REGULATORY PROCESSES IN THE CONTROL OF CHOLESTEROL SYNTHESIS IN RAT LIVER

by

M.J. JAMES, B.Ag.Sc. (Hons)

Being a Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy.

* * * * * * * *

Department of Animal Physiology,
University of Adelaide,
South Australia.

December, 1976.

TABLE OF CONTENTS

Page

1. Preface 1

2. Introduction and literature review 3

2.1 Pathway of cholesterol biosynthesis 3

2.2 Regulation of hepatic cholesterol synthesis 7

2.2.1 "Zeitgeber" - circadian rhythm 9

2.2.2 Dietary cholesterol 14

2.2.3 Fasting 24

2.2.4 Bile acids 26

2.2.5 Cyclic AMP 28

2.2.6 Circulating hormones 29

2.3 β-Hydroxy-β-methylglutaryl CoA reductase - physical properties 32

3. Materials and methods 35

3.1 Animals 35

3.2 Reagents 35

3.3 Measurement of hepatic cholesterogenesis 36

3.4 Measurement of hepatic HMG-CoA reductase activity 36

3.5 Synthesis of [3-^{14}C]HMG-CoA 38

3.6 Measurement of blood corticosterone level 40

4. Simplified assay for HMG-CoA reductase 41

4.1 Introduction 41

4.2 Method 42

4.3 Results and discussion 43

TABLE OF CONTENTS (Continued)

Page

5. Corticosterone and the control of hepatic cholesterol synthesis 45

5.1 Introduction 45

5.2 Methods 46

5.3 Results 47

5.3.1 Normal diurnal rhythms 47

5.3.2 Effect of continuous darkness 47

5.3.3 Effect of continuous light 48

5.3.4 Relationship between HMG-CoA reductase activity and cholesterol synthesis 49

5.3.5 Relationship between blood corticosterone and cholesterol synthesis/HMG-CoA reductase activity 49

5.4 Discussion 51

6. Dietary feedback control of hepatic cholesterol synthesis - intracellular mechanism 57

6.1 Introduction 57

6.2 Methods 60

6.3 Results 61

6.4 Discussion 63

7. Concluding remarks 72

Bibliography

SUMMARY

This thesis makes some new approaches to reconciling diverse findings on cholesterogenic homeostasis.

(a) The effect of corticosterone on hepatic cholesterol synthesis has been investigated in detail. The diurnal variations in blood corticosterone levels, hepatic cholesterol synthesis *in vitro* and hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase activity were examined in adult, male rats housed under continuous darkness, continuous light, and normal light regimes. Both continuous darkness and continuous light altered these three rhythms from those observed under normal lighting.

Regression analysis showed that (i) under normal light the levels of hepatic cholesterol synthesis and reductase activity at sacrifice were found to correlate with blood corticosterone level 16-20 hours earlier, and (ii) that continuous light abolished the relationship between HMG-CoA reductase and cholesterol synthesis from [1-^{14}C]acetate found in normal light and continuous darkness, so that in this situation the reductase was apparently not the rate-controlling enzyme of the cholesterol pathway.

(b) The existence of a feed-back response by hepatic HMG-CoA reductase to dietary cholesterol is well established, but the intracellular mechanism of this response is not known. Activation energies of this membrane-bound enzyme prepared from the livers of normal-fed and cholesterol-fed rats have been measured. Cholesterol-feeding markedly increased the activation energy (E_a) of the reductase

from 19.5 to 41.2 Kcal/mol/^{o}K, and this result is discussed in terms of altered membrane fluidity. It is suggested that dietary cholesterol exerts its rapid inhibition upon hepatic, microsomal HMG-CoA reductase by modifying the lipid microenvironment of this enzyme and hence affecting configuration and activation energy.

Electron spin resonance (ESR) spectroscopy studies of the effect of cholesterol-feeding on the fluidity of hepatic endoplasmic reticulum, were also begun. These are related to the nature of the intracellular effector responsible for the inhibition of reductase activity after cholesterol-feeding.

(c) During the course of these studies, a new and rapid method for the determination of HMG-CoA reductase activity, was developed.

DECLARATION

I certify that this thesis does not incorporate, without acknowledgement, any material previously submitted for a degree or diploma in any university, and that to the best of my knowledge and belief it does not contain any material previously published or written by another person, except where due reference is made in the text.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Dr. John R. Sabine, for invaluable discussion and direction. His enthusiasm for this project and his understanding of the general topic of cholesterol homeostasis has been a continual source of encouragement.

I am especially grateful to Professor W.V. Macfarlane for his criticism and assistance with the writing of this thesis.

Dr. John Raison has given extensive advice and assistance with the electron spin resonance spectroscopy study reported in this thesis.

The expert typing of Pat Watson is gratefully acknowledged.

Aspects of the work presented in this thesis have been reported elsewhere:

Adrenal hormones and the control of hepatic cholesterol synthesis.
M.J.James and J.R.Sabine
Proc. Aust. Biochem. Soc. *6*, 42 (1973).

A membrane-mediated response of hepatic β-hydroxy-β-methylglutaryl CoA reductase to dietary cholesterol.
M.J.James and J.R.Sabine
Clin. Exp. Pharmacol. Physiol. *2*, 73-74 (1975).

The intracellular mechanism responsible for dietary feedback control of cholesterol synthesis.
J.R.Sabine and M.J.James
Life Sci. *18*, 1185-1192 (1976)

ABBREVIATIONS

HMG	β-hydroxy-β-methylglutaric acid
CoA	coenzyme A
K_m	Michaelis constant
VLDL	very low density lipoprotein
LDL	low density lipoprotein
HDL	high density lipoprotein
IP	intra-peritoneal
IV	intra-venous
G-I	gastrointestinal
cAMP	cyclic adenosine monophosphate
E_a	activation energy
G	gauss
MVA	mevalonic acid

1. PREFACE

WAITE INSTITUTE
LIBRARY

1. PREFACE

Development of the various strands of knowledge which lead to understanding of cholesterol biosynthesis, has had a predictably slow time course.

Although Gardner and Landner (1914) and Landner (1915) concluded that living organisms can not synthesize cholesterol, Channon gave an account in 1925 of an exhaustive balance study which showed that cholesterol is synthesized by intact rats. By 1942, the novel tool of radioisotope labels was used by Bloch and Rittenberg to show that acetate is a major contributor to both the aliphatic chain and the tetracyclic moiety of the cholesterol molecule. By 1960, most of the reactions of cholesterol synthesis from acetate had been outlined (e.g. Popják and Cornforth, 1960), mainly through the studies of Bloch, Lynen, Cornforth and Popják, an excellent historical account of which is given by Bloch (1965).

