bantu tribe consuming less fat. A subsequent more extensive study, in which 18 population groups in seven countries were compared, provided a strong positive linear correlation ($r = 0.89$) between intake of saturated fat and serum cholesterol concentration (Keys et al., 1970). The International Atherosclerosis Project (Scrimshaw and Guzman, 1968) showed that the rank order of serum cholesterol concentrations was similar to that of the percentage energy derived from total fat.

Evidence in favour of a hypercholesterolaemic role for saturated fat has also been provided by the study of geographical differences in dietary fat intake and by the observation of
migrant populations. The serum cholesterol levels of primitive populations are substantially lower than those of Western industrialised societies. In these groups, a significant relationship between dietary fat, serum lipid levels and coronary heart disease is apparent (Mendez et al. 1962; Truswell 1968; Sinnett and Whyte, 1973). In individuals migrating from primitive areas associated with a low incidence of coronary heart disease to urbanized areas with a higher incidence, there is a consistent relationship between alteration in dietary fat consumption and serum lipid levels. Keys et al. (1958) noted an apparent gradient for saturated fat, blood lipids and coronary event rate, increasing from indigenous Japanese men to migrant Japanese in Hawaii and Los Angeles to Caucasians. Those observations have been confirmed in similar populations (Kato et al., 1973; Worth et al., 1975; Robertson et al., 1977).

Serum lipids are influenced by total fat intake, by dietary cholesterol and by the nature of dietary fatty acids. Attention has largely been focussed on the degree of unsaturation of these fatty acids, as will be considered in further detail below, but it should also be noted that their chain length (Hashim et al., 1960; Grande et al., 1970) and their stereoisomeric nature (Vergroesen, 1972) are important.

Beveridge et al., (1955), using liquid formula diets in human volunteers, demonstrated considerable reductions in serum cholesterol concentration by feeding a corn oil/margarine mixture which supplied either 28% or 59% of the total energy. Supplementation of the diet with equivalent amounts of butter resulted in significant serum cholesterol elevation. It is thus generally
accepted that saturated and polyunsaturated fats have opposite effects on serum cholesterol levels and several equations have been derived to predict this response when altering the proportions of these two types of fat in the diet. Keys et al. (1957; 1965) employed the equation: cholesterol = 2.74ΔS - 1.31ΔP and Hegsted et al. (1965): cholesterol = 2.16ΔS - 1.65ΔP + 6.66C - 0.53. In these equations, S and P represent changes in the percentage energy derived from saturated and polyunsaturated fatty acids respectively; C is the dietary cholesterol in mg%. From these equations, it can be concluded that on a weight basis, saturated fats have a hypercholesterolaemic effect which is twice as great as the influence of polyunsaturated fats in the opposite direction.

Experimental and epidemiological data in support of the hypocholesterolaemic action of polyunsaturated fat is continually being published. Thus, Sacks et al. (1975) reported that vegetarians displayed lower serum cholesterol concentrations than a control group. The consumption of polyunsaturated meats by a volunteer group of young adults was also shown to be associated with a reduction in serum lipids (Hodges et al., 1975). However, these workers qualify their results by noting that substitution of these polyunsaturated products is only effective in "responsive persons", i.e. those with initially higher serum lipid levels. Similar results were reported by Stein et al. (1975), Brown et al. (1976) and Salel et al. (1977). Further recent evidence emanates from the study of Hill and Wynder (1976), who achieved a reduction in serum cholesterol levels in young, healthy volunteers by increasing
the polyunsaturated to saturated fat ratio of the diet from 0.3 to 1.2.

The considered importance of dietary cholesterol per se as an aetiological agent in hyperlipidaemia has tend to wax and wane, thus the original contention (Engelberg and Newman, 1943) that cholesterol intake was a major determinant of serum levels, was modified when the more obvious effects of total dietary fat were recognised, together with the inter-individual variation in response to cholesterol intake (Keys et al., 1956). Nevertheless, Mattson et al. (1972) reported that in subjects ingesting a diet containing 317 mg of cholesterol per 1000 Kcal, serum cholesterol concentrations increased by 25% over those measured on a cholesterol-free diet. However there would appear to be a threshold level of intake (500-600 mg per day) beyond which additional dietary cholesterol is without effect (Beveridge et al., 1960; Connor et al., 1961). This has recently been confirmed by Slater et al., (1976), who showed that feeding of extra eggs (2 per day to young men, 1 per day to older men), in addition to a normal diet, resulted in no significant increase in serum cholesterol.

Experimental studies in animals also tend to support the relationship between dietary lipid intake and serum concentrations. Much of the work has been performed in rabbits, following the initial studies of Anitschkow and Chalatow (1913) who produced lesions resembling those seen in human atherosclerosis by administering diets high in cholesterol. Although these diet-induced lesions have been reproduced by other workers, the use of such an experimental model has been extensively criticised as not being
analogous to the human condition and representing a storage phenomenon rather than the true degenerative change of atherosclerosis (Roberts and Straus, 1965; Wissler and Vesselinovitch, 1968).

However, by feeding semi-synthetic diets, high in saturated fat but without additional cholesterol, to the rabbit, it is possible to induce hypercholesterolaemia and atherosis (Lambert et al., 1958; Wigand, 1959). Using this model, the substitution of linoleic-rich polyunsaturated oils in the diet can be shown to reduce serum cholesterol concentrations and protect against atherosclerosis (Malmros and Wigand, 1959; Moore and Williams, 1964). The severity of these lesions has been shown to be inversely proportional to the iodine value of the ingested fat (Kritchevsky, 1970).

Mechanism of Action of Fat-Modified Diets

The mode of action of these diets, particularly regarding the hypocholesterolaemic effect of polyunsaturated fats, remains controversial. Possible mechanisms have been reviewed by Grundy and Ahrens (1976). These fats could act via a) a reduction in cholesterol absorption, b) a decrease in endogenous cholesterol synthesis, c) an increase in faecal excretion of cholesterol as neutral sterols or bile acids, or by a combination of these. Spritz and Mishkel (1969) have suggested that the effect of polyunsaturated fat might be to modify lipoprotein structure and there is some evidence to support this concept. Detailed consideration of this postulated mechanism is however considered to be beyond the scope of this thesis and has been recently
reviewed by Jackson et al. (1978).

Dietary Lipid and Cholesterol Absorption

Data derived from experiments on cholesterol absorption is equivocal. In the rat (Byers and Friedman, 1958) and in man (Pinter et al., 1964) polyunsaturated fat has been shown to increase cholesterol absorption. Conversely, Wood et al. (1966) reported a decreased absorption of this sterol in men fed polyunsaturated fats. Other workers have not shown any change in the rat (McGovern and Quackenbush, 1973), in normal human subjects (Nestel et al., 1974) or in patients suffering from hyperlipidaemia (Grundy, 1975).

Dietary Lipid and Cholesterol Synthesis

Investigations into cholesterol synthesis in man and experimental animals fed the two types of fat have been equally controversial, with reports of an increase (Wood and Migicovsky, 1958), a decrease (Wiech et al., 1967) and no change (Ahrens, 1957; Grundy and Ahrens, 1970; Nestel et al., 1974).

Dietary Lipid and Cholesterol Excretion

Human Studies

In man, faecal neutral sterol excretion has been measured under conditions of an increased intake of polyunsaturated fat. An increased excretion was reported by several workers (Wood et al., 1967; Moore et al., 1968); others have not observed this increase, despite a reduction in serum cholesterol concentration
Many investigations have included measurements of bile acid excretion and perhaps the most widely studied hypothesis is that polyunsaturated fats may enhance the conversion of cholesterol to bile acids. Abnormalities in bile acid excretion have been observed in patients suffering from primary disorders of lipid metabolism. Using isotopic techniques, Miettinen et al. (1967) observed low faecal bile acid excretion in patients with familial hypercholesterolaemia. However, the relationship is complex as demonstrated by the work of Hellstrom and Lindstedt (1966) and Kottke (1969), who found that whilst bile acid synthesis was subnormal in patients suffering from primary hypercholesterolaemia, it was abnormally high in those with combined hypercholesterolaemia and hypertriglyceridaemia. Similarly, Einarsson and Hellstrom (1972) reported differences in bile acid turnover in patients with different types of hyperlipoproteinaemia; lower turnovers were observed in Type II (hypercholesterolaemia) compared with Type IV (hypertriglyceridaemia) subjects. Similar differences were observed by Sodhi and Kudchadkar (1973), who also noted that faecal excretion of bile acids was lower in Type II than Type IV subjects.

Several investigators have suggested that the inclusion of mainly unsaturated fats in the diet results in an increased excretion of bile acids (Gordon et al., 1957; Haust and Beveridge, 1958; Goldsmith et al., 1960; Antonis and Bersohn, 1962). However, others have reported diminished (Ali et al., 1966) or unchanged excretion (Avignon and Steinberg, 1965). Nevertheless,
some of these experiments were carried out on small groups with varying degrees of initial cholesterolaemia, which as mentioned above may itself influence bile acid metabolism. Moore et al. (1968) studied normal subjects given a polyunsaturated fat diet (40% safflower oil as compared to 40% butterfat) and found an increase in both faecal bile acid and neutral sterol excretion. However, differences in cholesterol content of the two diets limits the interpretation of this study. Connor et al. (1969) performed similar experiments, using cholesterol-free diets and also observed a significant increase in bile acid excretion in response to polyunsaturated fat feeding. More recently, Nestel et al. (1973; 1974) have shown that whilst faecal steroid excretion, including bile acid excretion, increases initially after feeding polyunsaturated fats, once a new steady-state condition is reached, steroid excretion is similar to that observed with saturated fat in the diet.

In a study involving the effects of polyunsaturated fat diets on bile acid excretion in hyperlipidaemic subjects (Grundy, 1975), results showed considerable variation, depending on the type of lipidaemia, in accordance with the findings of Einarsson and Hellstrom (1972) and Sodhi and Kudchodkar (1973).

Animal Studies

Investigations of the interaction between dietary lipid and bile acid metabolism in animals have generally be designed to examine the effects of cholesterol feeding. In general, it can be concluded that in animals such as the rat, who show little hypercholesterolaemia in response to cholesterol feeding,
there is a pronounced increase in bile acid excretion (Wilson, 1962; Wilson, 1964; Uchida et al., 1977). Conversely, in species displaying hypercholesterolaemia, bile acid excretion is virtually unchanged (Hellstrom 1965; Beher et al., 1970). The effects of administration of diets rich in corn oil to rats has also been examined (Beher et al., 1970; Kim et al., 1976), no change in bile acid turnover was reported.

In view of the role of the enzyme cholesterol \( \alpha \)-hydroxylase in the control of bile acid synthesis, it would seem logical to examine the effect of variation in the dietary lipid component at this site. The influence of cholesterol feeding on the activity of this enzyme has been studied. An increase in activity was observed by some workers (Boyd et al., 1969; Mitropoulos et al., 1973; Raicht et al., 1975), whereas others have failed to detect any significant change (Bjorkhem and Danielsson, 1975). To date, the influence of the degree of saturation of the fat on the activity of this enzyme has not been examined. Such an effect might be anticipated in view of the known relationship between essential fatty acids and mixed function oxidase metabolism.

**Effects of Dietary Lipid on Mixed Function Oxidase Metabolism**

The mixed function oxidase enzymes are associated with the lipoprotein membranes of the hepatic endoplasmic reticulum; 30-55% of the dry weight of these membranes is lipid, comprised largely of phospholipids (Siekevitz 1963; Glaumann and Dallner, 1968), which are of prime importance to the integrity of the enzyme system. Chaplin and Mannering (1970) observed a decrease
in ethylmorphine and hexobarbital metabolism together with a depression of Type I binding following treatment of microsomes with phospholipase C. Likewise, extraction of microsomal phospholipid with iso-octane also resulted in a depression of type I binding (Leibman and Estabrook, 1971), indicating that this form of binding is dependent on phospholipid. An absolute requirement for phosphatidylcholine in a reconstituted enzyme system was demonstrated by Coon and his associates (Lu and Coon, 1968; Strobel et al., 1970). In addition to this specific function, lipids are also essential for the maintenance of the integrity of the membrane itself, as indicated by the observation that the phenobarbitone-induced increase in microsomal phospholipid, triglyceride and cholesterol is of a similar order to that of the protein component (Glaumann and Dallner, 1968).

The effects of dietary lipid on mixed function oxidase metabolism have been recently reviewed (Wade and Norred, 1976; Kato, 1977). The majority of investigations have examined the changes associated with manipulation of polyunsaturated fatty acids, particularly the essential fatty acid content of the diet. Experiments in which saturated fat was fed to rats have yielded equivocal results. Caster et al. (1970b) fed saturated fats at the level of 15-35% of total energy and observed an increase in aniline hydroxylation but no change in hexobarbital metabolism. Conversely, Hietanen et al. (1975) reported a decrease in aryl hydrocarbon hydroxylase activity but little effect on the O-demethylation of p-nitroanisole in rats fed 34% cocoa butter. In accordance with this, Agradi et al. (1975) demonstrated a decrease in benzo[a]pyrene hydroxylase and cytochrome P-450.
concentration in male rats fed a diet containing 10% saturated fat but observed no change in aniline metabolism.

In general, supplementation of fat-free diets with polyunsaturated fatty acids results in an increase in mixed function oxidase activity. Caster et al. (1968) observed a loss of drug metabolising ability in linoleate-deficient rats, which could be reversed by the addition of 3% linoleic acid to the diet. Similarly, rats fed diets deficient in unsaturated fatty acids show a diminished cytochrome P-450 content, (Marshall and McLean, 1971; Kaschnitz, 1970; Norred and Wade, 1972). The activity of NADPH-cytochrome c reductase is largely unaffected by essential fatty acid deficiency (Wade et al., 1972; Norred and Wade, 1976).

The effects of essential fatty acid deficiency on the kinetics of some mixed function oxidase reactions were examined by Norred and Wade (1972). The apparent Vmax's for metabolism of aniline, hexobarbital and ethylmorphine were increased by supplementation of a fat-free diet with corn oil. However, the apparent Km's for the reactions were unaffected, with the exception of aniline and hexobarbital, which, although not influenced by 3% corn oil were elevated by a diet containing 10% corn oil. This has led Wade and Norred (1976) to conclude that increases in mixed function oxidase metabolism induced by dietary lipid are largely a function of increased enzyme concentration rather than any qualitative alteration in the enzyme system.

Data derived from in vivo experiments, notably those measuring hexobarbital sleeping times, are conflicting. Most
workers (Caster et al., 1970a; Kaschnitz, 1970; Century, 1973) have found this parameter to be slightly increased or unchanged. However, more recent experiments (Wade and Norred, 1976) showed a reduction in sleeping time in both rats and mice fed diets supplemented with corn oil.

The effect of dietary lipid on induction of mixed function oxidase enzymes has also been investigated. Microsomal preparations derived from rats maintained on diets deficient in essential fatty acids were shown to have a reduced capacity for induction by phenobarbital (Century and Horwitt, 1968; Marshall and McLean, 1971). In order to permit maximal induction of cytochrome P-450 synthesis in response to phenobarbitone administration, Marshall and McLean (1969; 1971) showed that the addition of herring oil, linoleic acid, 0.1% oxidised cholesterol or sitosterol was necessary. Supplementation with coconut or olive oil was without effect. They thus postulated a "permissive effect" for linoleic acid which they consider might be a function of the degree of the unsaturation of the fat or of its susceptibility to peroxidation.

From this review of the literature, it can be concluded that adequate amounts of polyunsaturated fats are required to maintain microsomal metabolism. Deprivation may result in quantitative and some qualitative changes in the components of the enzyme system.

Selection of an Animal Model for Dietary Experiments

A major problem in the investigation of the interaction between dietary and serum lipid levels and atherosclerosis is
the selection of a suitable animal model. The subject has been frequently reviewed (Roberts and Straus, 1965; Kritchevsky, 1974; Clarkson et al., 1976). As mentioned earlier, the New Zealand white rabbit has been used extensively in these studies, as it is susceptible to the dietary induction of hyperlipidaemia and atheroma.

Other species such as the rat and dog are generally more resistant to the development of atheromatous lesions (Roberts and Straus, 1965; Kritchevsky, 1974). Thus although it is possible to engender atherosclerosis in these species, mechanical, chemical or immunological injury to the arterial tissue is usually needed as an adjunct to the atherogenic diet (Gutstein et al., 1963; Bajwa et al., 1971; Testa et al., 1975). The validity of results emanating from these experiments is doubtful as the conditions are aphysiological. The diet-induced changes in the rabbit would suggest this species as a better model although it is vegetarian and the constitution and distribution of the induced atheromatous plaques are dissimilar to those seen in man.

In recent years, the trend in atherosclerosis research has been towards the use of non-human primates such as the monkey or baboon. These animals have the advantage of being omnivorous and to exhibit a progression and distribution of lesions similar to man. However, there is a considerable inter-species variation in response to diet and some workers have found it necessary to use additional methods to induce atheroma (Corey et al., 1974).

Scott et al. (1972) outlined the properties which an ideal
animal should possess. They suggested that it should be possible to rapidly induce atheroma in the animal, without recourse to expensive or extremely unphysiological means; the animal should be of sufficient size to provide tissue for multiple studies; it should be easily available, inexpensive and easy to house and feed; the induced lesions should resemble those seen in man. Thus, whilst overall the monkey would seem a suitable model, the expense and housing difficulties encountered with this species are considerable and in other respects, the rabbit satisfies many of the above-stated criteria.

**Aim of Present Experiment**

Despite the acknowledged influence of dietary lipid on bile acid metabolism, there is little available information regarding its effect on cholesterol 7α-hydroxylase activity. It could be postulated that alterations in the concentration or conformation of this enzyme might affect the fate of cholesterol and consequently its availability for tissue deposition. As a corollary to this, factors such as diet, which are known to modify cholesterol and lipid metabolism, might be expected to influence the nature of the enzyme. Thus, considering the association between lipid and mixed function oxidase metabolism generally, an experiment was designed using the New Zealand white rabbit as the experimental model. The effect of feeding semi-synthetic diets containing either saturated or polyunsaturated fats on certain parameters of cholesterol and bile acid metabolism were examined. Additionally, in an attempt to elucidate the heterogeneity of cytochrome P-450 with respect
to its involvement in the cholesterol \( \Delta^7 \)-hydroxylase system, levels of cytochromes and some typical mixed function oxidase enzymes were measured.
Materials

Specific chemicals were supplied as follows:

Enzymes and Cofactors

Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), its reduced form NADPH, glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (Type XII) were purchased from Sigma Chemical Company, London. 3β-hydroxysteroid dehydrogenase (0.5 units/mg, from Pseudomonas testeroni) was obtained from P-L Biochemicals Inc. (International Enzymes Ltd., Windsor, Berks.).

Radiochemicals

\[ ^{14}C \text{Cholesterol} \text{ (50mCi/mmol)} \] was supplied by the Radiochemical Centre, Amersham, Bucks.

Bile Acids

Sodium glycocholate was purchased from Maybridge Research Chemicals, Tintagel, N. Cornwall.

Substrates

Biphenyl was obtained from B.D.H. Ltd., Poole, Dorset and was recrystallised from absolute ethanol. Ethylmorphine hydrochloride was purchased from May and Baker Ltd., Dagenham, Essex and cytochrome c (Type VI) from Sigma.

Other Chemicals

2- and 4-hydroxybiphenyls, formaldehyde and bovine serum
albumin were obtained from B.D.H. Ltd. XAD-7 resin (Rohm and Haas Ltd., Croydon, Surrey) was kindly donated by Dr. S. Barnes (Medical Unit, The Royal Free Hospital, London). Most other chemicals were supplied by B.D.H. Ltd., Fisons (Fisons Scientific Apparatus, Loughborough, Leics.), or as specified in the text.

Gases were supplied by B.O.C. Ltd., Crawley, Sussex and solvents were obtained from Fisons. Water was glass-distilled and stored in polypropylene containers.

Methods

I Serum Analyses

(a) Serum Total Cholesterol

Serum total cholesterol concentrations were measured using a reagent kit (Biochemica Test Combination No. 15969, Boehringer Mannheim GmbH, Mannheim, Germany), based on the method of Watson (1960). This is an adaptation of the Liebermann-Burchard colour reaction, involving the reaction of cholesterol in acetic anhydride with concentrated sulphuric acid and in the presence of dimethyl benzene sulphonic acid to form a coloured complex, the absorbance of which is measured at 578 nm using a Cecil model CE 272 U.V. spectrophotometer (Cecil Instruments Ltd., Cambridge).

(b) Serum Triglycerides

Serum triglyceride concentrations were measured using reagent kit (Biochemica Test Combination No. 15395, Boehringer
Mannheim GmbH). This uses the method of Wahlefeld (1974), involving the following series of reactions:

\[
\text{Triglyceride} \xrightarrow{\text{Esterase}} \text{Glycerol} + \text{Fatty acid.}
\]

\[
\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-1-phosphate} + \text{ADP}.
\]

\[
\text{ADP} + \text{PEP} \xrightarrow{\text{Phosphokinase}} \text{Pyruvate} + \text{ATP}.
\]

\[
\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}.
\]

The NAD generated is measured at 340 nm using the S.P. 1800 spectrophotometer.

(c) Serum Total Bile Acids

A modification (Barnes and Chitranukroh, 1977) of the assay system of Murphy et al. (1970) was utilised. Bile acids are isolated from serum by a liquid-solid extraction and measured by an enzymic-fluorimetric method. The enzyme used is 3-hydroxy steroid dehydrogenase, derived from Pseudomonas testeroni, which was originally used for the quantitative assay of androgenous steroids. It is also applicable to bile acids hydroxylated in the 3-position. The reaction may be presented as:

\[
\text{Steroid alcohol} + \text{NAD}^+ \xrightarrow{\text{3-hydroxy steroid dehydrogenase}} \text{Steroid ketone} + \text{NADH} + \text{H}^+.
\]

The reaction is encouraged to proceed to the right by the
addition of a ketone-trapping agent semicarbazide hydrochloride and the NAD is determined fluorimetrically.

**Extraction Procedure**

The resin, XAD-7 is a non-ionic acrylic polymer in the form of 350-500μm beads.

It was soaked overnight in 5 volumes of distilled water to remove the sodium chloride and sodium carbonate added as a preservative. The fine particles were removed by repeated swirling and decanting, so that the remaining beads sedimented in 20-30 seconds. The resin was then extracted by stirring for 2-3 hours, firstly with methanol, followed by chloroform: methanol (1:1 v/v) and finally with methanol. Thorough washing with water was then required until the chloroform odour disappeared. This resin was stored under water until required.

After use the resin was soaked in aqueous methanol (1:1 v/v) and regenerated by repeating the solvent extraction procedure.

XAD-7 resin (1g) was placed in a glass 10 ml sovirel tube fitted with a screw cap (V.A. Howe and Co, Ltd., London, SW6). A 1ml aliquot of each test serum was added to separate tubes, followed by 0.1M sodium hydroxide, to give a final volume of 9ml. An extraction blank was set up using 1g of XAD-7 and 9ml of sodium hydroxide only.

The tubes were mixed using a Rollermix (Luckham Ltd., Burgess Hill, Sussex) at room temperature (20°C) for 60 minutes. After mixing, the supernatant was removed by aspiration on a water pump. In order to maintain a pH of 10 at all stages
throughout the washing procedure, a 2ml volume of dilute sodium hydroxide (0.004M) was added, each tube was mixed briefly and the supernatant discarded. A 6ml volume of methanol was added to the resin and the contents of the tubes were mixed for a further 20 minutes on the Rollermix. The methanolic supernatant was transferred to a 25ml "pear-shaped" flask, the resin extracted into a second 6ml of methanol and the supernatants combined. The methanol was removed in vacuo using a rotary evaporator at 50°C.

**Enzymic Assay**

The following incubation system was set up:

<table>
<thead>
<tr>
<th>NAD</th>
<th>Reagent A</th>
<th>Extract</th>
<th>Extract</th>
<th>Serum</th>
<th>Serum</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>Blank^1</td>
<td>Blank^2</td>
<td>Blank</td>
<td>Test</td>
<td></td>
</tr>
</tbody>
</table>

| Reagent A (ml*) | 2.0 | - | 2.0 | - | 2.0 | - |
| Reagent B (ml**) | - | 2.0 | - | 2.0 | - | 2.0 |
| 50% aqueous | 0.05 | 0.05 | - | - | - | - |
| methanol (ml) | - | - | - | - | - | - |
| Serum Extract (ml) | - | - | - | 0.05 | 0.05 | - |
| Blank Extract (ml) | - | - | 0.05 | 0.05 | - | - |
| Standard (ml) | - | - | - | - | - | 0.05 |

*Reagent A*/100ml

- 94.5ml sodium pyrophosphate buffer 0.1M pH 10.2
- 5.0ml methanol (A.R.)
- 4mg semicarbazide hydrochloride
- 6mg βNAD
** Reagent B /100ml

As reagent A plus 0.5mg (0.25iu) \(3\beta\)-hydroxysteroid dehydrogenase.

These reagent mixtures were prepared immediately prior to use. Standards were prepared by diluting a stock solution of sodium glycocholate in 50% aqueous methanol (1mg/ml) to cover a range of concentration from 10.25 to 205\(\mu\) mol/litre (5-100\(\mu\)g/ml). Tubes were set up in duplicate as above and mixed on a "Whirlimixer." Incubation was carried out at 37\(^\circ\)C, in an atmosphere of air, in a shaking incubator (Mickle Laboratory Engineering Co., Gomshall, Surrey) with a shaking rate of about 80 oscillations per minute for 45 minutes.

The reaction was terminated by plunging the tubes into an ice-water slurry (0\(^\circ\)C). The tubes were subsequently maintained at this temperature whilst the fluorescence intensity was measured. The fluorescence of the solutions was read using a Perkin Elmer Spectrofluorimeter Model MPF3, with a xenon lamp, 2 monochromators and quartz cuvettes of 1cm light path, excitation wavelength 365nm, emission wavelength 457nm.

II Liver Analyses

(a) Preparation of Liver Fractions

Rabbits were killed as described in section V between 08.00 and 09.00 hours, the livers rapidly excised and the gallbladders detached. The livers were immediately placed in ice-cold buffer (modified Bucher medium: 10.8g \(\text{KH}_2\text{PO}_4\) ; 3.9g KOH ; 1.0g \(\text{MgCl}_2\) \(6\text{H}_2\text{O}\) ; 0.372g \(\text{EDTA}\) per litre). Any adhering fat or connective tissue was removed from the liver which was blotted dry and
weighed. After 'scissor-mincing', a rough homogenate was prepared in 1 volume of ice-cold buffer using a Silverson mixer-emulsifier (Silverson Machines Ltd., Waterside, Chesham, Bucks.). An aliquot of this was taken and a further homogenate prepared in 2 volumes of ice-cold buffer using 4 return strokes of a Size C Potter-Elvejhem type, teflon-glass homogeniser (A.H. Thomas and Co., Philadelphia, U.S.A.) power driven at 2,950 r.p.m. The homogeniser was rinsed with a further volume of ice-cold buffer and the resultant 20% homogenate centrifuged in polypropylene tubes at 20,000 g av. for 15 minutes at 2°C (M.S.E. "Highspeed 18", 8 x 50ml angle rotor at 12,900 r.p.m.). The supernatant, subsequently referred to as the microsomal supernatant and comprising the microsomal and soluble cell fractions was used for the determination of several enzyme activities.