Control over hepatic cholesterol synthesis by dietary cholesterol was demonstrated in 1951 by Gould and this homeostatic feedback has long been investigated by Siperstein (1960, 1965, 1966, 1970). But detailed studies of cholesterogenic regulation are comparatively recent and this latest component of cholesterol biosynthesis - its regulation - is now an equivocal area of research; the imbroglio of related literature perhaps reflects the ultimate complexity of physiological control maintained over cholesterol biosynthesis.

This thesis attempts to relate diverse findings concerning homeostasis through new approaches to two longstanding problems of control: (a) the control of hepatic cholesterol synthesis by adrenal corticosteroids, and (b) the mechanism of feedback control by dietary cholesterol over hepatic cholesterol synthesis.

2. INTRODUCTION AND LITERATURE REVIEW

2. INTRODUCTION AND LITERATURE REVIEW

2.1 Pathway of cholesterol biosynthesis

In the following review the pathway of sterol biosynthesis will be described because it is basic to the following study. But a statement of enzymic reactions would only duplicate material found in many reviews and textbooks (e.g. Gaylor, 1974; Sabine, 1977). For this reason sections of interest to the following study will be emphasized and expanded while other sections, although essential to sterol biosynthesis, will be limited to a brief description of enzymic transformations.

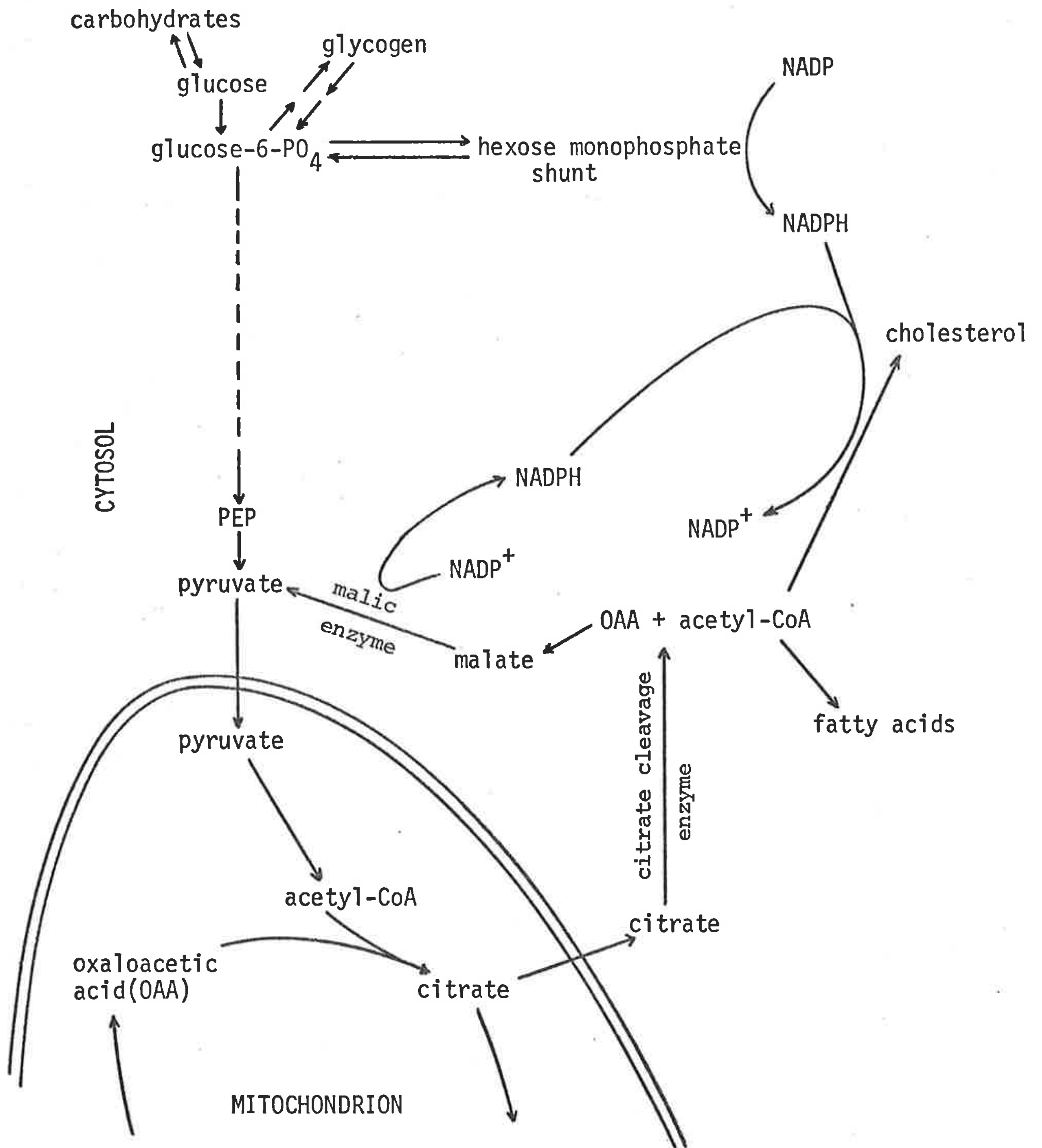
β-Hydroxy-β-methylglutaryl coenzyme A reductase is a major controlling agent in this pathway and is central to this investigation. An account of this enzyme follows in section 2.3.

Formation of Mevalonate

Acetyl-CoA is an end-product of glycolysis and a biological precursor of cholesterol. It can therefore be considered as the initial compound in the cholesterol biosynthetic pathway at which catabolism ends and anabolism begins.