Further centrifugation at 105,000 g av. for 60 minutes at 2°C (M.S.E. "Superspeed 50", 8 x 25ml angle rotor at 40,000 r.p.m.) yielded a clear supernatant which was discarded and a microsomal pellet which, after rinsing to remove residual supernatant, was resuspended with 2 return strokes in a size B homogeniser in buffer to give a 25% suspension.

All procedures were performed as rapidly as possible and at a temperature of 0-4°C. All fractions were maintained at 0°C prior to use.

(b) Measurement of Microsomal Protein

The method of Lowry et al. (1951) was used; 0.2ml aliquots of microsomal suspension were diluted to 10ml with 0.5M NaOH,
from which further 0.5ml aliquots were taken and mixed with 5ml of freshly prepared Lowry reagent (2% Na₂CO₃, 1% CuSO₄, 2% Na K tartrate, 100 : 1 : 1 by volume). After at least 10 minutes, 0.5ml of Folin-Ciocalteau Phenol reagent (diluted 1:1 with water) was added and immediately vortex mixed. After allowing 30 minutes for development, the blue colour was measured at 720nm in the Cecil spectrophotometer. All determinations were carried out in duplicate and blanks (0.5M NaOH) and standards (25-100 g bovine serum albumin in 0.5M NaOH) were treated identically.

(c) Measurement of Total Lipid

Extraction

Lipid was extracted by a modification of the method of Bligh and Dyer (1959).

Duplicate 2ml aliquots of the "rough homogenate" were homogenised with 38.0ml chloroform: methanol (2:1, v/v). The resultant homogenate was filtered through glass wool. A 3.2ml volume of methanol followed by 5.3ml of water was added to 10ml of the clear filtrate in a universal container and mixed by inversion. The mixture was then centrifuged in a bench centrifuge at approximately 2,000 r.p.m. for 5 minutes. The upper aqueous layer was removed by aspiration at the water pump.

Estimation of Total Lipid

A 5ml aliquot of the chloroform layer was taken into a weighed 25ml conical flask. The extract was evaporated in a
stream of nitrogen and the flask desiccated overnight over silica gel, under vacuum. The flask was then reweighed to give the weight of lipid by difference.

(d) **Measurement of Total Liver Cholesterol**

The lipid residue was taken up in 3ml of absolute ethanol. Duplicate 100μl aliquots were transferred to test tubes and evaporated to dryness in a water bath at 50°C, in a stream of nitrogen. The cholesterol content of the extract was determined using the same method as for serum.

(e) **In Vitro Enzyme Assays: General Considerations**

1. All incubation systems were prepared in duplicate on ice.
2. Incubations were carried out in an atmosphere of air in a shaking incubator, with a shaking rate of about 80 oscillations per minute.
3. An M.S.E. "Mistral 6L" centrifuge with a 6 x 1L swing-out rotor was used for all centrifugations.
4. For assays based on colorimetric measurement of the reaction product there was a linear relationship between optical density and amount of product formed over a concentration range greater than the extremes of variability encountered.

(f) **Ethylmorphine N-Demethylase**

N-demethylation is a typical reaction of the microsomal mixed function oxidase system. The formaldehyde produced is measured by the method of Nash (1953). The incubation
system, (Holtzman et al., 1968), was set up as follows:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (ml)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Semicarbazide solution (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cofactor solution (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Substrate (ml)</td>
<td>0.2</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>Microsomal supernatant (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Standard (ml)</td>
<td></td>
<td></td>
<td>0.1*</td>
</tr>
</tbody>
</table>

* denotes added after the incubation.

Incubation volume = 1.8 ml.

Buffer: Tris-HCl, 0.3M pH 7.4

Semicarbazide solution: 2% (w/v) aqueous solution, adjusted to pH 7.0 with NaOH.

Cofactor solution: Each 0.2 ml of buffer contained NADP 2μmol, glucose-6-phosphate 20μmol and magnesium as MgCl₂, 10μmol.

Substrate: Ethylmorphine hydrochloride, 75mM aqueous solution.

Standard: Aqueous formaldehyde 5μmol/ml, standardised using an extinction coefficient of 8 mM⁻¹ cm⁻¹ for the HCHO-Nash reagent complex.

Incubation was for 10 minutes at 37°C, the reaction was terminated by the addition of 1 ml 15% (w/v) zinc sulphate, after placing the tubes on ice. After addition of substrate and standard to the appropriate tubes, 1 ml of saturated barium hydroxide was added to each tube and the contents mixed. All tubes were then centrifuged at 2,000 r.p.m. for 15 minutes at 4°C. A 2 ml aliquot
of clear supernatant was transferred into clean tubes to which a 2ml volume of freshly prepared Nash reagent (0.4%(v/v) acetylacepone in 4M ammonium acetate) was added. After heating at 37°C for 40 minutes with shaking, the resultant yellow colour was measured at 412nm using the Cecil spectrophotometer.

(g) Biphenyl 4- and 2-Hydroxylase.

Biphenyl undergoes hydroxylation at a number of positions, the major metabolite being 4-hydroxybiphenyl. Small quantities of 2-hydroxybiphenyl are generated and both isomers can be measured simultaneously, since at pH 5.5, they exhibit different fluorescence spectra. The following incubation system (Creaven et al., 1965) was used:

<table>
<thead>
<tr>
<th></th>
<th>Test Blank</th>
<th>20H Standard</th>
<th>40H Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cofactor solution in</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer (ml)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>1.15% KCl (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Substrate (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Microsomal supernatant (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2-hydroxybiphenyl standard (ml)</td>
<td>-</td>
<td>-</td>
<td>0.5*</td>
</tr>
<tr>
<td>4-hydroxybiphenyl standard (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* denotes added after incubation.

Incubation volume = 2.5ml.

Buffer: Tris-HCl, 0.05M pH 8.1
Cofactor solution: each 1.0ml contained NADP 1µmol, glucose-6-phosphate 15µmol, MgCl₂ 10µmol.

Substrate: Biphenyl 12mM in 1.15% KCl containing 2.5%(w/v) Tween 80 (polyoxyethylene sorbitan monoleate).

Standards: 2-hydroxybiphenyl 12µg/ml in 10%(v/v) aqueous ethanol.
4-hydroxybiphenyl 30µg/ml in 10%(v/v) aqueous ethanol.

All tubes were incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 0.5ml of 4M HCl after placing the tubes on ice, substrate and standard being added to the appropriate tubes. After the addition of 7ml of n-heptane, all tubes were extracted for 15 minutes on a rotary shaker. Any emulsion formed was broken by centrifugation at 2,000 r.p.m. for 15 minutes. A 2ml aliquot of each heptane layer was transferred to clean tubes, 5ml of 0.1M NaOH added and the tubes extracted for a further 15 minutes on the rotary shaker. After centrifugation at 2,000 r.p.m. for 15 minutes, the heptane layer was aspirated off at the water pump. Hydroxybiphenyls in the NaOH phase were stable in the dark at 4°C for at least 24 hours.

A 2ml aliquot of the NaOH phase was pipetted into a fluorimeter cuvette and the pH brought to 5.5 by the addition of 0.5ml of 0.25M succinic acid. 2- and 4-hydroxybiphenyls were determined using the Perkin Elmer spectrofluorimeter at 290nm (excitation) and 410nm (emission) and 276nm (excitation) and 332nm (emission) respectively.

Under the conditions employed, 4-hydroxybiphenyl fluoresces slightly at the wavelength maxima of 2-hydroxybiphenyl. It is thus necessary to read the 4-hydroxybiphenyl standard and all test samples and blanks at both wavelength maxima and apply
a correction factor for subtraction from the 2-hydroxy readings of all test samples and blanks as follows:

\[
\frac{4\text{-OH biphenyl reading} \times \text{conc. 4\text{-OH biphenyl standard}}}{4\text{-OH biphenyl standard reading}} \times \frac{\text{conc. 4\text{-OH biphenyl standard}}}{\text{conc. 4\text{-OH biphenyl standard}}}
\]

(h) \text{NADPH-Cytochrome c Reductase}

The absorption of reduced cytochrome c was followed at 550nm, (Gigon et al., 1969).

Two 1cm glass cuvettes were set up as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl 0.02M in 1.15% KCl, pH 7.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Potassium cyanide 3mM</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytochrome c 0.15mM</td>
<td>1.0</td>
</tr>
<tr>
<td>Microsomal suspension, 25% in Tris/KCl as above</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The cuvettes were placed in the forward cell compartment of the SP 1800 spectrophotometer. After zeroing the recorder, 0.2ml Tris/KCl was added to the reference cuvette. After allowing 2 minutes for equilibration to ambient temperature, 0.2ml of NADPH solution (1.5mM) was added to the test cuvette. The increase in absorption at 550nm was followed and from the initial, linear phase of the reaction, cytochrome c reductase activity was calculated using an extinction coefficient for reduced cytochrome c of 27.7mM$^{-1}$cm$^{-1}$ (Mahler and Cordes, 1966).
(i) **Cytochrome b\(_5\)**

A modification of the method of Omura and Sato (1964) was used. A 1ml aliquot of microsomal suspension (as in (h) above) and 2ml of Tris/KCl were pipetted into each of two lcm glass cuvettes. A small amount (approximately 1-2mg) of sodium dithionite was added to the test cuvette and the reduced minus oxidised difference spectrum recorded between 390 and 500 nm using the SP 1800 spectrophotometer. Cytochrome b\(_5\) content was calculated using an extinction coefficient of \(171 \text{mM}^{-1}\text{cm}^{-1}\) for the extinction difference between 409 and 426nm.

(j) **Cytochrome P-450: Carbon Monoxide Ligand**

In its reduced form, the cytochrome P-450-CO complex absorbs strongly at 450nm. Measurement of this spectral maximum enables quantification of the cytochrome (Omura and Sato, 1964).

Test and reference cuvettes were set up as in (i) above and both reduced with a small amount of sodium dithionite. CO was bubbled through the contents of the test cuvette for 30 seconds and the difference spectrum between 390 and 500nm recorded as previously. Cytochrome P-450 content was calculated using the extinction coefficient \(91 \text{mM}^{-1}\text{cm}^{-1}\) for the extinction difference between 450 and 490nm.

(k) **Measurement of Cholesterol 7\(\alpha\)-hydroxylase activity**

The method employed was based on that of Berseus et al. (1969).

**Incubation**

The incubation system was as follows:
<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal supernatant (ml)</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Boiled Microsomal supernatant (ml)</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Cofactor solution* (mL)</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Substrate** (mL)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Acetone (mL)</td>
<td>0.02</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Cofactor solution /ml modified Bucher medium

15μmol Glucose-6-phosphate
25μmol NADP

** [4-14C] Cholesterol 1μCi/ml absolute ethanol.

The incubation was carried out in 25ml conical flasks. A 0.5ml volume [4-14C] cholesterol was added to each flask and the ethanol evaporated to dryness in a water bath at 50°C in a stream of nitrogen. The residue was taken up in the acetone and test and control incubates set up as above. Flasks were incubated at 37°C, with shaking, for 60 minutes.

Extraction Procedure

The reaction was terminated by the addition of 5ml of methanol, the mixture transferred to a 15ml glass tube with a screw cap and the flask washed out with a further 5ml of methanol. Neutral lipid was subsequently extracted according to the method of Hattersley (1975) (Fig.2.1). Extraction recovery was of the order of 80-90%, being checked by sampling at each stage of the extraction procedure.

The combined lipid extract was transferred to a 25ml "bear-shaped" flask and evaporated to dryness in vacuo using a
Incubation Medium

Add 2 x 5ml methanol, extract 10 minutes, centrifuge 2000 r.p.m., 10 minutes.

Supernatant ↔ Pellet

Mix 10 minutes centrifuge 5 minutes, 2000 r.p.m., discard upper layer.

Chloroform Extract

Add 10ml chloroform, extract 10 minutes, centrifuge 2000 r.p.m., 10 minutes.

Pellet

Add 5ml H₂O, mix 5 minutes, centrifuge 2000 r.p.m., 10 minutes. Discard upper layer.

Lipid Extract

Add 5ml hot ethyl acetate, extract 10 minutes, centrifuge 2000 r.p.m., 10 minutes.

Pellet discarded

Fig. 2.1 Extraction Procedure for Cholesterol \( \Delta^{\alpha} \)-hydroxylase Assay
rotary evaporator at 30-40°C.

**Thin-Layer Chromatography of Extract**

Analysis of the radioactive products formed was carried out by thin-layer chromatography (TLC) using Silica gel G (Scheicher and Schüll, W. Germany) on plates measuring 20cm x 20cm, with a gel layer 0.25mm thick. The solvent system employed was toluene: ethyl acetate (7:13 v/v). Ascending chromatography in saturated air was used, allowing approximately 50 minutes for development at room temperature (20°C) or until the solvent had reached a distance of 17-18cm from the edge of the plate (15-16cm from the origin). Duplicate plates were set up as follows.

The residues obtained from the extraction of test and control incubates were redissolved in 250μl of chloroform and a 25μl aliquot of each applied as narrow bands to the origin of both plates. To the second plate only, was applied a similar band of 7α-hydroxy cholesterol (Cholest-5-ene-3α,7α-diol), so that the two bands (standard + extract) overlapped slightly, thus allowing more certain identification of the product.

After chromatography, the second plate was visualised by spraying with a 20% (w/v) solution of dodecomolybdophosphoric acid in absolute ethanol, followed by gentle heating. The small quantities of metabolites generated, made identification of the bands by a thin-layer radioactive scanner impossible. Similarly, autoradiography was not possible as time was limiting. Thus, each chromatoplate was divided into 0.5cm x 2.0cm zones from 0.5cm below the origin to the solvent front. Each zone
was scraped into a polypropylene "mini-vial". (G.D. Searle Ltd., High Wycombe, Bucks.), to be counted individually, thus enabling a histogram to be constructed, showing the distribution of radioactivity on the plate.

Radioactivity - Counting Procedures

The following counting conditions were used:

Either a) 0.05ml of sample (aqueous or non-aqueous) was transferred to 'mini-vials' and diluted with 0.4ml of distilled water. A 4ml volume of Toluene: Synperonic (B.D.H.) scintillant was added (2:1, v/v Toluene: Synperonic, 5.5g/litre P.P.O) and mixed on a "Whirlimixer" to give a clear gel.

or b) segments of gel scraped into 'mini-vials' from the plates were shaken vigorously with 5ml of 'Cab-O-Sil' scintillant (5.5g P.P.O, 4.5g Cab-O-Sil, (Packard), in 1 litre of toluene).

Each vial was "counted" using an LKB Wallac 1210 Ultrobeta liquid scintillation counter, for a period of 10 minutes. The scintillation counter was programmed to correct automatically for quenching by the external standard channels ratio method. In the case of the aqueous and non-aqueous samples, efficiencies of counting were of the order of 85-97%.

III Histology

After removal of adventitious fatty tissue, the hearts and aortas were fixed in formalin (10% (v/v) formaldehyde in 0.05M sodium phosphate buffer). Each aorta was subsequently stained with Sudan Black B (1.5% (w/v) in 70% (v/v) ethanol).
IV Statistics

Where multiple determinations were made, results were expressed as the arithmetic mean ± the standard error of the mean. To establish significant differences between the means (minimum of 3 individual values), the student's t-test was used.

V Animals

Male New Zealand white rabbits, 8 weeks old (900-1400g), supplied by Ranch Rabbits (Crawley Down, Sussex) were used. The animals were randomly assigned to the two experimental groups, six to each group. They were housed individually in aluminium cages (46cm x 49cm x 61cm), on aluminium grids over sawdust which was changed twice a week.

Animals were allowed access to diet and tap water ad libitum at all times. The temperature and relative humidity of the animal house were regulated to 22°C and 50% respectively. The lighting was time-switched on at 06.30 hours and off at 18.30 hours. Measurements of body weight and food intake were made at regular intervals, no allowance was made for spillage of diet, as this was minimal and relatively constant among animals. Initially, animals were maintained on stock diet (RGP pellets, Labsure Animal Diets, supplied by J. Lillico and Sons, Reigate, Surrey). The experimental diets were gradually introduced to the animals over a pre-experimental period of 6 weeks by grinding the commercial diet and the successive addition of 25%, 50% and 75% of the test diets, followed by repelleting. Each dilution of diet was fed for 2 weeks.

The rabbits, which were divided into Group A (20% butterfat)
and Group B (20% corn oil), were fed for a total of 41 weeks, i.e. an experimental period of 34 weeks.

**Dietary Treatment**

The diets used were semi-synthetic basal diets, supplemented with either corn oil or butterfat, similar to those employed by Moore and Williams, (1964). The formulation of these diets is summarized in Tables 2.1 to 2.3.

For Group A, 24 parts of butter, equivalent to 20.6 parts dry weight, were added to 80 parts of the basal diet and for Group B, 20 parts of corn oil were added. Diets were mixed at weekly intervals, using a Hobart food mixer (Model A 200), mixing the dry ingredients for 15 minutes at the slowest speed, adding the oil or melted butter whilst mixing and finally mixing for 5 minutes at medium speed. For addition to diets, butter was melted in a water bath at 60°C. The experimental diets were subsequently mixed with tap water (approximately 500ml/kg diet) and passed through the mincing attachment of the Hobart mixer. The resultant pellets were dried overnight at 40-45°C. To prevent oxidation of the diet as far as possible, small batches were prepared and stored at 4°C.

**Experimental Procedure**

At intervals throughout the experiment, blood was withdrawn from the animals by marginal ear vein puncture. Samples were collected in non-heparinised glass bottles, allowed to stand for at least 60 minutes and centrifuged using a bench centrifuge for 10 minutes to separate the serum. Aliquots of serum were
Table 2.1 Composition and Gross Energy Value of Diets Fed to Rabbits

<table>
<thead>
<tr>
<th>Percentage Composition (by weight):</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>24.0^</td>
<td>20.0^</td>
</tr>
<tr>
<td>+ Wheat Starch</td>
<td>16.2</td>
<td>16.3</td>
</tr>
<tr>
<td>‡ Sucrose</td>
<td>9.9</td>
<td>10.0</td>
</tr>
<tr>
<td>▲ Casein</td>
<td>24.8</td>
<td>25.0</td>
</tr>
<tr>
<td>+ Methyl cellulose</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+ Potassium acetate</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>+ Magnesium oxide</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>+ Sodium chloride</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>* Choline chloride</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Mineral Mixture</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin Mixture</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Gross energy value (Kcal/100g Diet) 532 532
(MJ/100g Diet) 2.23 2.23

Percentage total energy supplied as fat 35 35
Percentage total energy supplied as starch 13 13
Percentage total energy supplied as protein 28 28

\^ Sigma +B.D.H. ‡ Tate and Lyle, Croydon, Surrey.

▲ Casein Industries, London, SW8 (Lactic acid casein)

\^ Equivalent to 20.6% dry matter, 'Lurpak'.

\^\^ 'Saladin', Van der Burgh and Jurghens Ltd., London.

+ Originally wheat straw, J. Lillico and Sons, Reigate, Surrey, replaced by purified cellulose at week 13 of the experiment.

\* As calculated by Moore and Williams (1964)
Table 2.2 Composition of Vitamin Mixture (per Kg mix)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin</td>
<td>100mg</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>16g</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>100mg</td>
</tr>
<tr>
<td>Meso-inositol</td>
<td>30g</td>
</tr>
<tr>
<td>p-aminobenzoic acid</td>
<td>70g</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>10g</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>10g</td>
</tr>
<tr>
<td>Cyanocobalamin</td>
<td>8mg</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>10g</td>
</tr>
<tr>
<td>Menadione sodium bisulph.</td>
<td>1.5g</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>10g</td>
</tr>
<tr>
<td>α-tocopheryl acetate</td>
<td>10g</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
<td>10g</td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>5mg</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>666mg</td>
</tr>
</tbody>
</table>

The base of this mixture was glucose.

Table 2.3 Composition of Mineral Mixture (per 10 Kg mix)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium lactate monohydrate</td>
<td>3202g</td>
</tr>
<tr>
<td>Ferric citrate trihydrate</td>
<td>355g</td>
</tr>
<tr>
<td>Di-potassium monohydrogen orthophosphate</td>
<td>2862g</td>
</tr>
<tr>
<td>Tricalcium orthophosphate</td>
<td>1620g</td>
</tr>
<tr>
<td>Monosodium dihydrogen orthophosphate dihydrate</td>
<td>1177g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>798g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>519g</td>
</tr>
<tr>
<td>Manganese sulphate tetrahydrate</td>
<td>60g</td>
</tr>
<tr>
<td>Cupric sulphate pentahydrate</td>
<td>15g</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>1.5g</td>
</tr>
<tr>
<td>Zinc carbonate</td>
<td>1.5g</td>
</tr>
</tbody>
</table>

* Supplied by Sigma  ** Supplied by Fisons  + Supplied by B.D.H.
stored at $4^\circ C$ for 1-5 days for analysis of lipids and at $-20^\circ C$ for 1-6 months for the determination of bile acids.

At the end of the experiment, the two groups, together with 5 control animals of the same age and maintained on laboratory chow, were killed by exsanguination via cardiac puncture followed by cervical dislocation. The animals were allowed free access to food up to the time of death. Serum was prepared as above and the livers and gall bladders removed together with the hearts and aortas extending to the point of division into the two common iliac arteries. Tissues were treated as described in sections II and III.
Results

a) Animal Weights and Food Intakes

During the course of the experiment, one animal from each group had to be killed. One was suffering from severe diarrhoea, which had started in the pre-experimental period and was not apparently related to the dietary treatment, whilst the other had severe otitis media. A second animal from Group A developed a large ventral hernia and was subsequently excluded from the experiment. Any experimental data obtained from these rabbits up to their time of death was eliminated from the mean values presented.

The original diet formulation contained 20% ground wheat straw as the source of fibre. However, due to the lack of any facilities for grinding the straw, it was decided to change the source of fibre to purified cellulose; this change was made at week 13 of the experiment.

From the growth curves (Fig. 2.2) it would appear that weight gain was adequate and similar in both groups during the first 12-13 weeks of the experiment. Both curves plateaued at this point, an event which corresponded with a decrease in food intake (Table 2.4) and could have been the result of the change in source of fibre. Between weeks 14 and 15 the growth curves of Group A and B crossed so that by week 22, the growth rate of the animals in Group A appeared to exceed those in Group B, although the difference was not statistically significant. Again, the food intake pattern corresponded to the change in growth rate, increasing in both groups beyond week 22.
Table 2.4  Mean Daily Food Intake of Rabbits During Experiment (g/rabbit/day)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2\textsuperscript{a} 3\textsuperscript{b} 4\textsuperscript{b} 5\textsuperscript{c} 6\textsuperscript{c} 7\textsuperscript{d} 8\textsuperscript{d} 9\textsuperscript{e} 10 11 12 13 14 15 16 17 18 19 20 21 22</td>
</tr>
</tbody>
</table>

A * 146 112 138 125 110 92 102 84 76 70 78 52 64 50 52 60 43 49 45 45 49

B ** 155 132 137 123 112 96 87 91 66 60 86 72 42 41 52 68 44 49 47 46 48

<table>
<thead>
<tr>
<th>GROUP</th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42</td>
</tr>
</tbody>
</table>

A * 58 56 58 62 64 66 68 69 63 59 55 61 54 59 69 65 62 68 64 66

B ** 53 50 52 55 54 60 46 63 56 54 50 57 57 55 49 55 49 57 59 61

\(a = \) stock diet  \(b = 25\%\) test diet  \(c = 50\%\) test diet  \(d = 75\%\) test diet  \(e = 100\%\) test diet

* 20\% butterfat,\((n=4)\)  ** 20\% corn oil,\((n=5)\)
but more noticeably in Group B.

b) **Serum Cholesterol Concentration**

As shown in Fig. 2.3, during the experimental period there was a pronounced elevation of mean serum cholesterol concentration in Group A, as compared to Group B which remained relatively constant. This difference was apparent (p<0.1) after only 4 weeks on the full experimental diets.

The reason for the increased cholesterol concentrations at week 20 is uncertain, but as it is common to both groups it is more likely to be due to factors independent of diet. An error in methodology cannot be discounted as the measurements could not be repeated due to insufficient serum.

The mean serum cholesterol level of the control group C in the terminal experiment was significantly lower than that of either experimental group (Table 2.6).

c) **Serum Total Bile Acid Concentration**

There was no significant difference between groups in total serum bile acids measured throughout the course of the experiment, with the exception of week 16 when the mean concentration in Group A was more than double that of Group B (p<0.01) (Table 2.5). However there was an apparent increase in this parameter in both groups during the experiment, although this was not statistically significant.

In the terminal experiment, the mean total serum bile acid concentration of Group C appeared to be lower than that of the experimental groups but again the difference was not
Fig. 2.3 Mean Serum Cholesterol Concentration of Rabbits Immediately Prior to the Pre-experimental Period and at Intervals During the Experiment

- Group A
- Group B

a = p<0.05
b = p<0.01
Table 2.5  Mean Serum Total Bile Acid Levels (mmol/l) of Rabbits at Intervals During the Experiment

<table>
<thead>
<tr>
<th>WEEK</th>
<th>GROUP</th>
<th>+A*</th>
<th>B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.96 + 0.23</td>
<td>1.55 + 0.29</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1.67 + 0.25</td>
<td>1.45 + 0.33</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.22 + 0.07</td>
<td>0.59 + 0.10</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.83 + 0.35</td>
<td>1.11 + 0.27</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1.20 + 0.42</td>
<td>0.99 + 0.23</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>2.90 + 0.29</td>
<td>3.46 + 0.76</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>2.24 + 0.33</td>
<td>3.07 + 0.84</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>2.43 + 0.42</td>
<td>2.42 + 0.46</td>
<td></td>
</tr>
</tbody>
</table>

Values expressed as mean ± standard error of mean

* 20% butterfat, (n=4)

** 20% corn oil, (n=5)

+ No significant difference between groups with the exception of week 16 (p<0.01.)
### Table 2.6 Effect of Dietary Treatment on Some Liver and Serum Parameters in Rabbits

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GROUP</th>
<th>PROBABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>3665 ± 168</td>
<td>3336 ± 142</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>110.0 ± 8.0</td>
<td>73.5 ± 17.3</td>
</tr>
<tr>
<td>Relative Liver Weight (g/100g body weight)</td>
<td>2.96 ± 0.09</td>
<td>2.14 ± 0.38</td>
</tr>
<tr>
<td>Microsomal Protein (mg/g liver)</td>
<td>21.1 ± 1.4</td>
<td>22.7 ± 2.3</td>
</tr>
<tr>
<td>Liver Total Lipid (mg/g liver)</td>
<td>36.0 ± 1.7</td>
<td>27.6 ± 2.4</td>
</tr>
<tr>
<td>Liver Total Cholesterol (mg/g liver)</td>
<td>6.65 ± 0.51</td>
<td>2.81 ± 0.52</td>
</tr>
<tr>
<td>Serum Cholesterol (mmol/litre)</td>
<td>15.5 ± 6.0</td>
<td>1.31 ± 0.52</td>
</tr>
<tr>
<td>Serum Triglyceride (mmol/litre)</td>
<td>3.22 ± 0.99</td>
<td>1.03 ± 0.59</td>
</tr>
<tr>
<td>Serum Total Bile Acids (μmol/litre)</td>
<td>2.43 ± 0.42</td>
<td>2.42 ± 0.46</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* 20% butterfat, (n=4)  ** 20% corn oil, (n=5)  *** commercial chow diet, (n=5)
significant (Table 2.6).

d) **Serum Triglyceride Concentration**

This parameter was measured only in the terminal experiment when the triglyceride concentration in Group A was significantly elevated above Group B and C. No difference was observed between Groups B and C (Table 2.6).

e) **Results of Terminal Experiment**

These results are summarized in Table 2.6.

On dissecting the animals, it was observed that those in Group A had greater deposits of intra-abdominal, peri-renal and peri-cardiac fat. The livers of these rabbits were much paler and significantly larger than those in Group B, but were comparable in size to those in Group C. When the weight of this organ was expressed relative to body weight, the difference between A and C became highly significant whereas that between the two experimental groups was less pronounced, though still significant.

The liver microsomal protein content did not vary significantly between groups but total liver lipid was substantially greater in the butter-fed animals than in either of the other groups. Similarly, there was a pronounced accumulation of cholesterol in the livers of these animals when compared to B and C.

**Cholesterol 7α-hydroxylase activity**

Table 2.7 shows the metabolism of [4-14C]cholesterol to
Table 2.7 Effect of Dietary Treatment on Hepatic Microsomal Cholesterol \( \alpha \)-hydroxylase Activity *in vitro*

<table>
<thead>
<tr>
<th></th>
<th>GROUP</th>
<th>PROBABILITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>pmol ([4-^{14}C]) Cholesterol metabolised/g liver/hour</td>
<td>67.5 ± 8.6</td>
<td>57.7 ± 17.4</td>
</tr>
<tr>
<td>pmol ([4-^{14}C]) Cholesterol metabolised/mg microsomal protein/hour</td>
<td>3.33 ± 0.66</td>
<td>2.56 ± 0.75</td>
</tr>
<tr>
<td>nmol ([4-^{14}C]) Cholesterol metabolised/total liver/hour</td>
<td>7.24 ± 1.08</td>
<td>4.59 ± 1.92</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* 20% butterfat, (n=4)

** 20% corn oil, (n=5)

*** Commercial chow diet, (n=5)
7α-hydroxycholesterol. Whilst the activities of this enzyme were significantly higher in the chow-fed controls compared with both experimental groups, differences between groups A and B were less obvious. There was however an indication of a slight reduction in activity in B as compared to A which became more pronounced when results were expressed on a total liver basis. However, the significance of data expressed in this way to metabolism in vivo is uncertain.

Mixed Function Oxidase Activities

Tables 2.8 and 2.9 show the concentrations of cytochromes P-450 and b5 together with the activities of NADPH-cytochrome c reductase and certain mixed function oxidase enzymes expressed per g liver or as specific activities. Both experimental groups displayed an apparent increased cytochrome concentration accompanied by a decrease in enzyme activities as compared to the control animals. However, these changes were not statistically significant, or only marginally so, which is probably a reflection of the small sample numbers.

A similar, but less pronounced difference was observed between the two experimental groups. The cytochrome levels and enzyme activities in the corn-oil fed animal being respectively higher and lower than in those fed butter. This was particularly evident with respect to cytochrome P-450 levels and biphenyl 2-hydroxylase activity.

Histological Examination of Aortic Tissue

Gross staining of the hearts and aortas of animals from
Table 2.8  Effect of Dietary Treatment on Some Parameters of Mixed Function Oxidase Metabolism
Expressed per g Liver

<table>
<thead>
<tr>
<th></th>
<th>GROUP</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
<td>C***</td>
<td></td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol)</td>
<td>25.5 ± 4.3</td>
<td>30.8 ± 3.1</td>
<td>20.4 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b5 (nmol)</td>
<td>20.5 ± 2.6</td>
<td>24.1 ± 3.2</td>
<td>15.5 ± 2.4a</td>
<td></td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol cytochrome c reduced/minute)</td>
<td>0.73 ± 0.07</td>
<td>0.92 ± 0.14</td>
<td>0.78 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td>16.8 ± 10.2+</td>
<td>9.2 ± 6.1+</td>
<td>31.2 ± 9.9</td>
<td></td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol produced/hour)</td>
<td>1.71 ± 0.33</td>
<td>1.94 ± 0.25</td>
<td>2.23 ± 0.42</td>
<td></td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μmol formaldehyde produced/hour)</td>
<td>4.36 ± 1.97</td>
<td>4.31 ± 0.87</td>
<td>5.38 ± 1.23</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* 20% butterfat, (n=4)  ** 20% corn oil, (n=5)  *** commercial chow diet, (n=5)  + n=3  a p<0.05 (BxC)
Table 2.9  Effect of Dietary Treatment on Some Parameters of Mixed Function Oxidase Metabolism

Expressed per mg Microsomal Protein

<table>
<thead>
<tr>
<th></th>
<th>GROUP</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome P-450 (nmol)</td>
<td></td>
<td>1.18 ± 0.13</td>
<td>1.36 ± 0.08</td>
<td>1.10 ± 0.13</td>
</tr>
<tr>
<td>Cytochrome b&lt;sub&gt;5&lt;/sub&gt; (nmol)</td>
<td></td>
<td>0.97 ± 0.09</td>
<td>1.05 ± 0.06</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (μmol cytochrome c reduced/minute)</td>
<td></td>
<td>34.5 ± 1.9</td>
<td>40.8 ± 4.4</td>
<td>43.6 ± 3.1</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase (nmol produced/hour)</td>
<td></td>
<td>0.85 ± 0.47&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.43 ± 0.31&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.81 ± 0.50</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase (nmol produced/hour)</td>
<td></td>
<td>79.0 ± 11.6</td>
<td>85.6 ± 10.5</td>
<td>128 ± 21</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase (μmol formaldehyde produced/hour)</td>
<td></td>
<td>0.20 ± 0.08</td>
<td>0.19 ± 0.03</td>
<td>0.30 ± 0.05</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* 20% butterfat, (n=4)  ** 20% corn oil, (n=5)  *** commercial chow diet, (n=5)  + n=3
each group revealed a greater degree of lipid degeneration of
the intimal surface of those from Group A as judged by the
extent and density of Sudan Black Staining. However it was
not possible to detect differences in aortas derived from
animals in groups B and C. Owing to the nature of the prepara-
tions, particularly with regard to the degree of background
staining, more sophisticated analysis of the severity of the
atheromatous lesions was not possible.
Discussion

In this experiment, a group of animals designated as controls, which were age-matched to the experimental animals and had been fed on laboratory chow, were killed at the same time as the experimental groups. The inclusion of such a group is arguable and the interpretation of observed differences is difficult as these animals were not maintained under identical experimental conditions and the diet formulations are very different. However the design of a "control" semi-synthetic diet is also problematical as it would necessitate the use of a diet excessively high in carbohydrate, which would further complicate interpretation of data. Thus, strictly speaking, comparisons should only be made between the experimental groups themselves, but the chow-fed group was included as it was considered of interest to establish the effect of the semi-synthetic diet per se on the parameters measured. This was of particular importance with respect to the mixed function oxidase metabolism data as much of the work reported in the literature has been carried out in experimental animals other than the rabbit.

The experimental animals appeared healthy but did not attain their full growth potential when compared with the control animals. Food intakes were 50-60% of those reported by Moore and Williams (1964) but were comparable to the findings of Funch et al. (1960). All three experiments employed identical semi-synthetic diets with the exception of the source of dietary fibre (see below). This phenomenon of a reduced weight gain and food intake in animals fed semi-synthetic diets has recently
been reported by Hamilton and Carroll (1976). It is possible that the high fat content of the experimental diets rendered them unpalatable. Arrington et al. (1974) reported reductions in the voluntary feed intakes of rabbits on increasing the fat content of the diet from 2% to 14%. However in these animals there was also an improved feed conversion and they conclude that the rabbits adequately tolerated the increase. Inclusion of a group of pair-fed control rabbits fed laboratory chow, would facilitate interpretation of the significance of the body weight data.

Feeding of the high butter fat diet resulted in an appreciable elevation in serum cholesterol. Once again, this finding was more similar to that of Funch et al. (1960) than to Moore and Williams (1964), who observed a less pronounced hypercholesterolaemia. The difference in dietary fibre would appear to account for these disparities. In the present experiment, although wheat straw was used initially, as in the experiments of Moore and Williams, purified cellulose was subsequently used, in common with the experiments of Funch and his colleagues. Moore (1967) showed that purified cellulose was less palatable and enhanced the hypercholesterolaemic effect of dietary lipid. Serum triglyceride concentration was also significantly increased in Group A compared to B as was total liver lipid and cholesterol. These findings are in accordance with those of previous workers using high fat diets without added cholesterol (Wigand, 1959; Funch et al., 1960; Moore and Williams, 1964) and confirm the establishment of the hypercholesterolaemic model.

There was no change in total serum bile acids between
Groups A and B during the course of the experiment, but there appeared to be an increase in both groups with time. This might reflect a gradual response to the administration of high fat diets, although the effect of age on bile acid metabolism cannot be excluded.

There is little information in the literature pertaining to the effect of variation in dietary lipid on levels of serum bile acids. This may in part reflect difficulties encountered in establishing a method of adequate sensitivity for measuring the low levels in serum. However, measurement of this parameter should provide information regarding the dynamics of the enterohepatic circulation of bile acids (LaRusso et al., 1974). As considered earlier, most experiments have examined the effects of lipid on the excretion and turnover of bile acids. Thus Beher et al. (1970) showed that administration of corn oil to rats had no effect on cholic acid turnover. Similarly, Kim et al. (1976) followed the oxidation of $[26^{-14}C]$ cholesterol as an index of rate of bile acid formation and found no difference between rats fed low fat, 15% corn oil or 15% butter diets. They did note however that corn oil increased the excretion of faecal labelled lipid, giving support to the earlier work of Gerson et al. (1961) and Wilson (1961). The lack of response in bile acid metabolism to saturated fat feeding in the present experiment is in accordance with the conclusions of Uchida et al. (1977). These workers were generally concerned with the effects of cholesterol feeding but they conclude that species such as the rabbit, which are prone to an hypercholesterolaemic response, display little change in bile acid metabolism.
Failure to observe an effect of polyunsaturated fat may indicate that the hypocholesterolaemic effect of these fats results from some other mechanism, as will be considered later.

At the end of the experiment, serum total bile acid levels in each experimental group were compared to those in the chow-fed animals. They were found to be slightly elevated in the latter group, but this was not statistically significant despite the concomitant significant increase in cholesterol 7α-hydroxylase activity. It would thus seem that feeding an unrefined chow diet as compared to a semi-synthetic diet results in an increase in bile acid synthesis, which in this experiment was associated with decreased serum and liver cholesterol levels. Other workers (Johansson, 1970; Kritchevsky et al., 1977a) have reported this effect on 7α-hydroxylation. Further support is given by the work of Fisher et al. (1976) who measured bile acid pool size in rats. In those fed semi-synthetic diets, it was reduced to 60% compared with animals fed commercial control diets; no qualitative differences in bile composition were observed. Although not measured in the present experiments faecal bile acid excretion has been repeatedly shown to be higher in animals fed commercial chow diets (Portman and Murphy, 1958; Grundy et al., 1965; Gustafsson and Norman, 1969; Kritchevsky et al., 1973a; Balmer and Zilversmit, 1974). Similarly, Kim et al. (1976) demonstrated an increased oxidation of [26-14C] cholesterol in rats on commercial feed compared to semi-synthetic diets.

The observed hypocholesterolaemic effect of commercial chow diets has been attributed to various components. Originally, Malmros and Wigand (1959) suggested that trace amounts of lipid
associated with the commercial feed might be important. Evidence contrary to this was produced by Kritchevsky and Tepper (1968), who showed that fat-extracted chow did not induce hypercholesterolaemia or atherosclerosis in rabbits. Similarly, addition of the extracted fat to a semi-synthetic diet did not prevent its hypercholesterolaemic action. The importance of the nature of the protein component has also been considered (Howard et al., 1965; Hamilton and Carroll, 1976). However, Kritchevsky et al. (1977b) have concluded that the effects of protein can be eliminated by alteration of the fibre component of a semi-synthetic diet.

The hypocholesterolaemic influence of the fibre content of commercial diets was examined earlier by Moore (1967) and Kritchevsky et al. (1973a). The ability of various types of fibre to bind bile acids has been demonstrated (Eastwood and Hamilton, 1968; Balmer and Zilversmit, 1974; Birkner and Kern, 1974; Kritchevsky and Story, 1974). From this it has been inferred that consumption of commercial chow diets may be associated with an enhanced bile acid excretion. Portman and Murphy (1958) reported that increasing the fibre content of purified diets from 5% to 20% resulted in a greater cholic acid production. Gustafsson and Norman (1969) found this to be unaffected by the addition of pure cellulose, demonstrating the importance of the type of dietary fibre. The difference between crude and purified fibre was confirmed by the experiments of Morgan et al. (1974), who investigated the effect of these two fibres on cholesterol 7α-hydroxylase activity. Crude fibre only was found to stimulate the activity of this enzyme.
Finally it has been suggested that rather than the fibre per se, trace contaminants of the unrefined fibre, such as sterols, which have been shown to be hypercholesterolaemic in both experimental animals and man (Swell et al., 1954; Best et al., 1955; Sachs and Weston, 1956; Kudchodkar et al., 1976), might be responsible. It would be of interest to investigate the effect of administration of such sterols on the activity of cholesterol 7α-hydroxylation in the type of model used in this experiment.

Thus, the mechanism of hypercholesterolaemia of commercial chow has been confirmed by this experiment. However that of the polyunsaturated fatty acids is still uncertain. Although the difference was small, the activity of cholesterol 7α-hydroxylation in the corn oil-fed animals was unexpectedly lower than in those fed butterfat. This is contrary to the finding of Mayer and Mayer (1975) who reported an enhancement of 7α-hydroxylation after feeding a polyunsaturated fat diet. However comparison with this experiment is difficult, as these workers used rats and only fed the diet for 14 days. Kritchevsky et al. (1977a) also found that feeding a diet rich in unsaturated fats led to a decrease in 7α-hydroxylation of cholesterol. As observed by Bjorkhem et al. (1978), these conflicting findings might be due to compositional differences in the fats, particularly with respect to their peroxide content. These workers found that whilst the small amounts of peroxides naturally present in the unsaturated triglycerides (triolein and trilinolein) had little effect on cholesterol 7α-hydroxylase, a diet consisting of peroxidised linoleic acid reduced its
activity. The peroxide content of the corn oil used in this experiment was not measured, although it would be expected to be low, owing to the presence of natural antioxidants such as \( \alpha \)-tocopherol. However mixing of the oil with the other constituents of the diet may have resulted in its interaction with certain pro-oxidants, thus increasing the overall peroxide value of the diet. Although oxidation of the saturated fat diet may also have occurred, its peroxide content would be unlikely to have increased, thus the possibility that the observed reduction in \( \alpha \)-hydroxylation was due to peroxides cannot be excluded. Bjorkhem et al. (1978) also compared the effect of diets containing triglycerides of different fatty acid composition on \( \alpha \)-hydroxylation. They again found that in rats fed trilinolein or triolein this was decreased compared with those fed tripalmitin or trierucin. However, they conclude that the level of cholesterol \( \alpha \)-hydroxylase activity was better related to the degree of absorption of the fat than to its unsaturation. They suggest that, as the reduced absorption of some fatty acids is coupled with a reduced absorption of bile acids, unabsorbed fatty acids could lead to increased \( \alpha \)-hydroxylation due to diminished feedback inhibition.

Data emanating from this experiment would indicate that the hypocholesterolaemic action of polyunsaturated fats is not mediated by an effect on cholesterol \( \alpha \)-hydroxylase, despite the observations by some workers of an increased bile acid excretion (see above). Ahrens and his colleagues maintain that the hypocholesterolaemic action is more likely to be due to a redistribution of tissue cholesterol (Spritz et al., 1965;
Grundy and Ahrens, 1970). If this were so, one might expect to see an increase in hepatic cholesterol concentration, a finding not apparent in the present investigation.

It may be that we have taken too simplistic an approach in assuming that any factor modifying serum cholesterol concentrations will act via cholesterol absorption, synthesis or excretion. This view was recently expressed by Sodhi and his colleagues (1977). They consider that cholesterol transport in plasma is dependent on the availability of lipoproteins and that the rate of entry or exit of the sterol to or from the lipoprotein may be more critical in determination of serum cholesterol concentration. Thus, it is possible that the influence of polyunsaturated fats on this parameter is mediated via lipoproteins. Evidence in support of this contention derives from studies on the effects of these fats on lipoprotein turnover, which suggest that they inhibit hepatic secretion of low density lipoprotein (Yeshurun et al., 1976). As discussed in Chapter 1, high density lipoprotein levels in serum would appear to have a strong negative correlation with incidence of coronary heart disease but to date few studies have investigated the effect of diet on this fraction of serum cholesterol. A possible area of further investigation could be the effect of polyunsaturated fat intake on high density lipoprotein cholesterol.

Interpretation of the observed changes in mixed function oxidase metabolism associated with the dietary treatments can only be tentative due to the lack of statistical significance. Comparison with studies in the literature is difficult, as most
investigators have used the rat as an experimental model and have examined metabolism on fat-free basal diets compared with those supplemented with saturated or unsaturated fats, (Caster et al., 1968), at levels lower than those used in the present study. In general, these experiments have shown lower levels of cytochromes and enzyme activities in rats fed saturated fat, findings which are at variance with the present experiment. Marshall and McLean (1971) observed that polyunsaturated fats enhanced the inductive effect of phenobarbitone on microsomal metabolism, it would therefore be of interest to repeat the present experiment in animals pretreated with an inducer such as phenobarbitone.

Several explanations may be offered for the observed increase in cytochrome concentrations accompanied by decreased mixed function oxidase and 7α-hydroxylase activities. Firstly, the fat feeding may have a specific inhibitory effect on a particular form of P-450 whilst actually inducing the bulk of the cytochrome. In order to verify this, it would be necessary to examine a much wider range of substrates under similar experimental conditions. Secondly, one might envisage a qualitative alteration in the cytochrome, which could alter the binding characteristics of the substrates. It would be of interest to examine the kinetics of this system under these conditions, particularly as there is some evidence of an influence of dietary fat on the Km's of these enzymes (Wade and Norred, 1976). It could be postulated that as fatty acids themselves act as substrates for cytochrome P-450, they might bind to the molecule, thus preventing metabolism of other exogenous substrates; this would explain the paradoxical findings of an increase in cytochrome
Metabolism of fatty acids via cytochrome P-450 occurs by the process of \( \omega \)-oxidation (Robbins 1961; Preiss and Bloch, 1964; Wada et al., 1968; Bjorkhem and Danielsson, 1970). It is known that short, medium and some long chain saturated fatty acids may be oxidised via this pathway, but the participation of unsaturated long chain fatty acids is apparently undocumented. The proportion of fatty acid oxidation normally occurring by this route is small and its physiological significances uncertain. Conditions of starvation and diabetes have been shown to enhance \( \omega \)-oxidation (Wada et al., 1971; Bjorkhem, 1973). Thus, Wada and his colleagues (1971) speculated that under those conditions, when fatty acids were utilised in preference to glucose and formation of ketone bodies was increased, \( \omega \)-oxidation might be induced. This would result in the production of succinyl-CoA from dicarboxylic acids, which could subsequently be used for oxidation of acetyl-CoA and gluconeogenesis in the livers of starved or diabetic animals (Wada and Usami, 1977).

It could be hypothesised that under conditions of high fat intake as in the present experiment, where ketone levels might be expected to be increased, a greater proportion of fatty acids might be oxidised by this pathway. Thus, although normal or increased amounts of cytochrome P-450 were available for metabolism, competition for binding sites by the fatty acids might inhibit metabolism of other substrates. To test this hypothesis, it would be of interest to measure the oxidation of \([^{14}C] \)labelled laurate or stearate in animals fed high fat or control diets.
A further alternative is that feeding of high levels of fats, whilst increasing amounts of the cytochrome, may unfavourably alter the composition of the microsomal membranes in such a manner that metabolism is reduced. This is consonant with the view of Stier (1976), who considers the cytochrome P-450 system to be dynamic. He suggests that the system may be coupled or decoupled by changes in the lipid environment of the membrane which could be induced by environmental factors such as nutrition or xenobiotics.

In summary, feeding a high level of polyunsaturated fats was shown to prevent the hypercholesterolaemia and aortic lipid accumulation seen in the saturated fat-fed rabbits but an increased bile acid synthesis, as demonstrated by an increase in cholesterol \( \Delta^7 \)-hydroxylase activity was not observed. Thus, in the rabbit, under these particular experimental conditions, the hypercholesterolaemic action of polyunsaturated fats is not due to an effect on bile acid biosynthesis.
Chapter 3

The Effects of Ascorbic Acid Depletion and Supplementation on Cholesterol and Foreign Compound Metabolism in the Guinea Pig
Introduction

Several vitamins have been observed to influence the concentration of plasma lipids. Supplementation with vitamin D in man has been shown to elevate serum cholesterol (Dalderup et al., 1965). Similarly, vitamin D-induced infantile hypercalcæmia is characterised by a marked hypercholesterolaemia (Forfar and Thompsett, 1959), and Linden (1974) has suggested that regular consumption of fish liver may predispose to an increased level of plasma cholesterol and risk of coronary heart disease. A deficiency of vitamin E has been reported to cause hypercholesterolaemia, possibly via a stimulation of cholesterogenesis (Eskelson et al., 1973). Some of the B vitamins have also been implicated. In pharmacological doses, nicotinic acid acts as an effective hypocholesterolaemic agent (Carlson et al., 1968). Similarly there are some reports of hypercholesterolaemia associated with deficiencies of biotin and pyridoxine (Scott, 1958; Pool et al., 1971; Spitzer et al., 1966). Probably the most widely studied vitamin with respect to lipid metabolism and coronary heart disease is ascorbic acid.

Myaniskova (1947) first observed a reduction in serum cholesterol concentration in hypercholesterolaemic patients, following the administration of ascorbic acid. In a study designed to determine human requirements for this vitamin, Krebs (1953) reported the occurrence of severe cardiac events in 2 out of 10 young adults on a scorbutogenic diet. These events occurred in the absence of signs of acute scurvy. Willis and his colleagues determined arterial ascorbate concentrations in three groups of patients at autopsy: (a) sudden traumatic deaths;
(b) those dying after supplementation with ascorbic acid prior to death; (c) routine hospital autopsies. They recorded minimal or undetectable levels of ascorbate in the latter group, none of whom had shown signs of clinical scurvy (Willis and Fishman, 1955). This localised depletion of ascorbate was apparent in areas of mechanical stress, areas known to be primarily susceptible to atheroma (Velican and Velican, 1976). These early studies suggested a fundamental similarity in mechanisms of vascular damage in both scurvy and atherosclerosis.

The influence of ascorbic acid in the pathogenesis of atherosclerosis is at least two-fold. Firstly, it is known that ascorbic acid is essential to the integrity of collagen biosynthesis in the vascular wall (Gore et al., 1965) and to glycosaminoglycan composition of the aorta (Nambisam and Kurup, 1975). Thus the development of structural abnormalities in the arterial wall is likely, (Willis, 1957; Gore et al., 1965). Secondly, this vitamin plays an important role in cholesterol metabolism, a relationship which has been the subject of innumerable investigations (reviewed by Krumdieck and Butterworth, 1974; Turley et al., 1976).

Animal Studies

The type of animal model used in many of these studies has resulted in conflicting data which is difficult to interpret. Animals such as the rat, hen and rabbit have been used (Kirk, 1973; Nockels, 1973). These species however are able to synthesise ascorbic acid. Thus, assuming their tissues were saturated with the vitamin, any parameters studied under
conditions of experimental hypercholesterolaemia should be independent of its influence. In man, there is an absolute dietary requirement for ascorbic acid, due to the absence of the enzyme L-gulonolactone oxidase in the biosynthetic pathway (Burns et al., 1956; Burns, 1957; Nishikimi and Udenfriend, 1976). The non-human primate, the guinea pig and several species of bird have a similar metabolic defect and are thus more suitable experimental models. Numerous studies have used the acutely scorbutic guinea pig, although the inanition induced by this condition tends to mask the more subtle metabolic effects encountered in chronic ascorbate deficiency. This may account for the conflicting results emanating from these studies (Bolker et al., 1956; Lahiri and Bannerjee, 1956; Bannerjee and Singh, 1958; Ginter et al., 1965).

It has been suggested that the study of cholesterol metabolism under conditions of chronic ascorbate deficiency is more relevant to man, as acute or clinical scurvy is rare in human populations. Latent or subclinical deficiencies, arising from a chronic dietary inadequacy of the vitamin, are however quite prevalent, notably in certain population groups such as the elderly (Booth and Todd, 1972) and those with a seasonal variation in ascorbic acid intake (Ginter et al., 1970). As a consequence, Ginter and his colleagues have developed an improved experimental model (Ginter et al., 1968), in which a condition of chronic deficiency or hypovitaminosis is induced in the guinea pig by the daily administration of 0.5mg of ascorbic acid, which is adequate to maintain food intake, some growth and to prevent the overt symptoms of scurvy. This model
is considered to simulate the latent deficiency state observed in man (Ginter, 1975a; Hughes, 1976).

Ginter demonstrated an hepatic accumulation of cholesterol in these animals (Ginter and Nemec, 1969; Ginter et al., 1969), an effect augmented by dietary supplementation with cholesterol which also led to aortic deposition of lipid (Ginter, 1975b). As the corollary to this, cholesterol feeding increased the ascorbic acid requirement of the guinea pig (Ginter and Zloch, 1972) and eventually resulted in a depletion of tissue ascorbic acid (Ginter, 1976).

**Human Studies**

In man, investigations of this relationship have yielded equivocal results. Clinical trials executed in Eastern European countries, where the vitamin was fed at levels approximately tenfold of those required to prevent scurvy, demonstrated a reduction of serum cholesterol in hypercholesterolaemic patients (Turley et al., 1976). In general, supplementation of normocholesterolaemic individuals with ascorbic acid did not alter serum cholesterol levels (Anderson et al., 1958; Sokoloff et al., 1966; Crawford et al., 1975), although Fix et al. (1974) and Kothari and Jain (1977) did observe a slight diminution. Furthermore, other workers were not even able to achieve any reduction in cholesterolama in hypercholesterolaemic subjects (Samuel and Shalchi, 1964; Peterson et al., 1975). Contrary to all these findings, Spittle (1971), using "megadoses" of ascorbic acid, succeeded in decreasing the serum cholesterol concentration in young, normocholesterolaemic subjects, but
reported its elevation in atherosclerotic patients. She attributed this latter finding to a mobilisation of arterial cholesterol, although this view was challenged by Morin (1972). All these studies can be criticised as they rarely provide information regarding the ascorbic acid status of the subjects concerned. Differing degrees of tissue saturation could contribute to the lack of conformity of the data. In support of this, Ginter et al. (1977) have postulated that ascorbic acid is probably only important as a hypocholesterolaemic agent in the presence of a chronic latent deficiency.