Hepatic cholesterol synthesis is an extra-mitochondrial process and since the bulk of cellular acetyl-CoA arises within the mitochondrion, consideration must be given to the flux of carbon through this extra-mitochondrial pool of acetyl-CoA (Figure 2.1). This is important since much of the work involving the measurement of cholesterol synthesis has employed ^{14}C-acetate as the substrate

Fig.2.1 Generation of extra-mitochondrial acetyl-CoA and NADPH

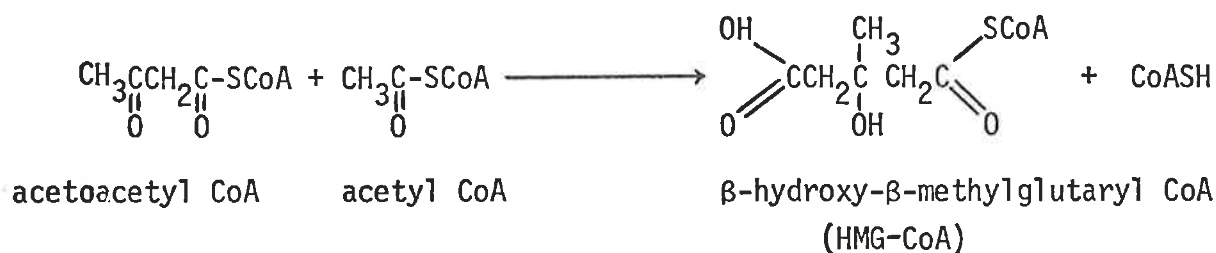


and this method assumes that the specific activity of the newly-formed ^{14}C-acetyl-CoA pool remains constant during the experiment. Furthermore, this method has been used for part of the present investigations (see section 5.4).

The mitochondrial wall is essentially impermeable to acetyl-CoA (Lowenstein, 1968) and major processes giving rise to acetyl-CoA (glycolysis, oxidation of fatty acids, and the breakdown of ketogenic amino acids) occur within mitochondria, at least during their final stages. This acetyl-CoA may traverse the mitochondrial membranes via citrate which is lysed in the cytoplasm by the citrate cleavage enzyme (Spencer *et al.*, 1964). In the rat, free acetate may even be a source of extra-mitochondrial acetyl-CoA by way of the cytosolic acetate thiokinase (Ballard, 1972); formation from pyruvate is also possible (Ballard, 1972). The efflux of acetyl-CoA from the cytoplasmic pool is into both cholesterol (Gaylor, 1974) and fatty acid synthesis (Martin and Vagelos, 1962). Thus alterations in fatty acid synthesis or in any of the processes responsible for extra-mitochondrial acetyl-CoA, may cause alterations in the rate of cholesterol synthesis by altering the size of the extra-mitochondrial pool of acetyl-CoA.

The conversion of acetyl-CoA to mevalonic acid is a sequence involving three enzymes (Figure 2.2). Condensation of two molecules of acetyl-CoA by acetyl-CoA acetyltransferase produces acetoacetyl-CoA which condenses with another molecule of acetyl-CoA in the presence of HMG-CoA synthetase and produces β-hydroxy-β-methylglutaryl CoA (HMG-CoA), the latter process being a well-defined, three-stage reaction (Miziorko *et al.*, 1975). HMG-CoA is then reduced

Fig.2.2 Formation of mevalonate



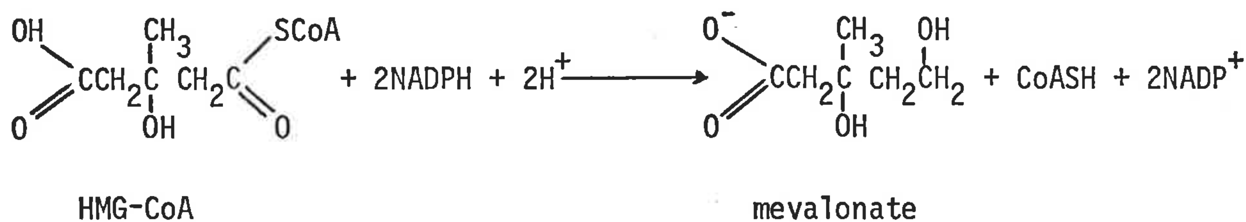


Fig.2.3 Mevalonate to "active isoprene"

$$\mathrm{^{-}O\!-\!C(=O)CH_2C(CH_3)(OH)CH_2CH_2OH} + \mathrm{ATP} \longrightarrow \mathrm{^{-}O\!-\!C(=O)CH_2C(CH_3)(OH)CH_2CH_2OPO_3H_2} + \mathrm{ADP}$$

mevalonate

5-phosphomevalonate

+

ATP

$$\downarrow$$

$$\mathrm{ADP} + CO_2 + \mathrm{CH_2{=}C(CH_3)CH_2CH_2OPO_3HPO_3H_2} \xleftarrow{\mathrm{ATP}\ +} \mathrm{^{-}O\!-\!C(=O)CH_2C(CH_3)(OH)CH_2CH_2OPO_3HPO_3H_2} + \mathrm{ADP}$$

3-isopentenyl pyrophosphate

("active isoprene")