Elwood et al. (1970a) attempted unsuccessfully to correlate plasma ascorbic acid and cholesterol concentration in normocholesterolaemic individuals. Nevertheless, Gatenby-Davies and Newton (1974), in a study undertaken in pastoral African tribesmen, did demonstrate a positive correlation between these two parameters, although the statistical treatment of their results has been criticised (Turley et al., 1976). Ginter and his coworkers (1970) noted a definite seasonal occurrence of hypercholesterolaemia which corresponded to times when food sources of ascorbate were limited in availability; this was in accordance with the work of Fyfe et al. (1968).

Mode of Action of Ascorbic Acid

The mechanism of the interaction between ascorbic acid and cholesterol metabolism has also been extensively investigated. An increase in absorption of cholesterol does not appear to account for the hypercholesterolaemia. Ginter and his colleagues (1969), using the chronically deficient guinea pig, followed
the distribution of an intragastric dose of $^{4-14}$C cholesterol. Compared to controls, activities in the blood and livers of these animals were low, whilst those of the gastrointestinal tract and stools were high. Rather than enhancing the process, ascorbate deficiency appeared to slightly inhibit cholesterol absorption, a finding consonant with that of Bronte-Stewart et al. (1963) in acutely scorbutic humans.

The role played by ascorbic acid in cholesterol biosynthesis is uncertain. In acutely scorbutic guinea pigs, increased biosynthesis has been observed (Becker et al., 1953), although Bolker et al. (1956) failed to confirm this. Similarly in chronically deficient guinea pigs and baboons, hepatic cholesterolgenesis, as measured by $^{1-14}$C acetate incorporation, was unchanged (Ginter and Nemec, 1969; Weight et al., 1974). More recently however, Kallner (1977) reported a preliminary finding of a reduction in hepatic hydroxymethylglutaryl CoA reductase activity in chronically deficient guinea pigs. They also found a reduction in cholesterol catabolism in the same animals and suggest that the observed hepatic cholesterol accumulation is a function of an ascorbate-induced defect in both pathways.

Studies investigating the role of ascorbic acid in bile acid biosynthesis have been more definitive, indicating a decreased biosynthesis in both acutely (Guchait et al., 1963) and chronically (Ginter et al., 1971) deficient guinea pigs. When $^{4-14}$C cholesterol was administered to these latter animals, there was a diminished excretion of label in the bile fraction of liver, gall bladder bile and faeces. Similarly, after administration of $^{26-14}$C cholesterol there was a lower
recovery of $^{14}\text{C}O_2$, which could be reversed by repletion with ascorbic acid (Ginter et al., 1972). Using this technique, Ginter (1973) estimated bile acid biosynthesis and showed that on a body weight basis, significantly less bile acids were synthesised by deficient animals (8.3mg/day) as compared to controls (11.8mg/day). The rate of synthesis was positively correlated with liver ascorbate concentration and as expected, negatively correlated with plasma and liver cholesterol concentration. In support of this, Hornig and Weiser (1976) have demonstrated a negative correlation between liver ascorbic acid and liver total bile acid content.

A further aspect of cholesterol metabolism has been explored by Iwamoto et al. (1978). They demonstrated an inhibition of bile acid absorption by ascorbic acid. The results of this study and those discussed above do not mutually exclude each other, as a reduction in bile acid absorption would enhance synthesis by releasing the feedback mechanism, although this might also be expected to enhance the biosynthesis of cholesterol per se.

The site and nature of the interaction between ascorbate and bile acid biosynthesis remains to be firmly established. Initial investigations concentrated on the mitochondrial side chain cleavage reaction. Guchait et al. (1963) demonstrated an enhanced synthesis of bile acids in vitro by guinea pig mitochondria isolated from livers of deficient animals and incubated with ascorbic acid; Kritchevsky et al. (1973b) using normal mitochondria, could not repeat this. Guchait's experiment has been criticised (Turley et al., 1976) in that
it may not have measured the normal biosynthetic pathway via 7α-hydroxycholesterol, but the alternative proposed by Mitropoulos and Myant (1967), where side chain cleavage is the initial reaction.

Ginter (1975b) has hypothesised that the rate-limiting enzyme of bile acid biosynthesis, cholesterol 7α-hydroxylase might be the site of action of ascorbic acid. In comparing the oxidation of $[26-^{14}C]$cholesterol and 7α-hydroxy cholesterol he observed that whereas the oxidation of cholesterol was significantly lower in the chronically deficient guinea pigs, that of the hydroxylated metabolite was unaffected. This indicated that the site of ascorbic acid intervention was at or before the rate-limiting enzyme. More recently, Bjorkhem and Kallner (1976) showed that 7α-hydroxylation in microsomes from chronically deficient animals was lower than that in supplemented animals but no stimulation by ascorbate added in vitro was found. This is contrary to the findings of Kritchevsky et al. (1973b) who reported a marginal in vitro stimulation.

Ascorbic Acid and Mixed Function Oxidase Metabolism

Ascorbic acid is known to play an important role in mixed function oxidase metabolism. Both in vivo and in vitro investigations have shown that acute ascorbate deficiency results in the impaired metabolism of a variety of xenobiotics and steroids (reviewed by Zannoni and Lynch, 1973; Zannoni and Sato, 1976). Reactions such as O- and N- demethylation and hydroxylation were decreased (50-70%), as were the individual electron transport components cytochrome P-450 (40%) and
NADPH-cytochrome c reductase (85%), after 21 days of deficiency. Repletion of the deficient animals with ascorbate for 6 to 10 days resulted in a restoration of enzyme activities (Zannoni et al., 1972). Studies with foetal guinea pig livers have shown a correlation between drug metabolism activities, the quantity of electron transport components and liver ascorbate concentration (Zannoni and Sato, 1975). In fact, weanling animals seem to be particularly sensitive to this effect of ascorbate deficiency (Sato and Zannoni, 1974).

The mechanism of the depression in mixed function oxidase metabolism is uncertain. Ascorbate deficiency has been shown to have a qualitative as well as a quantitative effect on cytochrome P-450. Type I and type II substrate binding spectra in microsomes isolated from deficient animals were shown to be atypical (Zannoni et al., 1972; Gundermann et al., 1973). The Km values for aniline, aminopyrine and p-nitroanisole were also higher (Zannoni et al., 1972). It has also been suggested that ascorbate deficiency might influence the phosphatidylcholine content of the enzyme system (Zannoni, 1977).

The capacity for induction of these enzymes is not impaired in ascorbate-deficient animals (Wade et al., 1972; Zannoni et al., 1972). Luft et al. (1972) showed that administration of δ-aminolevulinic acid, a precursor of haem synthesis, led to increased cytochrome levels in deficient guinea pigs, indicating the participation of ascorbic acid in haem biosynthesis.

In view of these findings, it would seem feasible that the site of action of ascorbic acid in bile acid biosynthesis could be cholesterol 7α-hydroxylase. Further indirect evidence in
this respect was contributed by Ginter (1975b), who showed a parallel increase in levels of cytochrome P-450 and the oxidation of $[26^{-14}C]$ cholesterol, following the administration of ascorbic acid to deficient guinea pigs.

Megadoses of Ascorbic Acid

The magnitude of the daily dose of ascorbic acid required to maintain normal cholesterol metabolism is still uncertain. Over the last decade, the concept of "megavitamin therapy" has emerged. The term "megatherapy" is itself rather ill-defined, but would imply ingestion of the vitamin in excess of the daily recommended intake (for ascorbate in man 30mg U.K. 45mg U.S.A.). Various authors however have different concepts of these levels. The original thesis for ascorbic acid "megatherapy" was proposed by Stone (1965) and elaborated by Pauling (1968), who concluded that the recommended intakes for this vitamin were set far too low. From studies in animals synthesising ascorbic acid, Stone proposed that intakes should be 50 to 500 times those recommended. However others such as Ginter (1975b) have employed levels of 1g per day as "megadoses".

Baker et al. (1971b) reported that only a small proportion of an administered labelled "megadose" was incorporated into body pools. Similarly, the administration of either 80mg or 1g of ascorbate per day to women resulted in similar leucocyte ascorbate levels (Baird et al., 1976). Thus a "megadose" is apparently not required for tissue saturation, in accordance with the conclusions of earlier workers (Chatterjee, 1967; Vitler, 1967), who suggested that a daily intake of 100-200mg
was adequate.

To date, it has been widely assumed that owing to its water soluble nature, ascorbic acid is excreted in the urine at high levels of intake and is thus harmless. However, information pertaining to its toxicity at high dose levels is accumulating. One associated problem is that on cessation of "megatherapy", certain individuals may manifest symptoms of overt scurvy (Rhead and Schrauzer, 1971). Indeed, the same workers reported that blood levels of the vitamin, in a subject given 1-3g daily for up to 54 months fell below control levels after discontinuation of therapy (Schrauzer and Rhead, 1973). However, studies in guinea pigs are at variance with these findings (Hornig et al., 1973; Nandi et al., 1973).

A further complication of "megadoses" of ascorbic acid was discussed by Brown (1973). He demonstrated a disturbance of bone metabolism in chicks, following the administration of high doses; supporting data in man however is lacking. "Megadoses" of the vitamin have also led to an increased urinary excretion of oxalic acid and in some individuals to the formation of urinary calculi (Briggs, 1976). Similarly uric acid stones were reported in man after the chronic daily administration of 8g of ascorbate (Stein et al., 1976). In addition, an haemolytic effect of ascorbic acid (5g/day) has been observed in healthy volunteers (Mengel and Green, 1976).

A further complication of ascorbic acid "megatherapy" has been proposed by Basu (1977). To some extent, ascorbic acid is metabolised to the sulphate in man (Baker et al., 1971a). Thus, by depleting sulphur amino acids, high levels of ascorbic acid may
potentially interfere with the metabolism of some foreign compounds and amino acids.

There is as yet little evidence available regarding the effect of "megadoses" of ascorbic acid on the mixed function oxidase enzyme system. However, it is recognised that high concentrations of ascorbate in vitro are capable of generating free radicals and stimulating lipid peroxidation (Ottolenghi, 1959; Thiele and Huff, 1960). One might therefore hypothesise that high concentrations of ascorbate in vivo could lead to an increase in peroxide-induced membrane damage and subsequent disruption of the microsomal enzyme system, thus altering the metabolism of both foreign and endogenous substances. Similar changes might occur in the absence of the vitamin, as paradoxically, ascorbate at lower concentrations protects against oxidation (Bielski et al., 1975). It could be envisaged that the vitamin might be required within a certain limited range of concentration to maintain normal membrane structure and function. Divergence from this concentration in either direction could result in aberrations within the membrane, ultimately interfering with the metabolism of foreign compounds and endogenous substances such as cholesterol.

In order to investigate this hypothesis, experiments were designed to examine the effects of various levels of deficiency and excess of the vitamin. Data regarding the modulatory effect of ascorbic acid on cholesterol 7α-hydroxylase activity is limited, the experimental design of the studies from which it is derived being open to criticism, particularly with respect to dietary control. It thus seemed desirable to conduct a more
definitive study, in which serum and hepatic concentrations of ascorbate, cholesterol and bile acids, together with indices of hepatic xenobiotic and bile acid metabolism were measured in the same animals.
Materials

These were as described in Chapter 2, with the addition of the following:

L-ascorbic acid for dietary administration was supplied by Fisons and for analytical purposes by B.D.H.

Activated charcoal (Norit) acid-washed and O-phthalaldehyde were supplied by Sigma.

Malonaldehyde bis (-dimethyl acetal) (1,1,3,3-tetramethoxy-propane), for use as a standard, was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Standard bile acids were obtained from Steraloids Ltd., Croydon, Surrey.

\[ \text{[} { }^{14}\text{C}] \text{ sodium glycocholate 16 mCi/mmol was supplied by the Radiochemical Centre, Amersham.} \]

NADP was obtained from P-L Biochemicals Inc.

Methods

These were also as described in Chapter 2, with the exception of the following:

I Blood Analysis

(a) Serum Total Cholesterol Concentration

Owing to the expense of the method used in Chapter 2, the O-phthalaldehyde method (Rudel and Morris, 1973) was employed.

This method involves a preliminary saponification of the serum, followed by extraction of the non-saponifiable lipid with hexane and reaction of the dried extract with O-phthalal-
dehyde to form a cholesterol-specific coloured product which absorbs at 550nm.

A 0.1ml aliquot of serum, 0.3ml of 33%(w/v) KOH and 3ml of 95%(v/v) ethanol were placed in a 15ml glass sovirel tube. After thorough mixing, the tube was placed in a heating block at 60°C for 15 minutes. A blank, consisting of 0.1ml of absolute ethanol and 0.1ml of cholesterol standard (1mg/ml absolute ethanol) were saponified in parallel (see page ). After cooling the tubes, a 10ml volume of hexane was added forcefully to each; followed by 3ml of distilled water. The tubes were shaken for 1 minute on a rotary shaker to ensure complete mixing. 1ml aliquots of each hexane layer were pipetted in duplicate into 10ml test tubes and the solvent evaporated under nitrogen.

A 2ml volume of 0-phthalaldehyde reagent (50mg 0-phthalaldehyde/100ml glacial acetic acid) was added to each tube, followed by thorough mixing to dissolve the residue. Approximately 10 minutes after this addition, 1ml of concentrated sulphuric acid was slowly added, immediately followed by vigorous mixing. The extinction of the solutions at 550nm was measured 10 to 30 minutes after addition of the acid, using the Cecil spectrophotometer.

(b) Serum Triglyceride Concentration

This was measured using a fully enzymatic, automated method No. 126012 (Boehringer Mannheim GmbH). This method is the same as that described in Chapter 2 (Wahlefeld, 1974).
(c) **Plasma Total Ascorbic Acid Concentration**

Plasma ascorbic acid was measured according to the method of Denson and Bowers (1961). Total ascorbic acid i.e. ascorbic acid, dehydroascorbic acid and diketogulonic acid is determined by coupling with 2, 4-dinitrophenylhydrazine, following oxidation of the ascorbic acid. The resultant orange-coloured addition compound is dissolved in sulphuric acid and absorbs at 520nm.

Plasma was prepared by collecting blood into polystyrene tubes containing EDTA (Teklab, Sacreston, Durham), mixing thoroughly and centrifuging for 10 minutes on a bench centrifuge.

Duplicate 0.5ml aliquots of plasma were transferred to small polystyrene tubes (LP3) containing 2ml of 5% (w/v) trichloroacetic acid. The tubes were thoroughly mixed and spun for 10 minutes on a bench centrifuge. The supernatants were transferred to clean LP3 tubes and subsequently stored overnight at 0-4°C or for up to 1 week at -20°C, prior to determination of the ascorbic acid.

A 1.5ml aliquot of supernatant was pipetted into a test tube containing 0.5ml of working 2, 4-dinitrophenylhydrazine reagent (2, 4-dinitrophenylhydrazine stock solution, 2.2% (w/v) in 10N sulphuric acid, 5% (w/v) thiourea, 0.6% (w/v) copper sulphate, 100:5:5 by volume). Samples and blanks (1.5ml 5% trichloroacetic acid) were set up in duplicate. Replicate ascorbic acid standards (4μg/ml 5% trichloroacetic acid) were prepared; duplicate aliquots of both of these were run in parallel with the samples and blanks. *After thorough

*The relationship between cholesterol and ascorbate concentration and extinction could be shown to be linear over a range of concentrations greater than the extremes of variability encountered.
mixing, all tubes were stoppered and incubated in a water bath at 37°C for 4 hours. After cooling in an ice-bath, a 2.5ml volume of ice-cold 65% (v/v) sulphuric acid was slowly added to each tube with shaking. The solutions were thoroughly mixed and allowed to equilibrate to room temperature (at least 30 minutes). The extinctions of all solutions were determined at 520nm using the Cecil spectrophotometer.

II Liver Analysis

(a) Preparation of Liver Fractions

This was as described in Chapter 2, with the exception of certain details.

The homogenisation medium was 0.02M Tris/1.15% KCl, pH 7.4. Homogenisation was carried out using a Potter homogeniser in 4 volumes of buffer, to give a 20% homogenate as described previously.

The microsomal pellet was obtained by centrifugation at 170,000 g av. for 35 minutes at 2°C (Beckman Model L5-65 ultracentrifuge, 8 x 25ml angle rotor, 50,000 r.p.m).

(b) Measurement of Total Lipid Content

The method used in Chapter 2, was modified for use on the 20% homogenate.

A 5ml aliquot of homogenate was mixed with 20ml of chloroform: methanol (1:1, v/v) and extracted for 10 minutes on the rotary shaker. After filtering through glass wool,
a 10ml volume of clear filtrate was added to 3.75ml of methanol followed by 6.25ml of water in a universal container and mixed by inversion. After centrifuging for 5 minutes at 2,000 r.p.m., the upper aqueous layer was removed by aspiration. Total lipid was estimated as described previously.

(c) **Measurement of Total Liver Cholesterol**

2ml of the chloroform layer from II(b) was evaporated to dryness in a "pear-shaped" flask in vacuo at 40°C, on a rotary evaporator. The residue was taken up in 200μl of absolute ethanol and 100μl aliquots saponified and treated as described for serum cholesterol.

(d) **Liver Ascorbic Acid Concentration**

5ml aliquots of liver homogenate were precipitated with 4 volumes of 5% (w/v) trichloroacetic acid, mixed and spun for 10 minutes in a bench centrifuge. 10ml volumes of supernatant were transferred to glass screw-capped vials and routinely stored at -20°C for up to 1 week. Acid-washed Norit (0.33g) was added to the supernatant and the mixture shaken and filtered (Whatman No 1, size 11). Ascorbic acid concentration was determined in duplicate 1.5ml aliquots using the procedure described for plasma.

(e) **Measurement of Cholesterol 7α-hydroxylase Activity**

As a result of work carried out in this department in optimisation of the conditions for this assay, the incubation system was modified as follows:
<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomal supernatant(ml)</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>Boiled microsomal supernatant(ml)</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>(\beta)-mercaptoethylamine 140mM(ml)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Substrate(ml)*</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Cofactor solution(ml)**</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Substrate: 0.5µCi \([4-^{14}C]\) cholesterol stabilised in Tween 80 (7.2mg) and dissolved in 2.0ml of 0.1M potassium phosphate buffer, pH 7.4.

** Cofactor solution: NADP, 5µmol and glucose-6-phosphate, 50µmol, dissolved in 1.0ml of distilled water.

The first 3 constituents were pre-incubated at room temperature in 25ml conical flasks for 10 minutes before the reaction was initiated by the addition of the cofactor solution. The flasks were subsequently incubated at 37°C for 60 minutes with shaking.

The extraction procedure and thin-layer chromatography of the extracts were as described previously. However single chromatoplates were run and the radioactive bands localised by autoradiography for 4-6 weeks, rather than by the colorimetric procedure. Counting conditions were as described previously.

(f) Measurement of p-Nitroanisole O-Demethylase Activity

An assay system based on that of Netter and Seidel (1964) was used, in which the formation of p-aminophenol is followed at 417nm.
Two 1cm glass cuvettes were set up as follows:

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl 0.05M, pH 7.85</td>
<td>1.4 ml</td>
</tr>
<tr>
<td>Microsomal supernatant</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>* Substrate</td>
<td>0.005 ml</td>
</tr>
<tr>
<td>MgCl₂ 0.1M</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

* Substrate: p-nitroanisole 500mM in dimethyl formamide, added after temperature equilibration.

The NADPH generating system was prepared in 0.05M Tris-HCl buffer, each 0.5ml contained NADP, 3mM and glucose-6-phosphate, 30mM (as microsomal supernatant was used, no glucose-6-phosphate dehydrogenase was added).

The cuvettes were placed in the forward cell compartment of the SP 1800 spectrophotometer, at 37°C, for 5 minutes to allow temperature equilibration. After zeroing the recorder, 0.5ml of buffer was added to the reference cuvette, and the reaction started by the addition of 0.5ml of NADPH generating system to the test cuvette. The increase in absorption at 417nm was followed and from the linear phase of the reaction, p-nitroanisole O-demethylase activity was calculated using an extinction coefficient of 6.8 mM⁻¹ cm⁻¹.

(g) Measurement of Microsomal Lipid Peroxidation

Lipid peroxidation was measured using a modification of the method used by Slater and Sawyer (1971). This is based on the in vitro measurement of malonaldehyde, the product of
peroxidation of polyunsaturated fatty acids, after its reaction with thiobarbituric acid. The following incubation system was used:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer* (ml)</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>Microsomal suspension (ml)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>0.02M Tris/1.15% KCl pH 7.4 (ml)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Cofactor solution** (ml)</td>
<td>0.20</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubation volume 2.5 ml

* Buffer = 65 mM KCl/57 mM Tris pH 8.3
** Each 0.2 ml of buffer contained NADP 2 μmol, glucose-6-phosphate 15 μmol and glucose-6-phosphate dehydrogenase 1 unit.

Duplicate tubes were incubated for 20 minutes at 37°C with shaking. The reaction was terminated by the addition of 5 ml of 10% (w/v) trichloroacetic acid and the tubes centrifuged for 10 minutes in a bench centrifuge.

A 2 ml aliquot of supernatant was mixed with 2 ml of 0.67% (w/v) thiobarbituric acid in a clean test tube and incubated for 10 minutes in a water bath at 100°C. Duplicate tubes were set up, together with blanks (10% trichloroacetic acid) and a range of standards (tetramethoxypropane, 0.25-2.5 μg/ml 10% trichloroacetic acid). The resultant pink colours were measured at 535 nm, using the Cecil spectrophotometer.

(h) Glucose-6-Phosphatase Histochemistry

Glucose-6-phosphatase activity can be examined histochemi-
cally in cryostat sections of liver and may provide qualitative
data on the intracellular alteration in enzyme distribution
and activity.

Small pieces of liver (approximately 4mm cube) were cut
from the centre of the left lobe and frozen on to microtome
chucks standing in a shallow dish containing an ethanol/solid
carbon dioxide freezing mixture. These tissue blocks could
be stored at -20°C for at least 48 hours, without detectable
loss of enzyme activity. 10μsections were cut using a cryostat
fitted with a rotary, rocking microtome (Bright Instruments,
Huntingdon), at -20°C and directly transferred to glass
microscope slides.

Enzyme activity was measured by the method of Wachstein
and Meisel (1956), in which inorganic phosphate released from
the substrate is "captured" by lead ions in the medium and
precipitated as lead phosphate. This is converted to the
readily visible black lead sulphide by treatment with ammonium
sulphide.

Sections of liver were incubated at 37°C for 30 minutes
in 50ml of solution containing Tris-maleate buffer pH 6.7,
40mM; lead nitrate 4.5mM; glucose-6-phosphate 1.5mM. After
incubation, slides were twice washed in distilled water and
treated as follows:
1) Dilute ammonium sulphide solution (2-4 drops stock B.D.H.
solution in approximately 50ml of distilled water), for
2 minutes.
2) Distilled water for 2 minutes.
3) 6% (v/v) formaldehyde solution for 2 minutes.
4) Distilled water for 2 minutes.
Sections were mounted in glycerol jelly and examined in a light microscope. Sites of glucose-6-phosphatase activity were stained dark brown.

III  Biliary Analysis

(a) Biliary Cholesterol Concentration
The cholesterol concentration of gall bladder bile was measured by a modification of the method for serum. 200μl of bile was saponified and extracted into 10ml of hexane, 3ml of the hexane layer was evaporated to dryness in a stream of nitrogen and the cholesterol content determined by the 0-phthalaldehyde method.

(b) Biliary Bile Acid Concentration
For determination of total bile acid concentration, bile samples were diluted with 50% aqueous methanol, pH 10.0 (1 in 250) and 50μl aliquots taken for assay using the enzyme-fluorimetric method described for serum in Chapter 2.

For a qualitative and quantitative assessment of the individual bile acids present, a method was developed which used a thin-layer chromatographic separation, followed by elution of appropriate bands from the plate and subsequent quantification using the same technique as above.

A 20μl aliquot of bile was applied as a narrow 1 cm band to the origin of a thin-layer plate (Silica gel G). Two solvent systems were used. A propionic acid: isoamyl acetate: water:
n-propanol (3:4:1:2, v/v); B 2,2,4-trimethylpentane: di-isopropyl ether: acetic acid: isopropanol (2:1:1:1, v/v) (Beke et al., 1976). Ascending chromatography in saturated air was used. The plate was run in solvent system A for approximately 60 minutes at room temperature (20°C), or until the solvent front had reached a distance of approximately 10 cm from the origin. The plate was removed from the tank and thoroughly dried in a cold air stream. A second development in solvent system A was followed by development in solvent system B for approximately 90 minutes at room temperature, or until the solvent front had reached 16-17 cm from the origin. The first 2 developments permit separation of the conjugated bile acids, whilst the third separates the free bile acids.

Plates were run in duplicate and after chromatography one was visualised by spraying with a solution of phosphomolybdic acid (20% w/v in absolute alcohol), followed by gentle heating. Identification of the individual bile acids was achieved by comparison with a mixture of conjugated and free standard bile acids ran on the same plate.

In order to quantify the component bile acids, appropriate bands on the second plate were scraped into 10ml glass sovirel tubes and extracted with 5ml of methanol: glycine-NAOH 0.005M pH 9.7 (3:1, v/v) for 10 minutes on a rotary shaker. The extraction was repeated with a further 5ml of buffered methanol, the combined extracts transferred to "pear-shaped" flasks and evaporated to dryness in vacuo using a rotary evaporator at 30-40°C. Residues were taken up in 200μl of 50% aqueous methanol, pH 10.0 and 50μl aliquots taken for assay. Total
recovery of the procedure was measured by the addition of a known quantity of $[^{14}\text{C}]$ sodium glycocholate to the bile, prior to chromatography and was of the order of 85-95%.

IV  **Aortic Histology**

Hearts and aortas were fixed in buffered formalin (10% v/v formaldehyde in 0.05M sodium phosphate buffer), dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections of approximately 5μ thickness were cut on a base-sledge microtome (M.S.E. Ltd.) and stained with haematoxylin and eosin (Drury and Wallington, 1967). Further pieces of tissue were embedded in gelatin and sections of 12-15μ thickness cut on a freezing microtome (Leitz) and stained with Oil Red O (Drury and Wallington, 1967).

V  **Dietary Analysis**

Ascorbic acid content of the guinea pig diet was determined after extraction with metaphosphoric acid by titration with a standardized dye 2,6-dichlorophenolindophenol, which is quantitatively reduced by L-ascorbic acid in acid solution.