$$\rightleftharpoons$$

$$\mathrm{CH_3C(CH_3){=}CHCH_2OPO_3HPO_3H_2}$$

dimethylallyl pyrophosphate

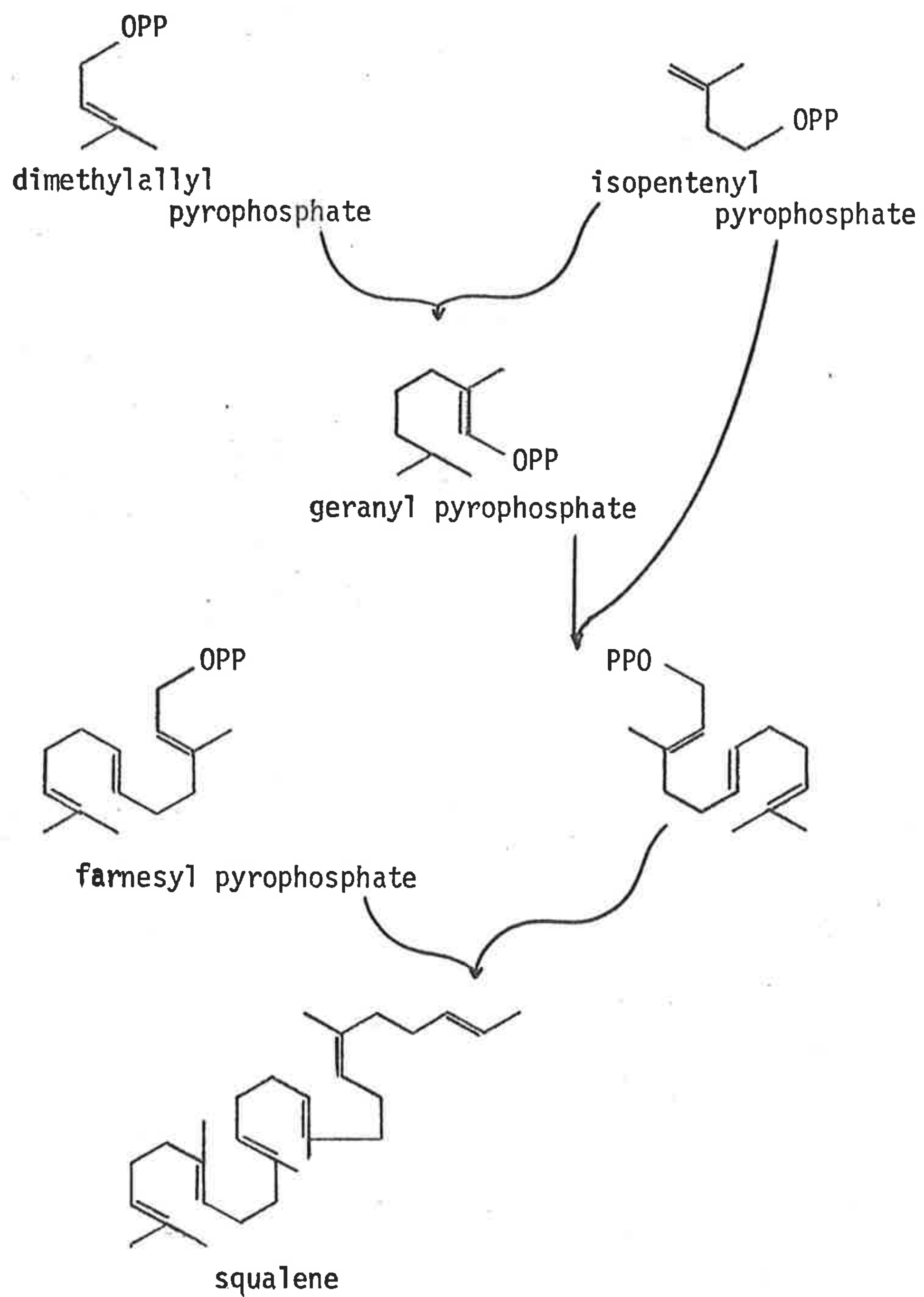
with the removal of the CoA moiety by HMG-CoA reductase (EC 1.1.1.34). resulting in mevalonic acid. (The absence of the CoA moiety in the product of this reaction formed the basis of a rapid assay for HMG-CoA reductase which was developed within this study - section 4). The first two enzymes are considered to be cytosolic (Gaylor, 1974) although some HMG-CoA synthetase activity may be associated with the microsomal fraction (McNamara and Rodwell, 1972). However, HMG-CoA reductase is bound to the endoplasmic reticulum, a property which is presented in this thesis as being important in the control of this enzyme by dietary cholesterol (section 6).

Mevalonate to Squalene

Within the cytosol, mevalonate undergoes two phosphorylations in the presence of ATP and the appropriate kinases, first to a 5-phosphate ester which is again phosphorylated at the 5 position resulting in pyrophosphomevalonate. A decarboxylation reaction follows to produce isopentenyl pyrophosphate which is isomerized to the dimethylallyl form, these two compounds being regarded as the biosynthetic isoprenoid units which are the monomers for a wide variety of isoprene compounds (Bloch, 1965) (Figure 2.3). Condensation between these two unit isomers yields geranyl pyrophosphate from which farnesyl pyrophosphate forms by the addition of another isopentenyl pyrophosphate unit (Cornforth, 1965; Popják, 1970) (Figure 2.4).

Squalene synthetase catalyses the dimeric coupling of two molecules of farnesyl pyrophosphate. However, this enzyme and all that catalyse the remaining transformations are membrane-bound (Gaylor, 1974) although a soluble, but non-enzymic, "sterol carrier protein" (SCP) is involved (Scallen, 1971; Ritter, 1973).

Fig.2.4 Formation of squalene



Squalene oxidation and cyclisation

In the presence of a soluble protein factor (SCP), membrane-bound squalene epoxidase catalyses the formation of squalene-2,3-epoxide. The following reaction in which squalene-2,3-epoxide is cyclised to lanosterol, is a most remarkable multi-valent transformation in which one proton is added, and several hydrogen atoms and methyl groups are rearranged (Figure 2.5). It is catalysed by a single enzyme, squalene-2,3-oxide sterol cyclase, which has been solubilised from microsomal particles and partially purified (Yamamoto and Bloch, 1970).

Lanosterol to Cholesterol

Lanosterol is the first cyclised intermediate in this sequence. The transformation of this compound to cholesterol involves removal of the 4α, 4β and 14α methyl groups by a novel mixed function oxidase (Gaylor, 1974), reduction of the double bond in the side chain, and transfer of the $\Delta^{8(9)}$ double bond to Δ^5 via Δ^7 and $\Delta^{5,7}$ intermediates. There are comprehensive reviews of these reactions (e.g. Gaylor, 1974; Sabine, 1977). Although these enzymic transformations have been investigated in detail, the major sequence of reaction in this multi-enzyme system is ill-defined. Studies with enzyme inhibitors (Steinberg and Avigan, 1960; Dvornik *et al.*, 1963) have shown intermediates in the pathway via 24-dehydrocholesterol and that via 7-dehydrocholesterol (Figure 2.6). Although the importance of each pathway is unknown, sterol carrier protein can regulate them (Ritter *et al.*, 1972).

Fig.2.5 <u>Cyclisation</u>

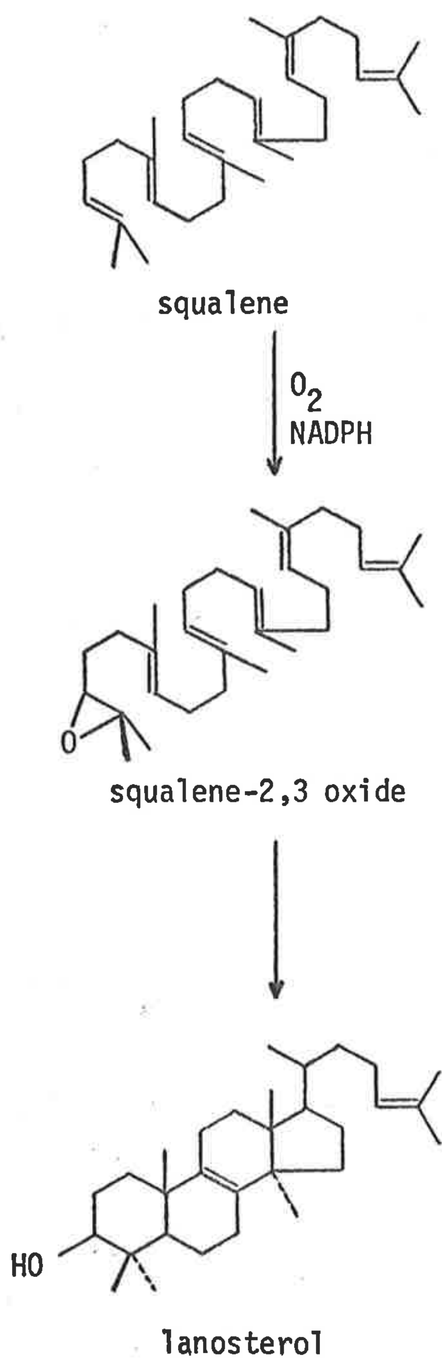
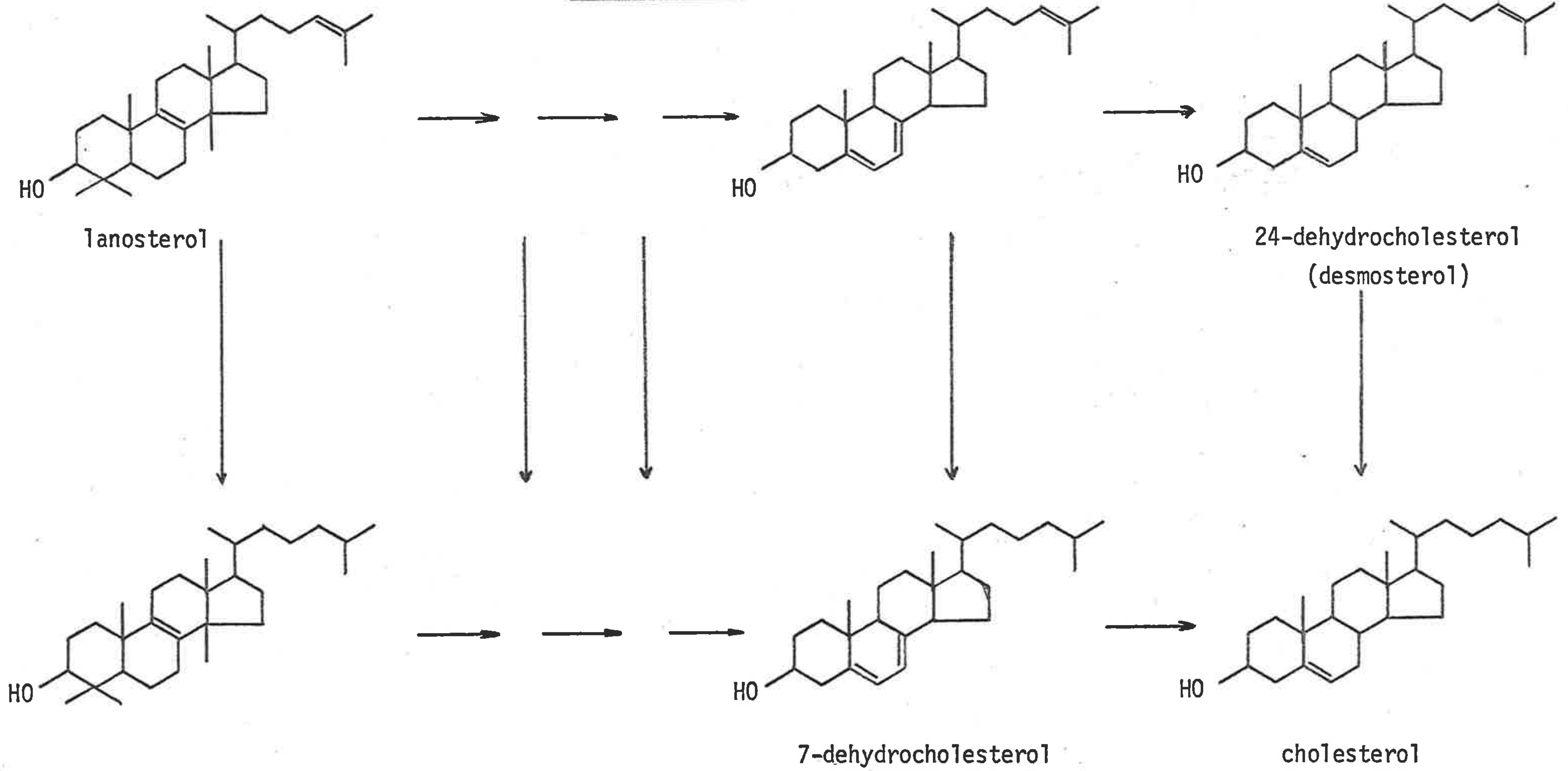


Fig.2.6 Lanosterol to cholesterol



Membrane Association

The cholesterol biosynthetic enzymes are variously associated with the endoplasmic reticulum (Figure 2.7). Sterol carrier protein and membrane involvement is consistent with the fact that the sequence from squalene to cholesterol involves water-insoluble intermediates. However, the association of HMG-CoA reductase with the endoplasmic reticulum is not consistent with this reasoning as both reactant and product are water soluble, with the reactions immediately before and after HMG-CoA reductase occurring in the cytosol.

2.2 Regulation of hepatic cholesterol synthesis

As outlined earlier, the synthesis of cholesterol by intact animals was first demonstrated in 1925 (Channon) and the liver was established as the major organ of synthesis in higher animals (Bloch *et al.*, 1946), but consideration and investigation of the regulation of hepatic cholesterol synthesis is comparatively recent. It is now apparent that the rate of cholesterol synthesis at any moment is the result of equilibration between at least three separate, but interacting, influences.