Duplicate 20g aliquots of diet were extracted by maceration in 350ml of 5% (w/v) metaphosphoric acid for approximately 1 minute in a liquidiser (Kenwood Instruments, Thorn Appliances, Havant, Hants). The resultant homogenate was spun for 10 minutes in a bench centrifuge. A 25ml aliquot of supernatant was transferred to a 50ml conical flask and 1ml of 50% (v/v) sulphuric acid followed by 2.9ml of formaldehyde were added. After thorough mixing, the solutions were allowed to stand for
8 to 10 minutes. They were subsequently titrated against a solution of 0.02% (w/v) dichlorophenolindophenol together with duplicate blanks of 5% metaphosphoric acid and standard ascorbic acid solutions (200μg/ml in 5% metaphosphoric acid). On addition of the dye it is decolored by ascorbic acid, the end point of the titration is taken when a pink colouration persists for at least 5-10 seconds.

Animals and Experimental Procedure

For all experiments, male guinea pigs of the Dunkin Hartley strain, 5-6 weeks old (300-400g), supplied by the Animal Virus Research Institute, Pirbright, Surrey, were used. They were housed in alloy cages, measuring 53 cm x 114 cm x 25 cm, over sawdust which was changed twice a week.

Animals were allowed access to diet and tap water ad libitum at all times. The temperature and relative humidity of the animal house was regulated to 22°C and 50%, respectively and lighting was time-switched on at 06.30 hours and off at 18.30 hours. Measurements of weight and food intake were made at regular intervals, no allowance was made for spillage of diet as this was found to be minimal and relatively constant between groups. The guinea pigs were maintained on stock diet (FD1 pellets, Labsure Animal Diets, supplied by J. Lillico and Sons, Reigate, Surrey) for 3-7 days to allow acclimatisation to the animal house conditions.
Experiment 1 (Chronic Deficiency)

The guinea pigs were randomly assigned to three experimental groups with 18 to each group, 9 to each cage.

The groups were A (ascorbate-deficient), B (control) and C (ascorbate-supplemented). All animals were fed on ascorbic acid-free pelleted guinea pig diet (Labsure Animal Diets, Christopher Hill Group Ltd., Poole, Dorset), shown by analysis to contain 2mg ascorbic acid/100g diet. Diets were supplemented by oral dosing with ascorbic acid dissolved in 20% (w/v) sucrose solution as follows: Group A 0.5mg/day; Group B 30mg/kg body weight/day (20mg/ml); Group C 300mg/kg body weight/day (200mg/ml).* At the beginning of the experimental period, Group A was totally depleted of ascorbic acid for 3 weeks, when signs of deficiency became apparent. During this period, these animals were dosed with sucrose solution alone. The animals were dosed orally for 5 out of 7 days, for the remaining 2 days, ascorbic acid was dissolved in the drinking water as follows: Group A 50mg/litre; Group B 600mg/litre; Group C 6g/litre,** assuming an approximate fluid intake of 30ml per animal per day.

The original experimental design was such that 6 animals from each group should have been killed after 6 and 12 weeks of treatment. The animals in both experimental groups would then have been given control levels of ascorbic acid in an attempt to reverse any induced changes. However, this design was necessarily modified and the 6 week interval omitted due to the loss of 6 animals from Group A at an early stage. These animals were suffering from eruptive lesions in the rear limbs, apparently arising from fractures sustained at an early post-natal stage and presumably exacerbated by the

* Increased to 333mg/ml at week 13; ** Increased to 9g/l at week 13.
deficiency of ascorbic acid.

A further difficulty was encountered in maintaining the Group A animals on 0.5mg ascorbate per day as suggested by Ginter (1968). It was impossible to maintain the body weight and food intake of these animals and overt signs of scurvy became apparent. To prevent these changes, a daily intake of 1.5mg ascorbate was required.

Six animals from each group were thus killed after 12 weeks of treatment and as a consequence of the experimental findings, the daily dose of ascorbate in Group A animals was again reduced to 0.5mg per day and that in Group C increased to 500mg/kg body weight per day.

At both time intervals, animals were killed after an 18 hour fast by exsanguination via cardiac puncture under mild ether anaesthesia, followed by cervical dislocation. Approximately 2.5ml of blood was collected for preparation of plasma as described above. The remainder was collected in non-heparinised bottles for preparation of serum. The livers, hearts and aortas of each animal were removed and treated as described in Chapter 2. Bile was collected from each gallbladder and stored at -20°C prior to analysis.
Results

Animal Weights and Food Intakes

Growth curves and food intake data are presented in Fig. 3.1 and Table 3.1.

Following depletion of ascorbic acid, the mean growth rate of the animals in Group A began to decline. Administration of a daily dose of 0.5mg ascorbate per animal did not prevent this decline when commenced at week 3. A dose of between 1.0 and 2.0mg per day was required to maintain food intake and growth and prevent obvious signs of deficiency in these animals. Thus by week 7, all animals in Group A were established on a daily dose of 1.5mg ascorbic acid. Although the difference in mean body weight between Groups A and B became statistically highly significant by week 5 of the experiment and remained so throughout, the actual food intakes and rates of growth were similar until week 17. At this point Group A was again totally depleted of ascorbic acid and despite the introduction of a daily dose of 0.5mg at week 20, these animals did not increase their food intake and began to lose weight.

With respect to Group C, mean body weight, growth rate and food intake did not differ from the control group until week 11 when mean daily food intake suddenly diminished to 89% of the control. It should be noted that the food intake of the control group also dropped at this time, but to a lesser extent. By week 14, food intake reached control levels but subsequently fell (79-88% control) until the end of the
Fig. 3.1 Growth Curves of Guinea Pigs Maintained on Ascorbic Acid (AA)-Deficient \( \triangle \) - \( \triangle \) (A) Control
\( \bullet \) - (B) and Ascorbic Acid-Supplemented \( \blacksquare \) - (C) Diets (Experiment 1)

Points represent mean values. Significant differences between means are shown: \( b = p < 0.01 \) \( c = p < 0.001 \)
<table>
<thead>
<tr>
<th>GROUP</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>34</td>
<td>36</td>
<td>36</td>
<td>27</td>
<td>28</td>
<td>49</td>
<td>43</td>
<td>44</td>
<td>45</td>
<td>48</td>
<td>46</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>44</td>
<td>50</td>
<td>53</td>
<td>48</td>
<td>31</td>
<td>38</td>
<td>36</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>B**</td>
<td>33</td>
<td>35</td>
<td>36</td>
<td>41</td>
<td>42</td>
<td>42</td>
<td>42</td>
<td>54</td>
<td>50</td>
<td>47</td>
<td>44</td>
<td>44</td>
<td>-</td>
<td>51</td>
<td>-</td>
<td>58</td>
<td>63</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>61</td>
<td>51</td>
<td>55</td>
</tr>
<tr>
<td>C***</td>
<td>33</td>
<td>38</td>
<td>37</td>
<td>40</td>
<td>42</td>
<td>42</td>
<td>45</td>
<td>50</td>
<td>46</td>
<td>50</td>
<td>39</td>
<td>-</td>
<td>56</td>
<td>-</td>
<td>53</td>
<td>50</td>
<td>50</td>
<td>52</td>
<td>53</td>
<td>54</td>
<td>51</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

* ascorbate-deficient group (1.5mg/day at 12 weeks; 0.5mg/day at 23 weeks)

** control group (30mg/kg body weight/day at 12 and 23 weeks)

*** ascorbate-supplemented group (300mg/kg body weight/day at 12 weeks; 500mg/kg body weight/day at 23 weeks)
experiment. This drop in food intake led to a small reduction in growth rate, although the difference in mean body weights was not statistically significant.

Biochemical Changes Occurring in Animals on the Ascorbate-Deficient Diet (Group A)

These data are presented in Tables 3.2-3.7.

After administration of the deficient regime for 12 weeks, there was a significant reduction in both plasma and liver ascorbate concentrations. After 23 weeks, there was an even greater reduction in these parameters.

Serum total cholesterol concentrations did not differ from control at 12 weeks. Liver cholesterol content was slightly elevated, although the difference was not statistically significant. Serum triglyceride, liver total lipid and biliary cholesterol concentrations showed no variation from controls. After 23 weeks however, serum total cholesterol became significantly elevated although the liver concentration was unchanged. Serum triglycerides were also raised but the difference was statistically non-significant. The other parameters of lipid metabolism were unaltered.

Both serum and biliary total bile acids were marginally reduced at 12 weeks; by 23 weeks the difference was no longer apparent. At 12 weeks the principal component bile acids were estimated both qualitatively and quantitatively but no changes were evident.
Table 3.2 Effect of Ascorbic Acid Depletion and Supplementation on Some Serum Parameters in the Guinea Pig (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>12 WEEKS</th>
<th>23 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>Ascorbic Acid (mg/100ml)</td>
<td>0.33 ± 0.11b</td>
<td>0.91 ± 0.14</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/litre)</td>
<td>1.28 ± 0.14</td>
<td>1.02 ± 0.13</td>
</tr>
<tr>
<td>Total Triglyceride (mmol/litre)</td>
<td>0.68 ± 0.09</td>
<td>0.71 ± 0.08+</td>
</tr>
<tr>
<td>Total Bile Acids (μmol/litre)</td>
<td>2.84 ± 1.02</td>
<td>4.14 ± 0.96</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard error of the mean

* Ascorbate-deficient   ** Control   *** Ascorbate-supplemented  + n=5    a=p<0.05; b=p<0.01

Except where otherwise stated, for A,B,C at 12 weeks, n=6

for A,B,C at 23 weeks, n=3, 6 and 6 respectively.
Table 3.3 Effect of Ascorbic Acid Depletion and Supplementation on Some Hepatic and Biliary Parameters in the Guinea Pig (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>12 WEEKS</th>
<th>23 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>Ascorbic Acid (mg/100g liver)</td>
<td>6.6 ± 1.2c</td>
<td>16.8 ± 1.5</td>
</tr>
<tr>
<td>Total Cholesterol (mg/g liver)</td>
<td>1.48 ± 0.19</td>
<td>1.09 ± 0.05</td>
</tr>
<tr>
<td>Total Lipid (mg/g liver)</td>
<td>87.4 ± 7.8</td>
<td>88.7 ± 4.2</td>
</tr>
<tr>
<td>Total Bile Acids (mg/ml bile)</td>
<td>14.3 ± 0.7+</td>
<td>17.2 ± 3.1+</td>
</tr>
<tr>
<td>Total Cholesterol (mg/ml bile)</td>
<td>34.1 ± 11.7</td>
<td>50.0 ± 10.7++</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean
* Ascorbate-deficient ** Control *** Ascorbate-supplemented + n=5, ++ n=4, +++ n=3
a=p<0.05  c=p<0.001
Except where otherwise stated, for A, B, C at 12 weeks n=6 and for A, B, C at 23 weeks n=3, 6, 6 respectively
Table 3.4  **Effect of Ascorbic Acid Depletion and Supplementation on the Principal Component Bile Acids in Gall Bladder Bile Expressed as a Percentage of Total Bile Acids Measured at 12 Weeks**

<table>
<thead>
<tr>
<th></th>
<th>Free Chenodeoxycholate</th>
<th>Glyco-chenodeoxycholate</th>
<th>Tauro-chenodeoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>3.14 ± 0.54⁺</td>
<td>57.0 ± 9.2⁺</td>
<td>21.5 ± 3.1⁺</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>2.01 ± 1.34⁺⁺</td>
<td>57.2 ± 7.6⁺⁺</td>
<td>14.9 ± 1.8⁺</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>4.21 ± 0.65</td>
<td>66.4 ± 5.3</td>
<td>9.52 ± 1.66</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient
** Control
*** Ascorbate-supplemented

Except where otherwise stated n=6 for all groups.
⁺ n=5
⁺⁺ n=4
Table 3.5  Effect of Ascorbic Acid Depletion and Supplementation on the Activity of Hepatic Cholesterol 7α-hydroxylase in the Guinea Pig (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>12 WEEKS</th>
<th>23 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>% CHOL [4-14C]</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>metabolised/hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol CHOL [4-14C]</td>
<td>10.4 ± 3.8</td>
<td>21.0 ± 7.5</td>
</tr>
<tr>
<td>metabolised/g liver/hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol CHOL [4-14C]</td>
<td>0.83 ± 0.36</td>
<td>1.51 ± 0.41</td>
</tr>
<tr>
<td>metabolised/mg microsomal protein/hour</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient  ** Control  *** Ascorbate-supplemented

Except where otherwise stated for A,B,C at 12 weeks n=6 and for A,B,C at 23 weeks n=3, 6 and 6 respectively

+ n=5
### Table 3.6 The Effect of Ascorbic Acid Depletion and Supplementation on Some Mixed Function Oxidase Enzymes and Related Parameters in the Guinea Pig (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>A</td>
<td>26.1 ± 2.2</td>
<td>33.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>31.3 ± 1.4</td>
<td>40.8 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>29.7 ± 2.1</td>
<td>36.0 ± 3.6</td>
</tr>
<tr>
<td>Relative liver weight (g/100g body weight)</td>
<td>A</td>
<td>3.99 ± 0.19</td>
<td>4.34 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.63 ± 0.11</td>
<td>3.67 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>3.63 ± 0.14</td>
<td>3.41 ± 0.25</td>
</tr>
<tr>
<td>Microsomal protein (mg/g liver)</td>
<td>A</td>
<td>14.4 ± 1.6</td>
<td>17.7 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>14.3 ± 0.3</td>
<td>17.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15.1 ± 0.9</td>
<td>15.1 ± 1.2</td>
</tr>
<tr>
<td>p-nitroanisole O-demethylase (nmol produced/g liver/hour)</td>
<td>A</td>
<td>61.3 ± 14.2++</td>
<td>43.5 ± 21.0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>64.4 ± 12.8++</td>
<td>30.0 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>55.6 ± 21.1++</td>
<td>34.1 ± 2.6</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase (nmol produced/g liver/hour)</td>
<td>A</td>
<td>173 ± 16b</td>
<td>340 ± 68</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>113 ± 11</td>
<td>108 ± 62</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>148 ± 10a</td>
<td>213 ± 67</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase (μmol produced/g liver/hour)</td>
<td>A</td>
<td>3.48 ± 0.32</td>
<td>7.98 ± 4.16</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>2.72 ± 0.34</td>
<td>5.01 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>2.82 ± 0.24</td>
<td>4.47 ± 0.44</td>
</tr>
</tbody>
</table>

*cont'd*
<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol/g liver)</td>
<td>A*</td>
<td>16.7 ± 1.8</td>
<td>16.9 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>B**</td>
<td>16.9 ± 1.3</td>
<td>18.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>C***</td>
<td>14.7 ± 1.7</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>Cytochrome b₅ (nmol/g liver)</td>
<td>A*</td>
<td>14.2 ± 1.0</td>
<td>12.1 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>B**</td>
<td>13.1 ± 0.7</td>
<td>12.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>C***</td>
<td>12.3 ± 1.5</td>
<td>12.1 ± 0.9</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (µmol cytochrome c reduced/g liver/minute)</td>
<td>A*</td>
<td>1.27 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64 ± 0.24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>B**</td>
<td>0.93 ± 0.07</td>
<td>0.75 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>C***</td>
<td>0.90 ± 0.10</td>
<td>0.64 ± 0.03</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient  ** Control  *** Ascorbate-supplemented

Except where otherwise stated for A,B,C at 12 weeks n=6
for A,B,C at 23 weeks n=3,6,6 respectively.

++ n=4
a = p<0.05  b = p<0.01
Table 3.7  The Effect of Ascorbic Acid Depletion and Supplementation on the Activities of Some Mixed Function Oxidase Enzymes in Guinea Pig Liver, Expressed per mg Microsomal Protein (Experiment 1)

<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12</td>
<td>23</td>
</tr>
<tr>
<td><strong>p-nitroanisole O-demethylase</strong> (nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4.24 ± 0.81**</td>
<td>2.32 ± 0.92</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4.62 ± 0.96**</td>
<td>1.72 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3.33 ± 1.09**</td>
<td>2.15 ± 0.31</td>
<td></td>
</tr>
<tr>
<td><strong>Biphenyl 2-hydroxylase</strong> (nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13.6 ± 3.4</td>
<td>19.1 ± 3.3b</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>7.2 ± 0.7</td>
<td>5.81 ± 2.82</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>9.9 ± 0.9a</td>
<td>15.6 ± 6.1</td>
<td></td>
</tr>
<tr>
<td><strong>Biphenyl 4-hydroxylase</strong> (nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>269 ± 61</td>
<td>419 ± 181</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>191 ± 24</td>
<td>293 ± 24</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>188 ± 14</td>
<td>314 ± 58</td>
<td></td>
</tr>
<tr>
<td><strong>Cytochrome P-450</strong> (nmol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.24 ± 0.22</td>
<td>0.92 ± 0.19</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.18 ± 0.09</td>
<td>1.02 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.98 ± 0.09</td>
<td>1.06 ± 0.15</td>
<td></td>
</tr>
<tr>
<td><strong>Cytochrome b&lt;sub&gt;5&lt;/sub&gt;</strong> (nmol)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.02 ± 0.21</td>
<td>0.68 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.91 ± 0.03</td>
<td>0.74 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.82 ± 0.08</td>
<td>0.82 ± 0.10</td>
<td></td>
</tr>
<tr>
<td><strong>NADPH-cytochrome c reductase</strong> (nmol cytochrome c reduced/minute)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>93.4 ± 14.2</td>
<td>95.1 ± 19.5b</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>67.8 ± 4.6</td>
<td>42.1 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>59.5 ± 5.8</td>
<td>44.1 ± 4.1</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

For explanation of table see Table 3.6
Liver weight and relative liver weight were unchanged after 12 weeks but after 23 weeks relative liver weight increased slightly, although this was not statistically significant. Microsomal protein content was unaffected by treatment at either time interval.

There appeared to be a gradation in activity of cholesterol 7α-hydroxylase between groups (C > B > A) after 12 weeks, although again these differences were not statistically significant. After 23 weeks this pattern was no longer apparent.

The activity of p-nitroanisole O-demethylase did not differ from controls at either time interval. In contrast, the activity of biphenyl 2-hydroxylase at 12 weeks was significantly increased. The activity of this enzyme was also increased at 23 weeks, although the difference was only statistically significant when expressed as specific activity. Biphenyl 4-hydroxylase was unaffected at either time interval.

Concentrations of cytochromes P-450 and b5 measured at both time intervals showed no variation from control values. Conversely, at 12 weeks, there was a significant increase in NADPH-cytochrome c reductase activity which became even more pronounced at 23 weeks.

Biochemical Changes Occurring in Animals Fed the Ascorbate-Supplemented Diet (Group C)

Data are presented in Tables 3.2-3.7.
The administration of a daily ascorbate dose of 300mg/kg body weight led to an elevation in both plasma and liver ascorbate concentration. Increasing the dose to 500mg/kg body weight did not result in any further increase in tissue ascorbate concentration, in fact for liver, the levels were slightly below the controls and less than when measured at 12 weeks.

Serum cholesterol concentration did not vary from control at either time interval. Liver total cholesterol content was significantly less than control at 12 weeks, a difference which was not apparent at 23 weeks.

Values for the other parameters of lipid and bile acid metabolism showed no variation from controls.

There were no differences in absolute or relative liver weight or microsomal protein content at either time interval.

As mentioned above, cholesterol 7α-hydroxylase activity displayed a slight but non-significant increase at 12 weeks but not at 23 weeks.

With the exception of biphenyl 2-hydroxylation activity, which showed a small but significant increase at 12 weeks only, no changes in the parameters of mixed function oxidase metabolism were observed.

The histological sections of the aortas from all 3 groups of guinea pigs were kindly examined by Dr. P. Grasso (Chief Pathologist, British Industrial Biological Research Association, Carshalton, Surrey). No evidence of lipid accumulation was apparent.
Experiment 2 (Chronic Deficiency)

In this experiment, the guinea pigs were randomly assigned to four experimental groups with 14 in each deficient group (A and B) and 12 in each of C (control) and D (supplemented). They were housed in groups of 12, 2 animals from each deficient group being housed separately. All animals were given the ascorbic acid-free pelleted diet and orally supplemented with ascorbic acid in sucrose solution as follows: Group A (ascorbic-deficient) 1mg/kg body weight/day (0.3mg/ml); Group B (ascorbic-deficient) 3mg/kg body weight/day (0.9mg/ml); Group C (control) 30mg/kg body weight/day (9mg/ml); Group D (ascorbate-supplemented) 1000mg/kg body weight/day (310mg/ml). Both groups A and B were initially depleted of ascorbic acid as in Experiment 1, although in this case, the period of depletion was only 2 weeks, no signs of scurvy, with the exception of weight loss in some animals, being apparent prior to supplementation. As previously, the ascorbic acid was administered in the drinking water for 2 days each week as follows: Group A 17mg/litre; Group B 50mg/litre; Group C 500mg/litre; Group D 16.7g/litre.

The experiment was designed to compare the effects of two low doses of ascorbic acid on cholesterol and mixed function oxidase metabolism, in view of the observed disparities between the results of Experiment 1, and those of Ginter (see introduction). All animals were dosed on a body weight basis; Group A being given a quantity of ascorbate equivalent to that used by Ginter (0.5mg/day), whilst Group B were given the equivalent of the deficient level used in Experiment 1(1.5mg/day). The dose level in the supplemented group was increased in view of the lack of
effect of the dose used in Experiment 1. Animals from each group were killed after 6 and 12 weeks of treatment, tissues being treated as described for Experiment 1. However, in this experiment, hearts and aortas were not removed.

Results

Animal Weights and Food Intakes

These data are presented in Fig. 3.2 and Table 3.8.

After 3 weeks of the deficient regime A, the growth rate of the animals in this group began to decline, corresponding with a decreased food intake. Although some growth was maintained throughout the experiment, the rate was significantly less than that of the control animals.

The animals on the second deficient regime B maintained a normal growth rate until week 7-8 when it began to decline, again corresponding with a decreased food intake.

The growth rates and food intakes of the supplemented group did not differ from the controls.

Biochemical Changes Occurring in Animals Fed the Ascorbate-Deficient Diets (Groups A and B)

These data are presented in Tables 3.9-3.13.

There was a significant reduction in both plasma and liver ascorbate concentration after 6 weeks of treatment. For plasma, this became even more pronounced after 12 weeks. There were no significant differences between the two deficient groups.

At both time intervals, there was an indication of a trend
Fig. 3.2 Growth Curves of Guinea Pigs Maintained on 0.3mg/kg Ascorbic Acid \( \triangle \) (A) 3.0mg/kg Ascorbic Acid \( \bigcirc \) (B) 30mg/kg Ascorbic Acid \( \bullet \) (C) and 1g/kg Ascorbic Acid \( \blacksquare \) (D) (Experiment 2)

Points represent mean values. Significant differences between means are shown: \( a = p \leq 0.05 \) \( b = p \leq 0.01 \)

- \( n=14 \) (Groups A and B)
- \( n=12 \) (Groups C and D)
- \( n=8 \) (Groups A and B)
- \( n=6 \) (Groups C and D)
Table 3.8  Mean Daily Food Intake of Guinea Pigs During Experiment 2. (grams/guinea pig/day).

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12 Over 12 weeks</td>
<td></td>
</tr>
<tr>
<td>A*</td>
<td>34 38 44 38 41 44 44 41 43 40 48 46</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>B**</td>
<td>34 39 52 46 47 48 48 45 45 50 50 50</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>C***</td>
<td>33 41 56 47 47 48 47 51 52 50 57 55</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>D****</td>
<td>33 40 54 46 47 48 48 52 54 48 53 53</td>
<td>48 ± 2</td>
</tr>
</tbody>
</table>

* ascorbate-deficient group (1mg/Kg body weight/day) n=14 weeks 1-6  
   n=8 weeks 6-12

** ascorbate-deficient group (3mg/Kg body weight/day) n=14 weeks 1-6  
   n=8 weeks 6-12

*** control group (30mg/Kg body weight/day) n=12 weeks 1-6  
   n=6 weeks 6-12

**** ascorbate-supplemented group (1g/Kg body weight/day) n=12 weeks 1-6  
   n=6 weeks 6-12
Table 3.9  Effect of Ascorbic Acid Depletion and Supplementation on Some Serum Parameters in the Guinea Pig (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Ascorbic Acid (mg/100ml)</td>
<td>0.23±0.04</td>
<td>0.32±0.06a</td>
</tr>
<tr>
<td></td>
<td>0.10±0.07+</td>
<td>0.17±0.10c</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/litre)</td>
<td>1.83±0.35</td>
<td>1.66±0.18</td>
</tr>
<tr>
<td></td>
<td>1.83±0.20</td>
<td>1.67±0.20</td>
</tr>
<tr>
<td>Total Triglyceride (mmol/litre)</td>
<td>0.72±0.11</td>
<td>0.63±0.11+</td>
</tr>
<tr>
<td></td>
<td>0.73±0.14</td>
<td>0.89±0.12</td>
</tr>
<tr>
<td>Total Bile Acids (µmol/litre)</td>
<td>5.46±0.35</td>
<td>5.66±1.27</td>
</tr>
<tr>
<td></td>
<td>0.69±0.32+</td>
<td>1.41±0.22</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard error of the mean.

* Ascorbate-deficient (1mg/Kg body weight/day)  
** Ascorbate-deficient (3mg/Kg body weight/day)  
*** Control (30mg/Kg body weight/day)  
**** Ascorbate-supplemented (1g/Kg body weight/day)  

Except where otherwise stated, n=6 for all groups.