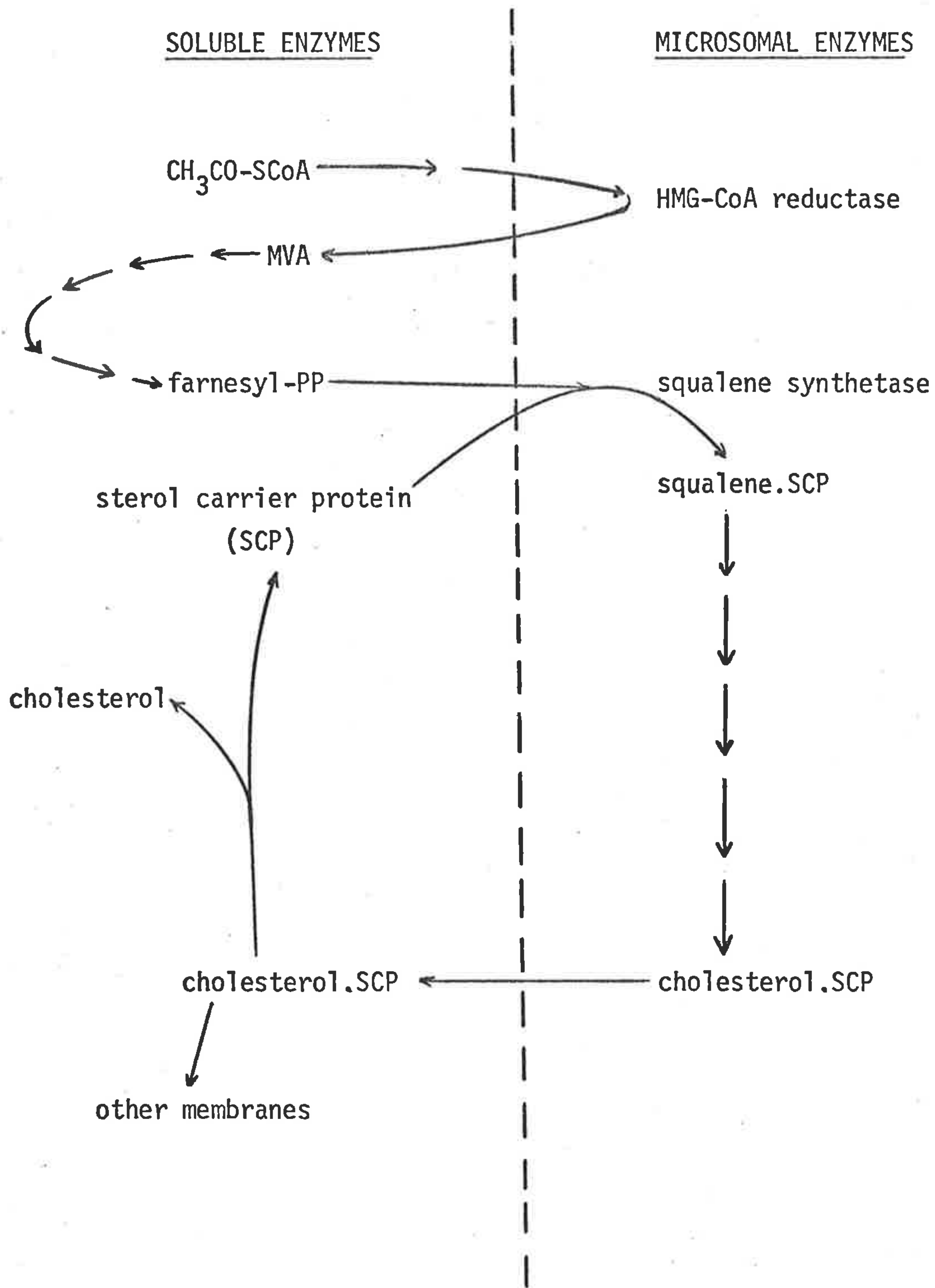
Hepatic cholesterol synthesis:

(i) varies diurnally according to the light:dark cycle

(ii) is inhibited by dietary cholesterol

(iii) is inhibited by fasting.

Fig.2.7 Membrane assocation



These three modes of regulation have now been extensively investigated, but cholesterol synthesis is probably further modified naturally by hormonal factors and other nutritional factors.

Normal liver homogenates and slices have a much greater capacity to synthesize cholesterol from mevalonate than from acetate (Gould and Swyryd, 1966). This implies that the rate-limiting step in the overall pathway lies between acetate and mevalonate. Furthermore, the level of mevalonate synthesis in normal slices corresponds very closely with the incorporation of ^{14}C-acetate into cholesterol (Siperstein and Fagan, 1966), which also indicates that the synthesis of mevalonate is rate-limiting for cholesterol formation. Bucher *et al.*, (1960) compared HMG-CoA reductase activity and the level of cholesterol synthesis in the livers of Triton-treated rats. Their data also indicate that the reductase is a rate-limiting enzyme. This same relation has been suggested in mouse liver (Kandutsch and Saucier, 1969).

The hypothesis that the reduction of HMG-CoA to mevalonic acid is a rate-limiting step in cholesterol synthesis, has generally led to the proposal that these three natural mechanisms of control have their effect at this reaction, catalysed by HMG-CoA reductase. Compared to other microsomal enzymes, the reductase has a remarkably rapid turnover ($t_{\frac{1}{2}}$ 1-2 hours; Edwards and Gould, 1974) which is an obvious advantage in fine regulation. The following discussions of each mode of control will therefore begin with consideration of evidence that control is effected through HMG-CoA reductase, although it will be seen that this model is not sufficient in all circumstances.

2.2.1 "Zeitgeber" - circadian rhythm

Kandutsch and Saucier (1969) observed a diurnal variation of cholesterol synthesis (and reductase activity) in mouse liver. This rhythm was shown to exist also in rat liver (Back *et al.*, 1969) and has now been demonstrated many times (Sabine, 1977). The maximum rate of synthesis occurs near midnight and has been found 3-10 times greater than that during the day (Sabine, 1977).

HMG-CoA reductase

It is "apparently mediated through HMG-CoA reductase" (Siperstein, 1970). At various times of the day Slakey *et al.* (1972) measured the activity of HMG-CoA reductase, the individual activities of six enzymes between mevalonate and squalene, and the rates of conversion of both acetate and mevalonate to cholesterol. Only the reductase activity and the rate of conversion of acetate to cholesterol showed a significant diurnal variation. Since the conversion of mevalonate to cholesterol showed no diurnal variation, not only the individual enzymes studied, but also those between squalene and cholesterol, can not be subject to diurnal variations. Therefore only the three enzymes (including HMG-CoA reductase) effecting conversion of acetyl-CoA to mevalonate can be responsible for the diurnal rhythm of cholesterol synthesis. Many other studies of animals have now shown that HMG-CoA reductase has a diurnal rhythm (Shapiro and Rodwell, 1969, 1972; Hamprecht *et al.*, 1969) coinciding closely in time with the demonstrated rhythm of cholesterol synthesis from acetate (Back *et al.*, 1969). But a lag between the peaks of cholesterol synthesis and

reductase activity prompted Back *et al.* (1969) to suggest that one or both of the enzymes prior to HMG-CoA reductase may participate in the rhythm of cholesterol synthesis although it is not known whether these enzymes (acetyl-CoA acetyltransferase, HMG-CoA synthetase) vary diurnally. Furthermore, Ramasarma (1972) has implied that the reductase may not be rate-limiting at midnight because the increase in cholesterol synthesis rate from noon to midnight is less than the increase in HMG-CoA reductase activity. Overall, it appears that the reductase may not be rate-limiting for the diurnal rhythm of cholesterol synthesis under all conditions of lighting and nutrition. The processes underlying HMG-CoA reductase diurnal variation have nevertheless received much attention.

Mechanism of rhythm

The diurnal rise in HMG-CoA reductase activity has been prevented by puromycin (Kandutsch and Saucier, 1969), (mouse liver) and cycloheximide (Shapiro and Rodwell, 1969) which indicates that the cyclic increase is a result of augmented reductase synthesis rather than activation of the enzyme. This indication was verified in a direct study by Higgins *et al.* (1971) who measured the incorporation of ^{3}H-leucine into highly purified HMG-CoA reductase. This showed the rhythm to be due to synthesis of new enzyme protein for approximately six hours, followed by complete cessation of synthesis for approximately fifteen hours. Regarding intracellular mechanisms of this phenomenon, it may be significant that Erickson and Gould (1974) have shown free cholesterol and cholesteryl esters to be associated with rat liver chromatin and they found that the relative amounts change markedly

during a diurnal period. Although Higgins *et al.* (1971) observed that the rate of degradation of HMG-CoA reductase was the same throughout all stages of the cycle, it is interesting that cycloheximide prevented not only the cyclic rise in enzyme activity, but also prevented the decline in activity after midnight (Shapiro and Rodwell, 1969); this implies the presence of a specific degradative or inactivating protein for HMG-CoA reductase.

A unique feature of HMG-CoA reductase rhythm was noted by Shapiro and Rodwell (1972). They observed that the zenith of the cycle actually comprised two distinct peaks with individual maxima separated by approximately 1½ hours. This prompted several possible explanations including the possibility of damped oscillations, an "overshoot" phenomenon which is then regulated by an excess of enzyme or end-product causing a suppression to levels so low that an increase again occurs, *et cetera*. (Note that this possibility can also apply as the reason for the cyclic rhythm of HMG-CoA reductase, or any cyclic rhythm.) However, Shapiro and Rodwell (1972) also observed this double peak in the reductase rhythm of fasted rats at a level one twentieth that of fed rats. Not only the general rhythm, but also its fine structure, therefore favours the hypothesis that the entire cyclic rhythm is internally programmed and independent of the level of enzyme or end-product.

Cholesterol-feeding abolishes the rise in HMG-CoA reductase activity seen from 6 p.m. to midnight (Shapiro and Rodwell, 1972) while fasting lowers the overall activity (5% of control) but

not the diurnal rhythm (Hamprecht *et al.*, 1969; Shapiro and Rodwell, 1972). As rats are intermittent feeders and as fasting is a powerful regulator of cholesterol synthesis, these factors could combine and result in a rhythmic variation of synthesis. But the persistence of the rhythm in fasted rats indicates that nutritional factors do not trigger the cyclic rhythm. The rhythm of HMG-CoA reductase seems therefore to be a permanent feature in all nutritional states (except cholesterol-feeding) although its amplitude is greatly increased after feeding.

This view is not supported by Dugan *et al.* (1972) who have found that the rhythms of both cholesterol synthesis and HMG-CoA reductase peaked at midday (12 hours out of phase) after controlled feeding for a short time at the beginning of the light period. Furthermore, the rhythm of cholesterol synthesis disappeared during one day of fasting (although the rhythm of HMG-CoA reductase persisted) and Dugan *et al.* (1972) concluded that the rhythm of cholesterol synthesis is dependent on feeding rather than lighting. This conclusion is not supported by the results of Hickman *et al.* (1972) and Bortz and Steele (1973) who found a persistence of the diurnal rhythm of cholesterol synthesis in the livers of fasting animals, once again implying that lighting rather than nutritional factors is the stimulus for the rhythm. That alternating light and dark is the "zeitgeber" of the reductase rhythm is shown by the results of Huber and Hamprecht (1972). They found that after the light-dark phase was shifted by 12 hours, the phase of the enzyme rhythm shifted accordingly within seven days.

Hormonal influence

As the "zeitgeber" appears to act through intrinsic rather than extrinsic (e.g. nutrition) mechanisms, some consideration has been given to hormonal influences on the diurnal rhythm. Hypophysectomy abolishes the diurnal variation of HMG-CoA reductase and a low level of activity results (Ness *et al.*, 1973; Edwards, 1973). But the radical treatment of hypophysectomy can give little information concerning the effect of discreet hormones on reductase activity. Edwards (1973) observed that hypophysectomized rats in addition ate much less food than intact animals and that they were no longer intermittent feeders. Such nutritional alterations are themselves potent modulators of HMG-CoA reductase activity.

Adrenalectomy has variously been reported to have wide ranging effects on cholesterol synthesis and HMG-CoA reductase activity. It has been reported to abolish the diurnal rhythm of cholesterol synthesis resulting in a high static rate (Hickman *et al.*, 1972; Sabine *et al.*, 1972) and to abolish the rhythm of HMG-CoA reductase activity resulting in a low static level (Edwards, 1973), yet Huber *et al.* (1972) have reported that the circadian rhythm of HMG-CoA reductase is unaffected by adrenalectomy.

Such anomalies may in due course be explained by consideration of insulin/glucagon and thyroxine effects in cholesterogenesis. Recent work by Nepokroeff *et al.* (1974) has demonstrated a clear role for the pancreas in the maintenance of the diurnal rhythm of HMG-CoA reductase. Reductase activity and the

amplitude of the diurnal variation of this enzyme are progressively reduced to low levels within one week after the onset of diabetes induced by streptozotocin. They have further shown the necessity of insulin for the diurnal rise of reductase activity since this rise is restored in diabetic rats by administration of insulin. But glucagon, dibutyryl cyclic AMP and hydrocortisone markedly inhibited the diurnal rise in normal rats. This suggests that the relative concentrations of insulin, glucagon, and glucocorticoids are important in the regulation of the diurnal rhythm of HMG-CoA reductase. But this system is further complicated by the report of Dugan *et al.* (1974), who used hypophysectomized-diabetic rats to show that L-triiodothyronine is necessary for the stimulation of the reductase by insulin. Therefore the final rhythm of HMG-CoA reductase (and cholesterogenesis) is the result of the interplay between at least three distinct hormonal systems.