+ n=5  ++ n=4  

\( a = p<0.05 \)  \( c = p<0.001 \)
Table 3.10  Effect of Ascorbic Acid Depletion and Supplementation on Some Hepatic and Related Parameters in the Guinea Pig (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th></th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>667±21\textsuperscript{b}</td>
<td>736±34</td>
<td>746±10</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>23.8±1.4</td>
<td>27.3±1.9</td>
<td>28.2±1.1</td>
</tr>
<tr>
<td>Relative Liver Weight (g/100g body wt.)</td>
<td>3.56±0.13</td>
<td>3.69±0.10</td>
<td>3.78±0.12</td>
</tr>
<tr>
<td>Ascorbic Acid (mg/100g liver)</td>
<td>6.30±0.87\textsuperscript{c}</td>
<td>5.54±0.93\textsuperscript{c}</td>
<td>15.4±1.0</td>
</tr>
<tr>
<td>Total Cholesterol (mg/g liver)</td>
<td>1.47±0.11</td>
<td>1.42±0.04</td>
<td>1.35±0.07</td>
</tr>
<tr>
<td>Total Lipid (mg/g liver)</td>
<td>79.9±4.4</td>
<td>71.7±2.8</td>
<td>70.7±2.7</td>
</tr>
<tr>
<td>Microsomal Protein (mg/g liver)</td>
<td>18.4±1.2</td>
<td>18.9±0.9</td>
<td>19.8±0.7</td>
</tr>
</tbody>
</table>

cont'd
<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 WEEKS</td>
<td>6 WEEKS</td>
<td>cont'd</td>
<td></td>
</tr>
</tbody>
</table>

In the Guinea Pig (Experiment 2) the 3.10 effect of ascorbic acid depletion and supplementation on some hepatic and related parameters.
Table 3.11  Effect of Ascorbic Acid Depletion and Supplementation on the Activity of Hepatic Cholesterol 7α-Hydroxylase in the Guinea Pig (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A*</td>
<td>B**</td>
</tr>
<tr>
<td>% [4-14C] Cholesterol metabolised/hour</td>
<td>0.15±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20±0.05</td>
</tr>
<tr>
<td>pmol [4-14C] Cholesterol metabolised/g liver/hour</td>
<td>21.7±1.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.8±7.5</td>
</tr>
<tr>
<td>pmol [4-14C] Cholesterol metabolised/mg microsomal protein/hour</td>
<td>1.21±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.60±0.46</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient (1mg/Kg body weight/day)

** Ascorbate-deficient (3mg/Kg body weight/day)

*** Control (30mg/Kg body weight/day)

**** Ascorbate-supplemented (1g/Kg body weight/day)

n=6 for all groups.  a = p<0.05  b = p<0.01
Table 3.12  Effect of Ascorbic Acid Depletion and Supplementation on Some Parameters of Mixed Function Oxidase Metabolism Expressed per g Liver (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th></th>
<th></th>
<th>12 WEEKS</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Cytochrome b₅ (nmol)</td>
<td>17.0±1.1</td>
<td>18.1±0.9</td>
<td>19.3±0.5</td>
<td>19.6±1.4</td>
<td>20.4±1.0</td>
<td>22.3±1.7</td>
</tr>
<tr>
<td>Cytochrome F₄₅₀ (nmol)</td>
<td>20.0±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.5±1.6</td>
<td>22.5±0.7</td>
<td>22.8±1.9</td>
<td>24.2±1.0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>27.8±1.0</td>
</tr>
<tr>
<td>Cytochrome c reductase (μmol cyt. c reduced/min)</td>
<td>0.70±0.06</td>
<td>0.81±0.04</td>
<td>0.85±0.05</td>
<td>0.92±0.08</td>
<td>1.10±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.28±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>p-Nitroanisole O-demethylase (nmol produced/hour)</td>
<td>42.3±2.5</td>
<td>50.4±1.6</td>
<td>43.2±7.3</td>
<td>53.2±2.3</td>
<td>59.5±5.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.1±9.6</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase (nmol produced/hour)</td>
<td>195±13</td>
<td>205±15</td>
<td>210±11</td>
<td>210±21</td>
<td>164±21</td>
<td>115±44</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase (μmol produced/hour)</td>
<td>3.32±0.48</td>
<td>3.36±0.39</td>
<td>3.48±0.43</td>
<td>3.62±0.38</td>
<td>3.65±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.53±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean. Groups are the same as in Table 3.11. n=6 for all groups.  
<sup>a</sup> = p<0.05  
<sup>b</sup> = p<0.01  
<sup>+</sup> = AxB p<0.05
Table 3.13  Effect of Ascorbic Acid Depletion and Supplementation on Some Parameters of Mixed Function Oxidase Metabolism Expressed per mg Microsomal Protein  (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th></th>
<th></th>
<th></th>
<th>12 WEEKS</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B**</td>
<td>C***</td>
<td>D****</td>
<td>A</td>
<td>B**</td>
<td>C***</td>
<td>D****</td>
</tr>
<tr>
<td>Cytochrome b$_{5}$</td>
<td>0.92±0.03</td>
<td>0.97±0.05</td>
<td>0.98±0.04</td>
<td>1.00±0.03</td>
<td>0.77±0.03</td>
<td>0.80±0.06</td>
<td>0.73±0.08</td>
<td>0.76±0.02</td>
</tr>
<tr>
<td>(nmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P-450</td>
<td>1.11±0.07</td>
<td>1.25±0.07</td>
<td>1.15±0.08</td>
<td>1.16±0.08</td>
<td>0.91±0.03</td>
<td>1.01±0.04</td>
<td>0.85±0.08</td>
<td>0.96±0.03</td>
</tr>
<tr>
<td>(nmol)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c reductase</td>
<td>38.4±3.0</td>
<td>42.7±1.5</td>
<td>43.3±3.3</td>
<td>46.9±3.3</td>
<td>41.4±1.3$^{a}$</td>
<td>46.2±1.4$^{b}$</td>
<td>35.3±2.7</td>
<td>40.0±0.6</td>
</tr>
<tr>
<td>(nmol cyt. c reduced/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Nitroanisole</td>
<td>2.33±0.16</td>
<td>2.71±0.31</td>
<td>2.22±0.43</td>
<td>2.76±0.23</td>
<td>2.26±0.25$^{a}$</td>
<td>2.69±0.50</td>
<td>3.15±0.18</td>
<td>3.32±0.29</td>
</tr>
<tr>
<td>O-demethylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>10.7±0.7</td>
<td>10.9±0.9</td>
<td>10.6±0.7</td>
<td>10.7±0.8</td>
<td>6.18±0.78</td>
<td>4.17±1.56</td>
<td>4.78±0.60</td>
<td>4.26±0.85</td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>184±29</td>
<td>176±15</td>
<td>174±19</td>
<td>188±23</td>
<td>138±11$^{b}$</td>
<td>129±13$^{a}$</td>
<td>93.9±3.1</td>
<td>90.1±3.1</td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean. Groups are the same as in Table 3.11

n=6 for all groups.  a = p<0.05  b = p<0.01  + AvB p<0.05
towards increasing total serum cholesterol concentration relative to the decreased daily intake of ascorbic acid but this relationship was not statistically significant. Liver cholesterol appeared somewhat elevated at both 6 and 12 weeks in deficient Group A, but the difference was not significant. Serum triglycerides and liver total lipid were unaffected by treatment.

At 6 weeks, serum and biliary total bile acids were unchanged; however at 12 weeks in both groups A and B there was a slight reduction in these parameters, although the difference was statistically non-significant.

Liver weight was slightly diminished in group A after both 6 and 12 weeks of treatment, the difference being statistically significant at 12 weeks, however the relative liver weight was unchanged. For group B, no change was seen in either parameter at 6 weeks but at 12 weeks there was some indication of a decrease in liver weight, which would accord with the body weight data. Microsomal protein content was unchanged at either time interval.

After 6 weeks of deficiency, the mean cholesterol \( \Delta \) -hydroxylase activity in Group A was significantly reduced but in Group B did not differ from the control group. After 12 weeks, there was some indication of a decreased activity in both deficient groups, but the difference was not significant.

The activity of p-nitroanisole 0-demethylase was unchanged in either deficient group at 6 weeks. By 12 weeks however, it was significantly reduced in Group A.

Biphenyl 2-hydroxylase activity was unaffected at 6 weeks; by 12 weeks, this enzyme was apparently slightly induced relative
to the controls, but not significantly so. Biphenyl 4-hydroxylase activity however was induced in both deficient groups at 12 weeks, although unaltered at 6 weeks.

Concentrations of cytochromes P-450 and b5 did not differ significantly from control when measured at either time interval. At 6 weeks no variation in cytochrome c reductase activity was observed but by 12 weeks this activity was significantly elevated in both deficient groups, particularly in Group B.

Hepatic in vitro lipid peroxidation and glucose-6-phosphatase histochemistry were unaffected by either level of deficiency.

Biochemical Changes Occurring in Animals Fed the Ascorbic-Supplemented Diet (Group D)

Data are presented in Tables 3.9-3.13

Plasma ascorbate levels were increased at both time intervals following the administration of 1g/kg ascorbic acid. Hepatic concentrations were increased above controls at 6 weeks but not at 12 weeks.

Serum and liver cholesterol concentrations, serum triglyceride, liver total lipid content, serum and biliary total bile acids were apparently un influenced by treatment.

Similarly there were no differences in absolute or relative liver weight or microsomal protein content at either time interval.

Cholesterol 7α-hydroxylase activity was unaffected by ascorbate supplementation. Similarly, the other parameters of mixed function oxidase metabolism were not influenced by this treatment, with the exception of cytochrome c reductase activity.
This was apparently induced after 12 weeks of treatment although unaltered after 6 weeks.

Hepatic in vitro lipid peroxidation and glucose-6-phosphatase histochemistry were unaffected by ascorbate supplementation.
Experiment 3 (Acute Deficiency)

The guinea pigs were randomly assigned to two groups: Group A (deficient) and Group B (control), with 5 in each group. They were housed as described previously and fed the ascorbate-free pelleted diet, supplemented orally as follows: Group A 20% sucrose solution alone; Group B 30mg ascorbic acid/kg body weight/day in 20% sucrose solution. For 2 days each week ascorbate was administered in the drinking water of the control animals at the level of 500mg/litre.

After 24 days on this regime, the animals were killed and the tissues treated as described in the previous experiment.

Results

Animal Weights and Food Intakes

The growth rate of the deficient animals began to decline after 1 week of depletion (Fig. 3.3) and by the third week, growth had virtually ceased, whilst food intake had fallen to approximately 70% of control. 3 of the deficient animals were emaciated and were showing scorbutic signs, including hair loss.

Biochemical Changes

These data are presented in Tables 3.14-3.17.

The deficient regime resulted in a pronounced reduction in both plasma and liver concentrations of ascorbic acid. Serum and liver total cholesterol concentrations were elevated by the acute deficiency, although the difference was only of marginal statistical significance. Serum triglyceride
Fig. 3.3 Growth Curves of Guinea Pigs Maintained on Ascorbic Acid-Deficient (▲—▲) and Control (●—●) Diets (Experiment 3)

Points represent mean values; n=5 for all groups

Significant differences between means are shown : a = p<0.05
Table 3.14  Effect of Acute Ascorbate Deficiency on Some Serum Parameters in the Guinea Pig (Experiment 3)

<table>
<thead>
<tr>
<th></th>
<th>A*</th>
<th>B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid (µg/100ml)</td>
<td>24.5 ± 8.7c</td>
<td>890 ± 77</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/litre)</td>
<td>2.08 ± 0.21</td>
<td>1.66 ± 0.08</td>
</tr>
<tr>
<td>Triglyceride (mmol/litre)</td>
<td>1.36 ± 0.52</td>
<td>1.06 ± 0.26</td>
</tr>
<tr>
<td>Total Bile Acids (µmol/litre)</td>
<td>1.67 ± 0.65</td>
<td>3.63 ± 0.97</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient (n=5)

** Control (30mg/Kg body weight/day) (n=5).

c = p<0.001
Table 3.15 Effect of Acute Ascorbate Deficiency on Some Hepatic and Related Parameters in the Guinea Pig (Experiment 3)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A*</th>
<th>B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>364 ± 34\textsuperscript{a}</td>
<td>485 ± 18</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>14.5 ± 1.3\textsuperscript{a}</td>
<td>19.0 ± 1.2</td>
</tr>
<tr>
<td>Relative Liver Weight (g/100g body weight)</td>
<td>4.00 ± 0.90</td>
<td>3.90 ± 0.17</td>
</tr>
<tr>
<td>Ascorbic Acid (mg/100g liver)</td>
<td>2.74 ± 0.54\textsuperscript{c}</td>
<td>17.7 ± 1.5</td>
</tr>
<tr>
<td>Total Cholesterol (mg/g liver)</td>
<td>2.03 ± 0.22</td>
<td>1.55 ± 0.06</td>
</tr>
<tr>
<td>Microsomal Protein (mg/g liver)</td>
<td>22.7 ± 2.6</td>
<td>24.9 ± 2.3</td>
</tr>
<tr>
<td>Biliary Total Bile Acids (mg/ml bile)</td>
<td>9.73 ± 1.51</td>
<td>11.4 ± 0.4</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient (n=5)

** Control (n=5)

\( a = p < 0.05 \quad c = p < 0.001 \)
Table 3.16  Effect of Acute Ascorbate Deficiency on Hepatic Cholesterol \( \alpha \)-hydroxylase Activity (Experiment 3)

<table>
<thead>
<tr>
<th></th>
<th>A*</th>
<th>B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>( % ) ( [4-\text{(^{14}\text{C}}] ) cholesterol metabolised/hour</td>
<td>0.28 ± 0.03</td>
<td>0.26 ± 0.02</td>
</tr>
<tr>
<td>pmol ( [4-\text{(^{14}\text{C}}] ) cholesterol metabolised/g liver/hour</td>
<td>40.7 ± 4.1</td>
<td>38.0 ± 2.3</td>
</tr>
<tr>
<td>pmol ( [4-\text{(^{14}\text{C}}] ) cholesterol metabolised/mg microsomal/protein/hour</td>
<td>1.87 ± 0.26</td>
<td>1.57 ± 0.12</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Ascorbate-deficient (n=5)

** Control (n=5)
Table 3.17  Effect of Acute Ascorbate Deficiency on Some Parameters of Mixed Function Oxidase Metabolism Expressed per g Liver (Experiment 3)

<table>
<thead>
<tr>
<th></th>
<th>A*</th>
<th>B**</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Nitroanisole O-demethylase</td>
<td>38.9 ± 6.1</td>
<td>53.7 ± 5.9</td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>15.9 ± 2.1</td>
<td>17.4 ± 1.6</td>
</tr>
<tr>
<td>(nmol produced/hour)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>3.67 ± 0.34</td>
<td>3.84 ± 0.36</td>
</tr>
<tr>
<td>(μmol produced/hour)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol)</td>
<td>13.4 ± 4.6^a</td>
<td>25.4 ± 1.5</td>
</tr>
<tr>
<td>Cytochrome b_5 (nmol)</td>
<td>15.1 ± 2.1</td>
<td>19.3 ± 0.8</td>
</tr>
<tr>
<td>NADPH cytochrome c reductase</td>
<td>1.51 ± 0.12</td>
<td>1.69 ± 0.80</td>
</tr>
<tr>
<td>(μmol cytochrome c reduced/minute)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

*  Ascorbate-deficient (n=5)

**  Control (n=5)

a = p<0.05
concentration was also slightly increased and serum total bile acids decreased, although again not significantly. Biliary total bile acids were unaffected.

Liver weight was significantly reduced in accordance with the decreased body weight. Microsomal protein content was unaltered by treatment.

The activities of cholesterol 7α-hydroxylase, biphenyl 2- and biphenyl 4-hydroxylase were not reduced by ascorbate deficiency. However, p-nitroanisole O-demethylase activity and cytochrome b5 content were marginally reduced. Cytochrome P-450 content was significantly decreased to approximately 50% of control. The activity of NADPH-cytochrome c reductase was unchanged.
Discussion

A major problem encountered in the design of these experiments was the selection of suitable dose levels for ascorbic acid. The daily requirement for this vitamin in the guinea pig remains uncertain. Williams and Deason (1967) showed that by the criterion of growth rate alone, the individual variation in requirement was considerable. In addition, there is still disagreement as to whether the requirement and recommended intake should be based on the level necessary to prevent scurvy plus a several-fold safety margin or on one which permits tissue saturation. An attempt to clarify the situation was made by Yew (1973). She studied a wide range of ascorbic intakes in young male guinea pigs (350g), observing their effect on growth rate, wound healing and recovery from anaesthesia and surgical stress. There was a pronounced individual variation in requirement, but she concluded that whilst optimal growth was achieved on an daily intake in excess of 5mg/kg body weight, recovery from stress necessitated ten times this quantity. Age and metabolic status of the animal also modifies its ascorbic acid requirement (Andrews and Brook, 1966; Hughes and Jones, 1971; Davies and Hughes, 1977). The problem of marked individuality in requirement in both guinea pigs and man has been discussed by Yew (1975).

Ginter employed a control dose of 10mg per day, irrespective of body weight (Ginter 1975b). In contrast, Bjorkhem and Kallner (1976) fed their control group pellets containing 450mg/kg ascorbic acid and allowed them free access to vegetables; no food intake data was presented, but it is probable that
these animals were receiving at least 25mg of ascorbate each day. In the present series of experiments, the control group received a daily dose of 30mg/kg body weight, which is of the same order as chow-fed guinea pigs and similar to the dose used by Ginter (1975b).

The level of chronic deficiency was also difficult to establish. Whilst in the first experiment Ginter's regime was followed (Ginter et al, 1968; Ginter et al., 1969; Ginter, 1975b), it was impossible to maintain the deficient animals on a daily dose of 0.5mg/day, without the appearance of scorbutic signs. Thus, for a 2-3 week period, larger quantities (1-3mg) were given, titrating the daily dose to the individual status of the animal. By week 6 of this experiment, all animals were maintained on a daily dose of 1.5mg and appearance and food intake were normal, in accord with Ginter's experiments. Similar problems were apparently encountered by Sulkin and Sulkin (1975) and Bjorkhem and Kallner (1976) who, using 0.5mg ascorbate per day, found that although body weight was maintained, no growth occurred and the teeth and fur displayed signs of scurvy. These differences may again reflect the variability in ascorbic acid requirement.

In the second experiment, a shorter initial period of depletion was employed, supplementation commencing prior to any sign of weight loss. In this way it was possible to maintain a group of animals on a dose level closer to that of Ginter, although all groups of animals in this experiment were dosed on the basis of body weight.

With respect to the magnitude of the "megadose" in the
supplemented animals, doses of 500mg, 1g and 4g have been used (Yew, 1973; Nandi et al., 1973; Sorenson et al., 1974; Veen-Baigt et al., 1975). Extrapolation of a human "megadose" to the guinea pig would only give a daily dose of 14-70mg/kg body weight, however the rationale behind such an extrapolation is questionable. Thus initially a dose ten times that of the controls was employed i.e 300mg/kg body weight.

After feeding the vitamin at this level for 11 weeks, there was no obvious effect on food intake or growth rate; the reduction in these parameters after this corresponded with a simultaneous but less drastic reduction in control food intake, thus the significance of this observation is debatable. Yew (1973), using a 500mg/kg dose, reported a slight reduction in growth rate as did Sorenson et al. (1974), using a 4g/kg dose. In the second experiment, a daily dose of 1g/kg for 12 weeks did not affect growth, in accordance with the findings of Nandi et al. (1973) and Veen-Baigt et al. (1975).

In both experiments, the animals on the high ascorbate regimes displayed an increase in plasma and liver ascorbate concentration only when measured at the first time interval. Keith and Pelletier (1974) studied the effect of increasing ascorbic acid intake on its organ concentration. Data for liver was not presented, but information pertaining to other organs indicated that ascorbic acid concentration only increased until the animal's requirement was reached. Tissue retention of ascorbic acid diminishes as a result of a decrease in metabolic activity (Davies and Hughes, 1977). These authors only considered the effect of nutrient deprivation on tissue
ascorbate retention but one could speculate that the high doses of ascorbate used might also induce some subtle alteration in metabolic capacity of the tissue. The observation that on the lower "megadose", this decrease was only apparent after 23 weeks, as opposed to 12 weeks on the higher dose, is concordant with this suggestion.

The effects of chronic ascorbic acid deficiency on the parameters of cholesterol metabolism measured in these experiments are somewhat at variance with other workers. In the first experiment, after 12 weeks of depletion, there was no change in serum total cholesterol concentration. This is in contrast to findings of Ginter (1975b) who reported that hypercholesterolaemia was evident after this time, although only presenting data from 16-28 weeks. Furthermore, serum cholesterol values for his control animals were substantially higher than those encountered here. However, there was an indication of an increase in serum cholesterol when the animals in experiment 1 were further depleted and in the most deficient animals (Group A) in experiment 2; liver cholesterol content was also elevated in these latter animals. In the acutely deficient animals (Experiment 3), the increase in both serum and liver cholesterol content was more pronounced, although the statistical significance was negated by the large individual variation. However, in general the results would indicate an inverse relationship between ascorbate intake and degree of cholesterolaemia. The experiments of Hornig and Weiser (1976) lend some support to this contention. They used a daily dose of 1.5mg per day of ascorbic acid (as the sodium salt) and observed some increase in cholesterolaemia,
although the difference was small. It could therefore be postulated that there is a critical threshold intake of ascorbic acid, necessary to maintain a particular liver concentration, which in turn might be essential to regulate or maintain normal liver cholesterol concentrations.

From these experiments, it is apparent that hypercholesterolaemia is only manifest at very low levels of intake, since in animals supplemented with 3mg/kg body weight/day, cholesterol levels were similar to controls. The above hypothesis is consonant with Ginter's view that the hypercholesterolaemic effect of ascorbate is dependent on its tissue concentration (Ginter et al., 1977) and that of Hornig and Weiser (1976), who suggest that cholesterol oxidation is only dependent on ascorbic acid status over a limited range of concentration. It is also possible that this "critical level" may vary between individual animals or between strains, thus contributing to the variability encountered in these and previous experiments.

Serum and biliary total bile acid concentrations showed little variation in either chronic experiment, except for a small reduction after 12 weeks depletion in both deficient groups in Experiment 2. In the acutely deficient animals, there was a reduction in serum bile acids and possibly in biliary bile acids although again the variation in individual values was great. From the data presented by Ginter (1975b) concerning $[26^{14}C]$ cholesterol oxidation, this is an unexpected finding, although perhaps consistent with the absence of any major effect on serum cholesterol levels. These findings are also at variance with those of Hornig and Weiser (1976), who reported
a significant reduction in total bile acids of liver, bile and small intestine in chronically deficient animals.

Ginter (1975b) has postulated that the site of action of ascorbic acid is at the \(7\alpha\)-hydroxylation of cholesterol. In the first experiment, the activity of this enzyme showed a slight reduction in the deficient group but by 23 weeks this was no longer apparent, an unexpected finding, as it coincides with an increase in serum cholesterol. In the second experiment, there was a reduced activity in the most deficient group (A) at 6 weeks. After further depletion, there was a trend towards a diminution in \(\alpha\)-hydroxylase activity at both levels of deficiency, perhaps indicative that at the higher level of supplementation, the effect of the deficiency on cholesterol \(\alpha\)-hydroxylation is only manifest after a longer period of depletion. In contrast to these findings, Bjorkhem and Kallner (1976) demonstrated a fifteen-fold decrease in the activity of this enzyme in chronically deficient female guinea pigs. It is possible that the sex difference may account for this disparity. However, the lack of any consistent or pronounced effect on cholesterol \(\alpha\)-hydroxylation in the chronically treated animals lends support to the concept of a critical threshold level of intake which conceivably varies between animals.

Unexpectedly, there was no observable change in \(\alpha\)-hydroxylase activity in the acutely deficient guinea pigs. This finding is difficult to explain but may indicate, as do the chronic experiments, that the catabolism of cholesterol may not be the only site of action of ascorbic acid. The observed accumulation
of cholesterol in the liver without any obvious effect on cholesterol catabolism is commensurate with the suggested interaction with cholesterol biosynthesis (Kallner, 1977). One problem in the interpretation of the 7α-hydroxylase data is the low activity encountered in the guinea pig, although similar levels were reported by Bjorkhem and Kallner (1976).

Hypertriglyceridaemia was apparent in the acutely deficient animals, and to some extent in the chronically treated ones; this phenomenon has been observed by others and has been attributed to a decrease in lipolytic activity (Ginter, 1978). Histological sections of the aorta were only examined in Experiment 1. There was no evidence of degeneration or lipid accumulation. This is contrary to the findings of Sulkin and Sulkin (1975), who reported changes ranging from endothelial proliferation to the formation of large musculofibrotic, atherosclerotic plaques in animals chronically depleted of ascorbic acid for over 100 days. Similarly, Ginter (1978) has reported pathological changes in the coronary arteries of these animals. The lack of any detectable change is again consonant with the concept of a critical threshold level of intake.

After feeding ascorbic acid at 300mg/kg body weight for 12 weeks, there was no difference in total serum cholesterol concentration. There was a reduction in liver cholesterol content, coinciding with a small increase in cholesterol 7α-hydroxylase activity and indicating a possible stimulation of cholesterol catabolism; however, serum and biliary bile acids were unchanged. Increasing the dose to 500mg or 1g/kg body
weight did not influence any of these parameters. Thus, excess ascorbate had no marked stimulatory effect on cholesterol 7α-hydroxylase activity, a finding in agreement with the view of Bjorkhem and Kallner (1976), who have questioned this concept. Attempts to demonstrate in vitro stimulation of the enzyme system have been unsuccessful (Kritchevsky et al., 1973b), although the experimental design of this study has been criticised.

Feeding either deficient or supplemental levels of ascorbate did not influence the pattern of bile acids in gall bladder bile. Information regarding qualitative changes in biliary bile acids in the ascorbate-deficient guinea pig is lacking, but in a recent study, Kallner (1977) examined the serum bile acid pattern in man after supplementation with ascorbic acid. His results suggested that there may be a decrease in chenodeoxycholate concentration after discontinuation of the supplements, although this is open to interpretation as the individual variation between subjects was great. Ginter (1977) showed that the formation of gallstones in hamsters could be limited by the administration of ascorbic acid. This could also be interpreted as indicative of an increase in chenodeoxycholate synthesis; although as Kallner (1977) points out, it could also result from a more general effect on the cholesterol: bile acid ratio.

As considered earlier, acute ascorbic acid deficiency markedly reduces mixed function oxidase metabolism. However, the effects of chronic deficiency on these enzymes has scarcely been considered. In the present series of experiments, chronic
ascorbate deficiency did not result in a reduction of any of the parameters of mixed function oxidase metabolism. This is contrary to the findings of Bjorkhem and Kallner (1976) who reported a 50% reduction of hepatic cytochrome P-450 in chronically deficient female guinea pigs.

An interesting finding, nonetheless, was the apparent induction of biphenyl hydroxylase and NADPH-cytochrome c reductase activities. In the first experiment, the 2-hydroxylase and cytochrome c reductase enzymes were elevated at both 12 and 23 weeks. In the second experiment, by 12 weeks, biphenyl 2-hydroxylase was slightly induced in the most deficient group and biphenyl 4-hydroxylation and cytochrome c reductase were increased in both deficient groups.

In Experiment 2, an attempt was made to explain this alteration in reductase activity. It is known that in addition to its involvement in drug oxidation, NADPH-cytochrome c reductase is an essential component of the enzymic lipid peroxidation system, which is partly responsible for the peroxidation of subcellular lipids (Hochstein and Emster, 1963; Orrenius et al., 1964; Pederson and Aust, 1972; Pederson et al., 1973); the two processes are thus closely related. Normally, induction of cytochrome c reductase activity would be expected to be associated with an induction in specific mixed function oxidase enzymes. Therefore, this observed independent increase in cytochrome c reductase activity was speculatively interpreted as implying a stimulation of the peroxidative function of the reductase, representing an uncoupling of electron transfer from cytochrome P-450 (Suarez et al., 1972). Furthermore,
increased biphenyl 2-hydroxylase activity has been suggested to be indicative of disturbed hepatic metabolism, associated with degranulation of the endoplasmic reticulum (Parke, 1977). A marked proliferation of smooth endoplasmic reticulum has been observed in guinea pigs suffering from "marginal" ascorbate deficiency (Sulkin and Sulkin, 1975), which accords with the finding of Experiment 1. It was hypothesised that the associated increase in biphenyl 2-hydroxylase and cytochrome c reductase activity might be indicative of a derangement in membrane structure, induced by ascorbate deficiency and possibly mediated by peroxidative damage. However, the results of the lipid peroxidation and glucose-6-phosphatase histochemistry studies do not support this hypothesis and the finding remains unexplained.

Labadarios (1975) demonstrated the ability of guinea pigs to synthesise ascorbic acid de novo when depleted of the vitamin and treated with prednisone. This was attributed to the induction of a microsomal reductase and confirmed the work of Odumosu and Wilson (1973), who observed the same phenomenon in untreated, deficient animals. The present finding of an induction of NADPH-cytochrome c reductase suggests that ascorbate deficiency might engender an adaptive response, in which there is a non-specific induction of microsomal reductases to facilitate synthesis of ascorbic acid. It is likely that the individual capacity for this response would show considerable variation which could further explain the inter-animal variability of response to ascorbate depletion.

These chronic experiments successfully demonstrated a discrepancy between changes engendered by either an acute or
chronic ascorbate deficiency, again leading one to speculate on the existence of a critical threshold concentration of ascorbate, beneath which aberrations in mixed function oxidase metabolism may occur. In order to confirm this and exclude the possibility of a strain variation in response to ascorbate deficiency, the acute experiment was carried out.

The results of this experiment confirmed those of previous workers, particularly the recent study of Sikic et al. (1977b), which also reported at least a 50% reduction in cytochrome P-450 content but no change in NADPH-cytochrome c reductase activity. However, although p-nitroanisole O-demethylation was slightly reduced, biphenyl hydroxylation was unaffected, perhaps indicating an effect on a specific type of P-450. In this way, demethylation might be altered whilst hydroxylation (biphenyl and cholesterol) remained unchanged. This idea is partially in agreement with that of Bjorkhem and Kallner (1976), who speculate that the ascorbate-induced defect is specific to the type of cytochrome P-450 responsible for cholesterol oxidation. They suggest however that ascorbate might be involved in the synthesis or breakdown of P-450 itself, although it is unlikely then to be specific to one type of the cytochrome. Furthermore, Rikans et al. (1977) were unable to demonstrate any disturbances in the enzymes of haem synthesis in response to ascorbate deficiency. The wide variation in individual response to deficiency reduced the statistical significance of the results and again emphasises the importance of the individual variation in the level of the vitamin required to ensure integrity of metabolism.

Supplemental ascorbic acid, at any of the levels studied,
had little effect on these parameters of mixed function oxidase metabolism. In the first experiment, there was a slight increase in biphenyl 2-hydroxylation and in the second, of NADPH-cytochrome c reductase activity. Sato and Zannoni (1974), using weanling guinea pigs, reported an induction of mixed function oxidase metabolism on supplementation with up to 200 mg ascorbate/kg body weight. Conversely Sikic et al. (1977a), in agreement with the present data, found no effect of supplemental ascorbate in young adult animals. They suggest that the disparity between their results and those of Sato and Zannoni might not only be accounted for by an age difference but also by the lack of consistency in the route of administration in the latter experiments. The present results also accord with studies carried out in man (Wilson et al., 1976), which showed that antipyrine and diphenylhydantoin plasma half lives were unaffected by "megadoses" of ascorbate of 4-5g per day. Ascorbic acid in high concentrations is recognised as a pro-oxidant, thus it might be expected to enhance non-enzymic lipid peroxidation (Hochstein et al., 1964). However, in vitro lipid peroxidation was unaffected in animals supplemented with 1g/kg body weight.

Thus in summary, over a limited range of ascorbic acid concentration, there would appear to be some type of dose-response relationship in mixed function oxidase metabolism, which affects the metabolism of both endogenous substrates such as cholesterol and of foreign compounds. Beneath a certain critical level of intake and thus of tissue concentration, metabolism may be impaired but this level is quite low and may well vary between
animals. Consequently, the effects of acute deficiency, i.e. total depletion, are entirely different from those of chronic deficiency, a finding which may have considerable implications to man (see final discussion). Supplemental ascorbate does not seem to induce mixed function oxidase metabolism, neither does it have any pronounced detrimental effect on the parameters of cholesterol metabolism measured here.
Chapter 4

The Effects of Dietary Iron Deficiency on Cholesterol and Foreign Compound Metabolism in the Rat
Introduction

There is a considerable body of epidemiological data associating various minerals with the incidence of hyperlipidaemia and coronary heart disease. Total cardiovascular mortality has been shown to be substantially higher in populations living in areas supplied by soft water than in those living in hard water areas (Schroeder 1960; Crawford et al., 1968; Crawford et al., 1971; Crawford, 1972; Masironi et al., 1972). However, although some workers have reported higher serum cholesterol levels in soft water areas (Anderson et al., 1969; Stitt et al., 1973), this is not a universal finding (Elwood et al., 1971; Bierenbaum et al., 1973). With respect to the specific minerals implicated, large supplements of calcium were found to be hypercholesterolaemic both in rabbits and in man (Iacono et al., 1960; Yacowitz et al., 1965; Bierenbaum et al., 1972). Similarly, the contribution of magnesium to the protective effect of hard water has been considered (reviewed by Seelig and Heggtveit, 1974).

Attention has also been focussed on several trace minerals. Schroeder et al. (1970) suggested that chromium deficiency was a causative factor in atherosclerosis. Vanadium deficiency is known to be associated with elevated serum lipid levels (Nielsen and Ollerich, 1973; Hopkins and Mohr, 1974). Silicon is also recognised as being hypercholesterolaemic (Schwartz, 1977). A deficiency of copper elevated serum cholesterol concentrations in rats and thus led Klevay (1975) to hypothesise that an absolute or relative (i.e. a high zinc/copper ratio) deficiency of copper might be a major aetiological factor in coronary
heart disease.

Finally, iron has been considered to play a role in lipid metabolism and the incidence of coronary heart disease. A large number of studies have demonstrated an association between hypolipidaemia, particularly hypocholesterolaemia, and various types of anaemia (Fessas et al., 1963; Westerman et al., 1964; Rifkind and Gale, 1967; Bazzano, 1969; Elwood et al., 1970b; Westerman, 1975; Seip et al., 1975; Seip and Skrede, 1977). It has been suggested that the lower incidence of coronary heart disease seen in developing countries might be attributable to the greater incidence of anaemia (Birnbaum and Levein, 1961; Rifkind and Gale, 1967). The same authors attributed the sex difference in occurrence of coronary heart disease to the lower haemoglobin level of premenopausal American women. Evidence in support of this derives from several case-control studies, where patients with coronary heart disease were shown to display higher haematocrits than their controls (Burch and DePasquale, 1962a; Burch and DePasquale, 1962b; DePasquale and Burch, 1963; Mayer, 1965). In 1970, Elwood et al., reported a significant correlation between serum cholesterol and haemoglobin levels in a large group of women, which could not be accounted for by the apparent dilution effect due to the increase in plasma volume. Correction of the anaemia led to a rise in cholesterol concentration, although this was not statistically significant.

Conflicting data have also been presented. Thus, Conley et al. (1964) observed that in a group of 200 patients with myocardial infarction, the mean haemoglobin level was slightly less than that of the control group. Initial interpretation
of results from the Framingham Study (Kannel et al., 1961) supported the association but on subjecting the data to further analysis, the correlation was not apparent (Truett et al., 1967). A recent report from the Los Angeles Heart Study (Abu-Zeid and Chapman, 1976) showed that neither haemoglobin or haematocrit were significantly correlated with the incidence of coronary heart disease.

Conversely, in experimental animals such as the rat and chick, iron has a hyperlipidaemic effect (Lewis and Iammarino, 1971; Guthrie et al., 1974; Sherman et al., 1977; Amine and Hegsted, 1971), which, at least in the rat, is also interdependent on other trace minerals such as zinc and copper (Klevay, 1973; Sourkes et al., 1968; Sherman et al., 1977).

Various mineral deficiencies are also known to influence the activity of the mixed function oxidase enzyme system. Deficiencies of both calcium (Dingell et al., 1966) and magnesium (Becking and Morrison, 1970b; Becking, 1976) depress the metabolism of various substrates, associated, in the case of magnesium, with lowered cytochrome P-450 and NADPH-cytochrome c reductase levels. Trace minerals are also of importance. In the rat, zinc deficiency reduces the rate of metabolism of various compounds such as pentobarbital and aminopyrine, after a prolonged period of depletion, probably via an effect on cytochrome P-450 (Becking and Morrison, 1970a). Mixed function oxidase activity is reduced by either deficiency or excess of dietary copper (Moffitt and Murphy, 1973). Paradoxically, a dietary deficiency of iron is associated with an increased activity of some mixed function oxidase enzymes.
Catz et al. (1970) reported that in adult mice, chronic iron deficiency significantly enhanced hexobarbital oxidation and aminopyrine N-demethylation, without any effect on aniline or benzo a pyrene hydroxylation, in vivo metabolism as measured by sleeping times, or on cytochrome P-450 content. Becking (1972; 1976) reported similar findings with respect to cytochrome P-450 content and aminopyrine metabolism in adult male rats but observed no change in pentobarbital metabolism in vivo or in vitro and an enhancement in both in vivo and in vitro aniline metabolism. These workers also fed a high iron diet to rats, without any deleterious effect on mixed function oxidase metabolism. This is in contrast to Wills (1972), who demonstrated a reduction of aminopyrine metabolism in iron-supplemented rats, which he attributed to increased lipid peroxidation. In man, only one study has examined the effects of iron deficiency on mixed function oxidase metabolism; no detrimental effect was observed (O'Malley and Stevenson, 1973).

Aim of Present Study

The epidemiological studies associating iron deficiency with a decreased incidence of coronary heart disease and hypercholesterolaemia, and its established inductive effect on mixed function oxidase metabolism, led to the hypothesis that the hypercholesterolaemia might be explained by an induction of cholesterol \( 7\alpha \)-hydroxylase. A series of experiments were thus set up to investigate the effects of iron deficiency in the rat on cholesterol and mixed function oxidase metabolism.
Materials

These were as described in Chapters 1 and 2, with the addition of the following: Drabkin's reagent and the carboxymethaemoglobin standard (57.2mg/100ml) were obtained from B.D.H. Ltd.

Methods

These were also as described in Chapters 1 and 2 with the addition of the following:

(a) Blood Haemoglobin Concentration

Haemoglobin was determined by the cyanomethaemoglobin method. Whole blood is treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate (Drabkin's reagent). The ferricyanide reacts with the haemoglobin to form methaemoglobin which in turn reacts with the cyanide to form cyanomethaemoglobin, the extinction of which can be measured.

A 20μl aliquot of blood was added to 4.0ml of Drabkin's reagent and mixed thoroughly. The tubes were centrifuged at 2,000 r.p.m. for 10 minutes in a bench centrifuge and after at least a further 4 minutes, the extinctions of the supernatants were measured at 540nm in the Cecil spectrophotometer. All determinations were carried out in duplicate and the extinctions of a blank (Drabkin's reagent) and a cyanomethaemoglobin standard containing 57.2mg cyanomethaemoglobin/100ml, equivalent to a haemoglobin concentration of 11.5g/100ml, were also measured.
(b) **Determination of Haematocrit**

The relative volume of red blood cells to plasma was determined as follows:

Duplicate heparinised capillary microhaematocrit tubes (M.S.E. Ltd., Crawley, Sussex) were three-quarters filled with blood, sealed at one end with a sealing material (Cristaseal, Hawksley and Sons Ltd., Lancing, Sussex) and centrifuged for 6 minutes at 12,400 r.p.m. in a microhaematocrit centrifuge (M.S.E. Ltd.).

The haematocrit value was determined as follows:

\[
\text{Haematocrit (\%)} = \frac{\text{Height of packed red cells (mm)}}{\text{Height of red cells and plasma (mm)}}
\]

(c) **Determination of Dietary Iron Content**

The concentration of iron in both diets was determined by atomic absorption spectrophotometry after wet ashing.

Duplicate 0.2g aliquots of each diet were digested with a mixture of 5ml of concentrated nitric acid and 1ml of perchloric acid in a 25ml conical flask, pre-washed with dilute hydrochloric acid. Duplicate blanks, consisting of 6ml of acid mixture only, were set up. The mixture was shaken and left to stand for 15 minutes and subsequently heated to approximately 100°C on a heating block, until the reaction subsided. The heat was gradually increased to 200-250°C and digestion continued until a white residue remained.

The residue was dissolved in a known quantity of 4.5M hydrochloric acid and the iron content determined by atomic absorption spectrophotometry using an IL 353 spectrophotometer.
Animals

In all experiments, male Wistar albino rats (Porton Strain, random bred in closed colony), supplied by the University of Surrey Animal Breeding Unit and aged 5 weeks (weighing 80-100g), were used. The animals were randomly assigned to test (iron-deficient) and control groups and housed in polypropylene cages with aluminium lids (65cm x 39cm x 20cm) on "Sebital" bedding (Plasticos Espanoles, S.A., Torrelavega, Spain, supplied by J. Lillico and Sons), which was changed at weekly intervals. Animal house conditions were as described in the previous Chapter. Prior to the administration of the experimental diets, the animals were maintained on Spratts Laboratory Animal Diet No 2. for 3-5 days to allow acclimatisation to the powder form of diet. The rats were then allowed access to glass-distilled water and to the experimental powdered diets, contained in stainless steel straight-sided bowls fitted with grids, ad libitum. Measurements of body weight (individual) and food intake (per cage) were made at regular intervals.

Diets

The iron-deficient diet used by Becking (1972), modified from that of McCall et al. (1962) was used. The compositions of the basic diet and the vitamin and mineral mixtures used are presented in Tables 4.1 - 4.3. The control and iron-deficient diets were shown by analysis to contain 223-250ppm and 9-15ppm of iron respectively. Diets were prepared, as described in
Table 4.1 Basal Iron-Deficient Diet (Becking 1972)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>(g/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Skim Milk</td>
<td>650</td>
</tr>
<tr>
<td>++ Sucrose</td>
<td>210</td>
</tr>
<tr>
<td>+++ Corn Oil</td>
<td>100</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>30</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
</tr>
<tr>
<td>Choline</td>
<td>2.2</td>
</tr>
<tr>
<td>Fat Soluble Vitamins</td>
<td>0.7ml*</td>
</tr>
</tbody>
</table>

(Control diet supplemented with iron as FeSO\(_4\) 220ppm (1.1g/Kg diet))

+ "Marvel" Cadbury Typhoo Catering Services, Bournville, U.K.
++ Tate and Lyle, Croydon, Surrey.
+++ "Saladin" Van den Berg and Jurghens Ltd., London.
* 40mg retinyl palmitate and 5mg ergocalciferol dissolved in 5ml 50% \(\alpha\)-tocopherol in soya oil.

Table 4.2 Mineral Mix (McCall et al., 1962)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>(g/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>200</td>
</tr>
<tr>
<td>NaI(_2) (\text{H}_2)(_2)O</td>
<td>0.022</td>
</tr>
<tr>
<td>MnSO(_4) (\text{H}_2)(_2)O</td>
<td>10.812</td>
</tr>
<tr>
<td>CuSO(_4) (\text{H}_2)(_2)O</td>
<td>2.722</td>
</tr>
<tr>
<td>Glucose</td>
<td>786.5</td>
</tr>
<tr>
<td>Substance</td>
<td>Amount (g/Kg)</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>0.5</td>
</tr>
<tr>
<td>Calcium Pantothenate</td>
<td>1.2</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.5</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Vitamin K</td>
<td>0.1</td>
</tr>
<tr>
<td>p-Aminobenzoic Acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0.1</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.02</td>
</tr>
<tr>
<td>Inositol</td>
<td>20.0</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>0.00015</td>
</tr>
<tr>
<td>Glucose</td>
<td>974.5</td>
</tr>
</tbody>
</table>
Experiment 1

Six animals were randomly assigned to each of two control and two experimental groups. The experimental diets were administered for 12 weeks, 6 animals from each group being killed after 6 weeks (la) of treatment and the remainder at the end of the experiment (lb).

Prior to commencement of the dietary regime and after 3 weeks of treatment, blood (approximately 100μl) was collected under mild ether anaesthesia from the tail vein of each rat into a 2ml heparinised tube (Seward Laboratory, London), for the determination of haematocrit and haemoglobin concentration.

At 6 and 12 weeks, the animals, which had been allowed free access to food prior to this, were killed by cervical dislocation between 8.30 and 9.30 am, following exsanguination from the abdominal aorta under mild ether anaesthesia. A small volume (100μl) of blood was collected as described above, the remainder being collected in non-heparinised glass bottles for preparation of serum. Livers were rapidly excised and treated as described in the previous Chapter.

Results

Animal Weights and Food Intakes

These data are presented in Figs. 4.1 and 4.2 and Tables 4.4 and 4.5.
Fig. 4.1  Growth Curves of Rats Maintained on Iron-Deficient (▲—▲) and Control (●—●) Diets (Experiment la)

Points represent mean values (n=6 for both groups).
No significant differences between means.
Fig. 4.2  Growth Curves of Rats Maintained on Iron-Deficient (△—△) and Control (●—●) Diets (Experiment 1b)

Points represent mean values (n=12, both groups at 6 weeks; n=6, both groups at 12 weeks). No significant differences between means.
### Table 4.4 Mean Food Intake (g/rat/day) of Control and Iron-Deficient Rats (Experiment la)

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>Mean ± SEM</th>
<th>Over 6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>10 10 12 13 15 15</td>
<td>12.5 ± 0.9</td>
</tr>
<tr>
<td>IRON-DEFICIENT</td>
<td>10 10 12 13 13 15</td>
<td>12.2 ± 0.4</td>
</tr>
</tbody>
</table>

n=6 for both groups.

### Table 4.5 Mean Food Intake (g/rat/day) of Control and Iron-Deficient Rats (Experiment lb)

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>Mean ± SEM</th>
<th>Over 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 12</td>
<td></td>
</tr>
<tr>
<td>CONTROL</td>
<td>7 12 12 14 13 16 16 16 17 18 18 17</td>
<td>14.7 ± 0.9</td>
</tr>
<tr>
<td>IRON-DEFICIENT</td>
<td>7 11 12 13 11 16 15 15 14 16 18 18</td>
<td>13.8 ± 0.9</td>
</tr>
</tbody>
</table>

1-6 weeks n=12 for both groups

6-12 weeks n=6 for both groups
In the 6 week experiment (la), there was some indication of a slightly decreased rate of growth in the deficient animals, although this was not statistically significant and not associated with any change in food intake. In the 12 week experiment (lb), growth rate and food intake did not significantly differ from the controls. The deficient animals from both experiments displayed a slight coarsening of the hair and lethargy.

Biochemical Changes Occurring in Animals Fed the Iron-Deficient Diet

This data are presented in Tables 4.6-4.9.

After 3 weeks of iron depletion in both experiments, haemoglobin and haematocrit values had fallen to 70-75% of control. By 6 weeks they had further decreased to approximately 60% of control; a longer period of depletion did not further diminish these parameters. Control values appeared to increase slightly over the first 6 weeks, presumably as a function of age.

Serum total cholesterol concentration rose slightly but not significantly after 6 weeks, although liver cholesterol content was unchanged. Conversely after 12 weeks, serum cholesterol levels were significantly diminished in the deficient animals although again there was no difference in hepatic concentration.

Serum total bile acid concentration did not differ from controls at either time interval.

Liver weight was significantly decreased at 6 weeks but not at 12 weeks. However, the weight of this organ relative to body weight was significantly lower at both time intervals.
Table 4.6  Effect of Iron Deficiency on Plasma Haemoglobin Concentrations and Haematocrit Values During the Experimental Periods

<table>
<thead>
<tr>
<th></th>
<th>la</th>
<th>lb</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEEKS</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Haemoglobin (g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T*</td>
<td>13.7 ± 0.5</td>
<td>11.0 ± 0.3c</td>
</tr>
<tr>
<td>C**</td>
<td>12.4 ± 0.4</td>
<td>15.4 ± 0.3</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T*</td>
<td>42.4 ± 1.2</td>
<td>32.7 ± 0.4c</td>
</tr>
<tr>
<td>C**</td>
<td>38.3 ± 1.5</td>
<td>45.3 ± 0.6</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.
* Iron-deficient (n=6)
** Control (n=6)
c =<p 0.001
Table 4.7  Effect of Iron Deficiency on Some Serum, Hepatic and Related Parameters in the Rat
(Experiments 1a and b)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*</td>
<td>C**</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>236 ± 7b</td>
<td>278 ± 7</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>8.21 ± 0.33b</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>Relative Liver Weight (g/100g body weight)</td>
<td>3.49 ± 0.06b</td>
<td>3.82 ± 0.06</td>
</tr>
<tr>
<td>Liver Microsomal Protein (mg/g)</td>
<td>12.6 ± 0.6</td>
<td>12.4 ± 1.1</td>
</tr>
<tr>
<td>Liver Total Cholesterol (mg/g)</td>
<td>1.58 ± 0.14</td>
<td>1.49 ± 0.11</td>
</tr>
<tr>
<td>Serum Total Cholesterol (mmol/litre)</td>
<td>1.87 ± 0.13</td>
<td>1.45 ± 0.15</td>
</tr>
<tr>
<td>Serum Total Bile Acids (μmol/litre)</td>
<td>2.08 ± 0.17</td>
<td>2.14 ± 0.13</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.
*  Iron-deficient  (n=6)
** Control  (n=6)

a = p<0.05    b = p<0.01
Table 4.8  Effect of Iron Deficiency on Hepatic Cholesterol 7α-Hydroxylase Activity in the Rat
(Experiment la and b)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*</td>
<td>C**</td>
</tr>
<tr>
<td>% $\left[ ^{14}C \right]$ Cholesterol metabolised/hour</td>
<td>1.82 ± 0.23</td>
<td>3.10 ± 0.67</td>
</tr>
<tr>
<td>pmol $\left[ ^{14}C \right]$ Cholesterol metabolised/g liver/hour</td>
<td>281 ± 36</td>
<td>481 ± 106</td>
</tr>
<tr>
<td>pmol $\left[ ^{14}C \right]$ Cholesterol metabolised/mg microsomal protein/hour</td>
<td>22.2 ± 2.3</td>
<td>40.7 ± 8.9</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Iron-deficient (n=6)

** Control (n=6)

<sup>b</sup> = p<0.01
Table 4.9  Effect of Iron Deficiency on Some Parameters of Mixed Function Oxidase Metabolism in the Rat

Expressed per g Liver (Experiment 1a and b)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T^* )</td>
<td>( C^{**} )</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol)</td>
<td>9.56 ± 0.94</td>
<td>9.10 ± 1.17</td>
</tr>
<tr>
<td>Cytochrome ( b_5 ) (nmol)</td>
<td>12.0 ± 0.5(^a)</td>
<td>9.19 ± 0.68</td>
</tr>
<tr>
<td>Cytochrome c reductase (( \mu )mol cyt. c reduced/minute)</td>
<td>0.54 ± 0.02</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>p-Nitroanisole O-demethylase (nmol produced/hour)</td>
<td>22.8 ± 4.8</td>
<td>20.7 ± 1.9</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase (nmol produced/hour)</td>
<td>189 ± 18(^a)</td>
<td>133 ± 8</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase (( \mu )mol produced/hour)</td>
<td>4.04 ± 0.29</td>
<td>3.34 ± 0.26</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Iron-deficient (n=6)

** Control (n=6)

\( a = \langle p \ 0.05 \)
Microsomal protein content was unaffected by treatment.

After 6 weeks, hepatic cholesterol \( \gamma \alpha \)-hydroxylase activity appeared to be lower in the deficient animals, although the difference was not statistically significant. Contrary to this, after 12 weeks the activity of this enzyme was significantly increased in these animals.

Cytochrome P-450 levels were unaltered after 6 weeks of deficiency, although there was an increase in cytochrome b\(_5\). p-Nitroanisole O-demethylase activity was unchanged but biphenyl 2-hydroxylase appeared slightly elevated in these animals. NADPH-cytochrome c reductase activity was not apparently influenced by treatment.

After 12 weeks of depletion there was no difference in either cytochrome levels but p-nitroanisole O-demethylation and biphenyl 2- and 4-hydroxylation were slightly induced, although the difference was not statistically significant. Again the reductase activity was unaffected.
Experiment 2

The animals were randomly assigned to a control and experimental group with 15 in each group, each housed in two cages containing either 7 or 8 animals.

The experimental procedure was as described for the first experiment, except that the animals were bled prior to starting the experiment but not after 3 weeks and were killed after 6, 8 and 12 weeks.

Results

Animals Weights and Food Intakes

These data are presented in Fig. 4.3 and Table 4.10.

The growth rate of the deficient animals was significantly reduced after 4 weeks, remaining so throughout the experiment. This phenomenon was unrelated to any significant alteration in food intake. As previously, these animals showed some coarsening of the fur and appeared slightly lethargic.

Biochemical Changes Occurring in Animals Fed the Deficient Diet

These data are presented in Tables 4.11-4.14.

After 6 weeks of the deficient regime, mean values for haemoglobin and haematocrit had fallen to 60-65% of control, further depletion did not result in a greater reduction of these values.

Serum total cholesterol concentrations were lower at 6 and 12 weeks, although the differences were not statistically significant. Conversely, at 8 weeks, this parameter was signifi-
Fig. 4.3 Growth Curves of Rats Maintained on Iron-Deficient (△—△) and Control (○—○) Diets (Experiment 2)

Points represent mean values; significant differences between means are as shown:  a = p<0.05  b = p<0.01

WEEKS
Table 4.10  Mean Food Intake (g/rat/day) of Control and Iron-Deficient Rats (Experiment 2)

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>Mean±SEM Over 12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>9</td>
<td>12</td>
<td>13</td>
<td>16</td>
<td>15</td>
<td>16</td>
<td>17</td>
<td>17</td>
<td>18</td>
<td>17</td>
<td>20</td>
<td>15.5±0.9</td>
<td></td>
</tr>
<tr>
<td>IRON-DEFICIENT</td>
<td>10</td>
<td>12</td>
<td>13</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>16</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td>14.6±0.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.11  Effect of Iron-Deficiency on Plasma Haemoglobin Concentrations and Haematocrit Values, Measured in All Animals Prior to Starting the Experiment and in Animals Killed During Experiment 2.

<table>
<thead>
<tr>
<th>WEEKS</th>
<th>0</th>
<th>6</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/100ml)</td>
<td>T* 14.7 ± 0.2</td>
<td>9.40 ± 0.25</td>
<td>9.77 ± 0.41</td>
<td>9.13 ± 0.53</td>
</tr>
<tr>
<td>C** 14.2 ± 0.2</td>
<td>15.2 ± 0.2</td>
<td>14.6 ± 0.3</td>
<td>14.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>T* 41.9 ± 0.5</td>
<td>31.0 ± 1.0</td>
<td>34.1 ± 1.2</td>
<td>32.2 ± 2.2</td>
</tr>
<tr>
<td>C** 44.9 ± 0.7</td>
<td>45.0 ± 0.9</td>
<td>45.1 ± 0.4</td>
<td>44.0 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.
*  Iron-deficient
**  Control
n=15 at 0 weeks  n=5 at 6,8,12 weeks.  c = p<0.001
Table 4.12  Effect of Iron Deficiency on Some Serum, Hepatic and Related Parameters in the Rat (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>8 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*</td>
<td>C**</td>
<td>T*</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>274 ± 7</td>
<td>295 ± 9</td>
<td>288 ± 8</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>9.22 ± 0.34</td>
<td>10.9 ± 0.7</td>
<td>9.20 ± 0.37(^b)</td>
</tr>
<tr>
<td>Relative Liver Weight (g/100g body weight)</td>
<td>3.36 ± 0.06(^a)</td>
<td>3.68 ± 0.13</td>
<td>3.19 ± 0.04(^a)</td>
</tr>
<tr>
<td>Microsomal Protein (mg/g)</td>
<td>24.2 ± 0.4</td>
<td>28.7 ± 2.0</td>
<td>14.5 ± 2.0</td>
</tr>
<tr>
<td>Liver Total Cholesterol (mg/g)</td>
<td>1.26 ± 0.09</td>
<td>1.60 ± 0.17</td>
<td>1.71 ± 0.15</td>
</tr>
<tr>
<td>Serum Total Cholesterol (mmol/litre)</td>
<td>1.42 ± 0.16</td>
<td>1.89 ± 0.15</td>
<td>2.20 ± 0.06(^a)</td>
</tr>
<tr>
<td>Serum Total Bile Acids (mol/litre)</td>
<td>1.28 ± 0.60</td>
<td>1.00 ± 0.11</td>
<td>1.75 ± 0.34</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Iron-deficient (n=5)  ** Control (n=5)

\(^a\) p<0.05  \(^b\) p<0.01
Table 4.13 Effect of Iron Deficiency on Hepatic Cholesterol 7α-Hydroxylase Activity in the Rat (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th></th>
<th>8 WEEKS</th>
<th></th>
<th>12 WEEKS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*</td>
<td>C**</td>
<td>T*</td>
<td>C**</td>
<td>T*</td>
<td>C**</td>
</tr>
<tr>
<td>% [4-14C] Cholesterol</td>
<td>2.82 ± 0.35</td>
<td>2.86 ± 0.43</td>
<td>2.02 ± 0.27</td>
<td>2.03 ± 0.44</td>
<td>2.76 ± 0.38</td>
<td>2.07 ± 0.35</td>
</tr>
<tr>
<td>metabolised/hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol [4-14C] Cholesterol</td>
<td>438 ± 55</td>
<td>444 ± 66</td>
<td>294 ± 40</td>
<td>297 ± 64</td>
<td>404 ± 55</td>
<td>302 ± 51</td>
</tr>
<tr>
<td>metabolised/g liver/hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pmol [4-14C] Cholesterol</td>
<td>18.0 ± 2.0</td>
<td>15.3 ± 1.6</td>
<td>22.5 ± 4.3</td>
<td>17.1 ± 4.0</td>
<td>30.0 ± 4.5</td>
<td>21.5 ± 3.3</td>
</tr>
<tr>
<td>metabolised/mg microsomal protein/hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.

* Iron-deficient (n=5)

** Control (n=5)
Table 4.14 Effect of Iron Deficiency on Some Parameters of Mixed Function Oxidase Metabolism in the Rat
Expressed per g Liver (Experiment 2)

<table>
<thead>
<tr>
<th></th>
<th>6 WEEKS</th>
<th>8 WEEKS</th>
<th>12 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T*</td>
<td>C**</td>
<td>T*</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol)</td>
<td>16.9 ± 0.5</td>
<td>19.2 ± 1.3</td>
<td>10.7 ± 1.2</td>
</tr>
<tr>
<td>Cytochrome b₅ (nmol)</td>
<td>16.0 ± 0.6</td>
<td>18.9 ± 1.0</td>
<td>12.5 ± 1.5</td>
</tr>
<tr>
<td>Cytochrome c reductase (μmol cyt. c reduced/min)</td>
<td>1.10 ± 0.03</td>
<td>1.12 ± 0.12</td>
<td>0.95 ± 0.10</td>
</tr>
<tr>
<td>p-Nitroanisole O-demethylase (nmol produced/hour)</td>
<td>28.2 ± 5.9</td>
<td>23.6 ± 4.9</td>
<td>27.6 ± 2.6 ±</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase (nmol produced/hour)</td>
<td>177 ± 8</td>
<td>160 ± 11</td>
<td>110 ± 13</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase (μmol produced/hour)</td>
<td>3.91 ± 0.13</td>
<td>3.94 ± 0.16</td>
<td>5.06 ± 0.21</td>
</tr>
</tbody>
</table>

Results expressed as mean ± standard error of mean.
* Iron-deficient (n=5)
** Control (n=5)
+ n=4 a = p<0.05
cantly increased in the deficient animals. Hepatic concentrations of cholesterol were generally uninfluenced by treatment.

Mean serum total bile acid concentration displayed little variation from control at any time interval.

Liver weight was significantly reduced by treatment at 8 and 12 weeks, although not at 6 weeks. However, the relative liver weight was significantly decreased at all 3 time intervals. Microsomal protein content was not significantly altered by treatment.

In this experiment, cholesterol \( \Delta \)α-hydroxylase activity showed little variation from control, although when expressed as specific activity there was an indication of a slight increase in the deficient animals.

Cytochrome P-450 and b5 levels were slightly but not significantly lower in the deficient animals at 6 and 8 weeks, whereas at 12 weeks the P-450 levels were somewhat higher in these animals. Cytochrome c reductase activities were unaffected by treatment.

After 6 weeks of treatment, there were no changes in the activities of the enzymes measured. However p-nitroanisole O-demethylation was significantly higher in the deficient animals at 8 and 12 weeks. Both 2- and 4- biphenyl hydroxylase activities were also greater at 8 weeks but the difference was not statistically significant. At 12 weeks, there was little variation in the activity of either hydroxylase.
Discussion

The iron-deficient regime caused some reduction in growth rate of the rats, although this finding was not consistent. Using similar diets, McCall et al. (1962) and Bailey-Wood et al. (1975) observed a decrease in body weight and growth rate whilst Becking (1972) reported no change in body weight. The general appearance of the animals was not markedly dissimilar to the controls, with the exception of a slight coarsening of the coat and a mild lethargy. This is in contrast to McCall et al. (1962) and Cusack and Brown (1965) who reported more severe skin, hair and behavioral changes.

The degree of deficiency attained in the present experiments was not as great as that achieved by other workers. On average, haemoglobin concentrations fell to approximately 60% of control after six weeks, with no further decrease; other workers have achieved decreases of 50-55%.

The finding of a decrease in liver weight, accords with the results of Bailey-Wood et al. (1975), who did not, however, observe the decrease in relative liver weight seen here, presumably because the reduction in body weight of their rats was more severe. Conversely, Cusack and Brown (1965) reported no change in liver weight.

The changes in total serum cholesterol concentration, observed here, were not consistent. In general, there was a trend towards a decrease in cholesterol with decreasing haemoglobin levels, but on two occasions, the converse of this was noted. Liver cholesterol concentrations did not differ from controls at any time. Few studies have examined the effects of nutritional
iron deficiency on serum lipid concentrations and no data is available for hepatic concentrations. Lewis and Iammarino (1971) reported a pronounced lipaemia in severely iron-deficient rats, associated with an increase in circulating chylomicrons and triglycerides, but with no change in cholesterol or phospholipid concentrations. Amine and Hegsted (1971) observed a similar type of hyperlipidaemia in both rats and chicks but also noted some depression in serum cholesterol concentration. These workers demonstrated an interaction between the type of dietary fat and the iron deficiency; hypertriglyceridaemia only being evident when hydrogenated vegetable oil or coconut oil was used and not with corn oil. This finding would explain the absence of any obvious lipaemia in the present series of experiments, where corn oil was the source of dietary fat. Guthrie et al. (1974) investigated the effect of maternal iron deficiency during pregnancy and/or lactation on the lipid metabolism of both dams and pups. The dams showed little change in serum triglyceride or cholesterol levels but the offspring had elevated serum triglyceride concentrations when iron depletion occurred during pregnancy or lactation or during both periods. However, cholesterol levels were elevated only in those depleted during both pregnancy and lactation, indicating the importance of iron stores in influencing the severity of the abnormality in lipid metabolism.

The mechanism accounting for these observed changes in lipid metabolism has not been investigated, although Lewis and Iammarino (1971) did report a decrease in lipoprotein lipase activity, which might account for the observed triglyceridaemia. In the present study, it was postulated that any change in serum
cholesterol concentration might result from an alteration in its catabolism to bile acids. However, serum levels of bile acids were largely unaffected by the deficient regime, although there were some changes in cholesterol \( \Delta \)-hydroxylase activity. Thus, after 6 weeks of depletion in the first experiment, there was an increase in serum cholesterol concentration, associated with a decrease in \( \Delta \)-hydroxylase activity. After 12 weeks, despite no further decrease in haemoglobin levels, the pattern was reversed i.e. there was a decrease in serum cholesterol associated with an increase in cholesterol \( \Delta \)-hydroxylation. In the second experiment however, despite a slight increase in serum cholesterol after 6 and 12 weeks of depletion, \( \Delta \)-hydroxylase activity only showed a very small increase at 12 weeks. The observed increase in serum cholesterol at 8 weeks was not accompanied by a corresponding decrease in enzyme activity.

These results may be interpreted in several ways. Firstly, it is possible that iron deficiency might also influence other pathways of cholesterol metabolism, such as its absorption or biosynthesis; this would merit further investigation. If the deficiency had opposing effects on these pathways, the variable results of these experiments and those of previous workers might be explained. Thus, different degrees of deficiency might influence serum cholesterol levels by either stimulating or inhibiting a particular pathway, although if this were so, hepatic cholesterol concentrations might be expected to vary.

An alternative explanation, which would better explain the changes in cholesterol \( \Delta \)-hydroxylase activity seen here, is that iron-deficiency might indirectly affect cholesterol
metabolism by influencing other nutrients. Severe iron-deficiency in the rat (Vitale et al., 1966) and in man (Velez et al., 1966) is known to induce folate deficiency, which in turn may reduce mixed function oxidase metabolism (Labadarios, 1975; Labadarios et al., 1978). Various authors have reported considerable interactions between iron and other trace minerals such as zinc and copper. Iron deficiency has been shown to lead to an increase in hepatic copper content (Sourkes et al., 1968; Symes et al., 1969) and as the corollary to this, a dietary deficiency of copper results in increased hepatic iron concentrations (Frieden, 1971). Klevay (1973) has demonstrated hyperlipidaemia in rats fed diets with a high zinc/copper ratio. However Sherman et al. (1977) demonstrated, in rats bred from iron-deficient dams, a hyperlipaemia which was associated not only with a decreased liver iron level but also with a decreased zinc/copper ratio, a finding opposed to that of Klevay. In a recent study, Allen and Klevay (1978) reported an increase in plasma cholesterol concentration, associated with an increased appearance of newly synthesised labelled cholesterol in plasma, in response to copper deficiency. They postulated that copper might act via an effect on lipoprotein or bile acid metabolism. Thus the observed modulatory effect of iron deficiency on cholesterol metabolism might result indirectly from its effect on hepatic concentrations of copper. In subsequent investigations, it would therefore be of interest to compare the effects of iron and copper deficiency on cholesterol metabolism, particularly with respect to cholesterol 7α-hydroxylase activity. It could be envisaged that copper status, particularly at the microsomal
level, might vary with different degrees of iron deficiency and thus variably affect cholesterol 7α-hydroxylase activity.

The effects of iron deficiency on mixed function oxidase metabolism seen here are not totally in accord with those reported by other workers. Generally, there was an increase in the metabolism of p-nitroanisole and biphenyl, although the magnitude of the effect was small, particularly for the latter substrate. As discussed earlier, this increase has been observed by other workers (Catz et al., 1970; Becking, 1972). The variable sensitivity of specific reactions to iron deficiency, as seen here, was also noted by Becking (1972). In rats, aromatic ring hydroxylations were more sensitive to the deficiency than aminopyrine N-demethylation. This author also speculates that the relative increase in copper content of the liver might influence these oxidative reactions, an idea consonant with that discussed above.

The relative increase in metabolism is difficult to explain. Labbé and Finch (1977) have shown that the activity of δ-aminolevulinic acid dehydratase, an enzyme of haem biosynthesis, is increased in dietary iron deficiency. One might postulate that an increased synthesis of cytochrome P-450 might explain the increase in mixed function oxidase metabolism. However, in the present experiment, in common with those of previous workers, levels of cytochrome P-450 and b5 and of NADPH-cytochrome c reductase were not markedly altered, although the cytochromes were slightly decreased in the second experiment.

Bailey-Wood et al. (1975) have suggested that the competition
by various organs for the limited available iron may reflect the metabolic capacity of the organ. This postulate explains their findings and those of Dallman and Goodman (1971), which showed a normal inductive response of hepatic microsomes to phenobarbitone treatment.

Becking (1972) suggested that the increased metabolism might be due to an alteration in substrate binding to cytochrome P-450 or on the rate of reduction of the P-450/substrate complex. He later expanded this hypothesis (Becking, 1976), by suggesting that the iron released from the liver during deficiency might alter the liver microsomal oxidation/reduction potential. This would result in a more rapid reduction of oxidation of the P-450/substrate complex and consequently an increased rate of metabolism. This hypothesis would be consonant with an increased rate of cholesterol \( \Delta^5 \)-hydroxylase activity.

Becking (1976) has also speculated on the importance of iron stores in controlling the rate of hepatic mixed function oxidase metabolism. He suggested that the relative ratio of ferric/ferrous iron might be relevant, as the ferric ions in ferritin (approximately 20\%) inhibited aniline hydroxylation and aminopyrine N-demethylation in vitro, whereas ferrous ions stimulated these enzymes. Similarly, ferric ions inhibited cytochrome P-450 activity in vitro whereas ferrous ions stimulated the enzyme (Fouts and Pohl, 1971).

It could be that the intracellular ferric/ferrous ion ratio is critical in the control of mixed function oxidase metabolism. Thus the lesser degree of deficiency achieved in the present experiments might be associated with a different
ratio of ferric/ferrous ions, due to a differing degree of depletion of iron stores, which could thus affect the metabolism of various substrates. Variations in this ratio might then also be important in controlling the activity of cholesterol 7\alpha-hydroxylase, hence offering a further explanation for the variable effects on serum cholesterol concentration.

In conclusion, dietary iron deficiency appeared to have a variable influence on serum cholesterol concentrations, associated with changes in cholesterol 7\alpha-hydroxylase activity. This variability could be explained by an interaction with other trace nutrients, particularly copper, as this is known to influence both cholesterol and mixed function oxidase metabolism. A further plausible explanation is that of the ferric/ferrous iron ratio, related to the degree of deficiency. If the degree of depletion had been more severe, iron stores would have been further depleted and the rate consequently reduced. As a result, stimulation of the mixed function oxidase system and perhaps of cholesterol 7\alpha-hydroxylase would be likely to occur. Thus, the possible involvement of iron stores in the control of cholesterol 7\alpha-hydroxylase activity would merit further study.
Chapter 5

General Discussion
The studies described in this thesis were designed to gain further insight into the interactions between certain dietary components and bile acid metabolism, and their relationship to the pathogenesis of atherosclerosis. As the results have been discussed in detail within each of the previous chapters, only the principal findings will be considered here.

**Dietary Lipid and Cholesterol Metabolism**

Firstly, under the experimental conditions employed, cholesterol \( \alpha \)-hydroxylase activity was significantly reduced in the fat-fed animals compared with the controls. Thus, the observed hypocholesterolaemic action of polyunsaturated fats cannot be attributed to an induction of bile acid synthesis. The effects of dietary fat modification on other aspects of cholesterol metabolism, such as its absorption and synthesis have already been investigated by previous workers but an area meriting further study is that of cholesterol transport via lipoproteins. In view of the recent publications concerning high density lipoprotein cholesterol, (see Chapter 1), it would seem important to examine the influence of dietary lipid on this lipoprotein.

The activity of cholesterol \( \alpha \)-hydroxylase was marginally lower in the rabbits given the polyunsaturated fat diet compared with those given saturated fat. As discussed earlier, the former diet might be expected to have a higher peroxide content, which could adversely affect microsomal metabolism by damaging the endoplasmic reticulum membranes. The influence of peroxide content of the diet and of dietary antioxidants on cholesterol \( \alpha \)-hydroxylase activity would merit further investigation.
The higher \( \gamma \)-hydroxylase activities in the chow-fed control animals compared, with those fed semi-synthetic diets, confirm the suggestion of previous authors that animals given unrefined diets display higher activities of this enzyme.

In both groups of rabbits on the high fat regimes there was an increase in cytochrome P-450 and \( b_5 \) concentrations accompanied by decreased mixed function oxidase enzyme activities. Several explanations for this anachronism may be postulated: 1) A high level of dietary fat, independent of the degree of saturation, may inhibit a specific type of P-450 whilst inducing the bulk of the cytochrome. To test this hypothesis, a wider range of substrates should be examined. As fatty acids are known substrates of cytochrome P-450 and are metabolised by \( \omega \)-oxidation, it would also be of interest to separate the cytochrome(s) by SDS-gel electrophoresis, after feeding diets containing variable types and quantities of fat, in order to ascertain if there were any specific inductions or inhibitions of different P-450 types. 2) The fats might bind to cytochrome P-450 and competitively inhibit the metabolism of exogenous substrates. The metabolism of fatty acids by cytochrome P-450 via \( \omega \)-oxidation occurs particularly in conditions where the animal has high circulating levels of ketone bodies. It is thus likely that when fat intake is high, metabolism of fatty acids via this pathway would be induced; therefore it would be of interest to measure \( \omega \)-oxidation under the experimental condition employed here. 3) It is also possible that a high level of dietary fat might unfavourably influence the composition of the microsomal membrane, thereby modifying metabolism; this possibility will be further considered below.
Ascorbic Acid and Cholesterol Metabolism

Secondly, further insight has been gained into the involvement of ascorbic acid in the metabolism both of cholesterol and of foreign compounds. In the guinea pig, there appears to be a threshold level of intake for ascorbic acid which plays some part in the control of cholesterol catabolism to bile acids; there is probably considerable variation in this requirement between individual animals. The level of intake below which aberrations in cholesterol metabolism become apparent in the guinea pig is very low. Therefore, it would seem important to determine this level of requirement in man, in order to confirm the contention of Ginter (1977), that a seasonal deficiency of ascorbic acid is of prime importance in the pathogenesis of hypercholesterolaemia. Although several human studies have now been carried out (Fix et al., 1974; Kothari and Jain, 1977; Kallner, 1977; Ginter, 1977), none are sufficiently extensive to define the range of intakes required in man to prevent hypercholesterolaemia. Rigorous dietary control would be necessary in such a study, in order to eliminate variability due to other dietary components such as lipid.

The results of the present study also indicate that in contrast to the effects of acute ascorbate deficiency, chronic deficiency has little effect on foreign compound metabolism, although again there may be a threshold level of requirement. This finding may also be of significance in man, as it has been suggested that drug metabolism may be impaired in the elderly or institutionalised populations (Zannoni, 1977), where ascorbate intake may be low and subclinical deficiencies apparent (Booth
and Todd, 1972). This assumption has been made on the basis of data emanating from studies in acutely deficient experimental animals. However, as the chronic deficiency state is closer to the human situation, the present results would suggest that drug metabolism is unlikely to be impaired in these people. Furthermore, when ascorbic acid was given at "megadose" levels no adverse effect on mixed function oxidase metabolism was observed.

The only alteration in mixed function oxidase metabolism in response to chronic ascorbate deficiency was the apparent induction of NADPH-cytochrome c reductase activity and to some extent of biphenyl 2-hydroxylase. However, although tentative explanations for this have been put forward (see Chapter 3), this finding remains unexplained.

Dietary Iron and Cholesterol Metabolism

Thirdly, in consideration of the role of dietary iron in cholesterol metabolism, the results from the present study are equivocal. Although iron deficiency modified cholesterol 7α-hydroxylase activity, the effect was not consistent. This could either be interpreted as being related to the degree of depletion of iron stores, or to interactions with other trace minerals such as copper. Both possibilities require further investigation, as discussed in Chapter 4.

Relevance of Experimental Findings to Man

Thus, the nutrients examined here all interact with mixed function oxidase enzymes which control the metabolism of
endogenous and exogenous compounds. However, when each nutrient is considered in isolation, the effects on the microsomal enzymes are small. Thus in attempting to assess the importance of these findings in man, two questions must be asked: 1) What is their relevance in terms of recommendations made to man? and 2) If an experimental model were established, in which all three nutrients and perhaps others such as fibre were modified, would the observed effect on metabolism be more dramatic and more closely represent that seen in man?

Recommendations with respect to dietary lipid are probably of most importance and are certainly most controversial. The present results fail to explain the hypocholesterolaemic effects of polyunsaturated fatty acids and lend support to the doubts recently expressed concerning the "lipid hypothesis" (McMichael, 1976; Mann, 1977; Kummerow, 1977; Cole, 1977; Kaunitz, 1977). Despite the prevailing uncertainties and scepticism regarding the efficacy and mechanism of dietary fat modification and regulation of lipid metabolism, various official bodies, both in this country and the United States (see Chapter 1), have made statements of nutrition policy. These have gradually become more positive regarding the recommended intakes for total, saturated and polyunsaturated fats.

Thus, in the United Kingdom, the D.H.S.S. (1974) were only able to recommend a reduction in total fat intake; however by 1977, the Royal College of Physicians did recommend an increase in the ratio of polyunsaturated to saturated fat. Similarly, in the United States, the American Medical Association (1972) made a conservative statement, recommending dietary lipid
modification only for persons falling into risk categories. Nevertheless, in the Dietary Goals proposed by the Senate Select Committee (1977), stringent "blanket" recommendations have been made for the whole population. These include a reduction of total fat intake to 30% of total energy and of cholesterol to 300mg per day and modification of the type of fat such as to provide equal proportions of saturated, monounsaturated and polyunsaturated fatty acids. These recommendations have been criticised by Harper (1978) and Reiser (1978); both authors refute the idea of mass recommendations, suggesting that to date, only individual treatment of patients with proven abnormalities of lipid metabolism is justified. The present results expand and support this view; although it is apparent that a deficiency or excess of the nutrients studied may influence cholesterol metabolism, at this stage the evidence is insufficient for these blanket recommendations.

The answer to the second question, i.e. "is the human disease of atherosclerosis the result of an interaction of both excesses of and deficiencies of particular nutrients, together with other environmental factors such as smoking and pollution and possibly stress?", can only be speculative. Atherosclerosis is now considered to be a multifactorial disease; the results of the present study support this, as all three nutrients examined influenced bile acid metabolism and the degree of cholesterololaemia, which is an accepted risk factor for atherosclerosis. In this study and in those of previous workers, these nutrients have all been shown to interact with cytochrome P-450. Both the synthesis and catabolism of cholesterol are dependent on P-450 and the
desaturation of fatty acids is regulated by a microsomal enzyme system in which cytochrome b₅, which is closely coupled to cytochrome P-450 (Schenkman et al., 1976), acts as the electron donor (Oshino et al., 1971; Oshino and Omura, 1973). Furthermore, various other factors which are associated with hyperlipidaemia and atherosclerosis also interact with this cytochrome. For instance, high levels of carbon monoxide, which are associated with an increased incidence of atherosclerosis in experimental animals (Astrup et al., 1970) and in man (Astrup et al., 1966), inhibit normal cytochrome P-450 hydroxylations (Orrenius and Ernster, 1964). Similarly, the excessive amounts of vitamin D often ingested by Western populations (Kummerow et al., 1976), are known to cause vascular hypercalcification (Seelig, 1969); hypercholesterolaemia and atherosclerosis (Parke et al., 1978) in experimental animals. Vitamin D₂ displays strong affinity binding for cytochrome P-450 (Cinti et al., 1976) and has been shown to decrease cholesterol 7α-hydroxylase activity in vitro (Rush, 1978). Oestrogens have been suggested as being protective against atherosclerosis on the basis of evidence that the susceptibility of women to cardiovascular disease increases after natural or surgical menopause (Oliver and Boyd, 1959; Kannel et al., 1976) and that diet-induced atheroma in animals can be retarded by oestrogen administration (Wissler and Veselinovitch, 1974) and again, oestrogens are metabolised by P-450 and could thus induce its synthesis.

Thus, many of the factors influencing atherosclerosis also influence or interact with cytochrome P-450. One could hypothesise
that this cytochrome plays a central role in the pathogenesis of this disease. In accordance with this, Stier (1976) has suggested that changes in the lipid environment of the endoplasmic reticulum membrane, induced by diet or exposure to chemicals, could lead to an uncoupling of the cytochrome P-450 system such that the cytochrome would subsequently act as a peroxidase. The process of lipid peroxidation results in the release of various reactive oxygen species such as hydroperoxides, which are known to inactivate enzyme systems associated with damage to endoplasmic reticulum membranes (Hrycay and O'Brien, 1971). These workers also showed that microsomal damage, induced by agents such as thiols or proteases, led to the conversion of P-450 to P-420, with a concomitant enhancement of peroxidase activity. Thus, it could be envisaged that a vicious cycle of uncontrolled membrane oxygenation might occur, both in the liver and at other sites, notably the arterial walls. Either (1) the endoplasmic reticulum could be adversely affected by diet or foreign chemicals, thus resulting in decoupling of the flavoprotein reductase-cytochrome-P-450 system such that the cytochrome would act as a peroxidase or (2) lipid peroxidation might be initiated, leading to membrane damage and conversion of P-450 to the peroxidase, resulting in further release of peroxides.

In this context, the three nutrients selected for investigation in the present study all influence lipid peroxidation (Wills, 1969; Rowe and Wills, 1976; Wills, 1972) and interact with each other in this process (Fujita, 1977; Hammer and Wills, 1978). Therefore it is possible that polyunsaturated fatty acids,
ascorbic acid and iron are vital to the structural and functional integrity of the endoplasmic reticulum membranes and their associated enzymes, over a limited range of concentration. Divergence from these critical concentrations could result in membrane damage and consequently inhibition of normal cytochrome P-450-mediated reactions, including cholesterol \( \Delta^4 \)-hydroxylation; pathological changes might then ensue. In man, the situation is likely to be highly complex, owing to numerous other factors which could also interact with the cytochrome, as discussed earlier.

In summary then, it is suggested that cytochrome P-450 could play a central role in the pathogenesis of atherosclerosis, which might be twofold. Abnormalities in diet and exposure to environmental contaminants could influence membrane integrity and inhibit the P-450-mediated oxidation of cholesterol, possibly leading to hypercholesterolaemia. Secondly, peroxidation might be engendered as a result of this damage, leading to a cycle of uncontrolled release of reactive oxygen species, perpetuating the damage both at the hepatic level and possibly affecting the integrity of the arterial walls. Thus, further investigation of the interaction of diet and foreign chemicals with this enzyme system is required, in the hope that further knowledge in this respect will give greater insight into prophylactic measures which could be adopted in atherosclerosis.
References
REFERENCES


American Medical Association (1972). Preventative Medicine, 1, 559.


Sirtori, C.R., Agradi, E., Conti, F., Mantero, O. and Gatti, E.