2.2.2 Dietary Cholesterol

That hepatic cholesterol synthesis in many higher animals is markedly suppressed by dietary cholesterol, has been demonstrated many times (Siperstein, 1970; Sabine, 1977) since the original observations of Gould (1951).

HMG-CoA reductase

Despite much indirect evidence that regulation by dietary cholesterol is at the level of HMG-CoA reductase (Bucher *et al.*, 1959; Siperstein and Guest, 1959, 1960), data from studies of the activity of the reductase itself are comparatively recent. Using

gas-liquid chromatography, Siperstein and Fagan (1966) demonstrated that 5% cholesterol in the diet of rats caused a marked inhibition of mevalonate synthesis while HMG-CoA synthesis was unaffected. Linn (1967a) was the first to report that mammalian reductase could be assayed by a thin-layer chromatographic method, which she then used to confirm that this enzyme was subject to regulation by dietary cholesterol (Linn, 1967b). Direct evidence was gathered by Shapiro and Rodwell (1971) who showed that after four hours of cholesterol feeding, the decline in cholesterol synthesis measured in liver homogenate closely paralleled the decline in HMG-CoA reductase activity. They concluded that "observed changes in HMG-CoA reductase activity thus appear to be an accurate reflection of changes in the intracellular rate of cholesterol synthesis".

Although the conversion of HMG-CoA to mevalonate is the major site of cholesterol feedback control, Siperstein and Fagan (1966) observed a modest decrease in the conversion of mevalonate to cholesterol after three days of cholesterol feeding (5% in the diet). Gould and Swyryd (1966) demonstrated a progressively greater inhibition of the reactions beyond mevalonate synthesis with prolonged feeding of cholesterol (almost complete inhibition after one year at 1% cholesterol in the diet). But they emphasize that the inhibition of mevalonate conversion to cholesterol developed more slowly than that of acetate conversion and appeared to be secondary to it. In contrast, cholesterol-feeding inhibits a reaction prior to that catalysed by HMG-CoA reductase, *viz.* HMG-CoA synthetase (White and Rudney, 1970). But this study also shows that the major effect

of cholesterol-feeding is on the reductase while the synthetase is only partially inhibited.

Nature of the inhibition

The molecular mechanism of the reductase inhibition which is activated by cholesterol in the diet, has long been a topic of conjecture and no single reported explanation has successfully combined all reported observations. An hypothesis which attempts to resolve apparent anomalies is presented and tested in section 6 of this study.

There has been speculation that an allosteric effect on the reductase was responsible for inhibition (Siperstein and Fagan, 1964; Harry *et al.*, 1973), but evidence for control by altered catalytic activity has been difficult to produce. No experiments with subcellular fractions have been able to demonstrate inhibition by possible effectors *in vitro*. Although these observations do not support the hypothesis, neither do they refute it and a possible explanation for the lack of inhibition *in vitro* is given in the following discussion on the nature of the intracellular effector. Furthermore, Shapiro and Rodwell (1969) reported that the Michaelis constant (K_m) was similar for HMG-CoA reductase from the livers of normal-fed and cholesterol-fed rats. This indicates that control does not involve a classical allosteric mechanism in which the effector alters the affinity of the enzyme active site for the substrate(s).

In part because evidence for inhibition of catalytic activity was lacking, the view that cholesterol-feeding leads to a repression of HMG-CoA reductase synthesis was investigated. Using a mathematical model to calculate the rate of synthesis of hepatic HMG-CoA reductase in cholesterol-fed rats, Rodwell (1972) and associates (1973) concluded that cholesterol-feeding decreases the amount, and not the activity, of the enzyme. But it is now clear that control by dietary cholesterol is a coalition of both models in which activity and amount of enzyme are both decreased.

The direct approach of measuring the actual amount of enzyme (by immunoprecipitation) after cholesterol-feeding has led Higgins and Rudney (1973) to propose a bimodal model for inhibition of HMG-CoA reduction to mevalonate, by dietary cholesterol. After cholesterol-feeding, they found an immediate inhibition of reductase activity independent of protein synthesis (4-8 hours), and then subsequent repression of enzyme synthesis (24-30 hours). An inhibition unrelated to protein synthesis was also reported by Edwards and Gould (1974) who administered cholesterol (in the diet and by stomach intubation) at midnight, and measured the cyclic decline of hepatic HMG-CoA reductase in normal and cholesterol-treated rats at a period when synthesis of the enzyme is known to have ceased (Higgins *et al.*, 1971). As cholesterol administration increased the rate of decline of HMG-CoA reductase during this period, they also concluded that exogenous cholesterol inactivated the reductase 2-8 hours after cholesterol administration (although an increased rate of reductase degradation is also consistent with their data).

The bimodal model of rapid and long-term inhibition must now be clear, although the inhibitory mechanisms of each phase have received little attention. Control of HMG-CoA reductase synthesis by a cholesterol-modulated transcription of its gene is an intriguing possibility. Erickson and Gould (1974) have shown that ingested cholesterol is associated with rat liver chromatin within 2 hours, and that cholesterol feeding also altered chromatin-bound free and esterified cholesterol ratios. Synthetic control at the level of the endoplasmic reticulum is suggested by the observations of Prónczuk and Fillios (1968) who reported that cholesterol feeding results in a decrease in the ribosomal polysomes and a consequent increase in monosomes through a ribosomal disaggregation which would result in a decreased capacity for protein synthesis.

As the inhibition of HMG-CoA reductase *in vitro* by cholesterol or cholesterol-rich lipoproteins has not been successful, and as the K_m's for the reductase from normal and cholesterol-fed rats are similar, the mechanism for inhibition of the activity of this enzyme has been ignored. Beg *et al.* (1973) have shown that HMG-CoA reductase activity is diminished in several *in vitro* liver systems preincubated with cAMP, and that isolated microsomal reductase activity is diminished by ATP, Mg^{2+} and a protein fraction of liver cytosol. The significance of this study is unclear, particularly as Raskin *et al.* (1974) have shown that 50-fold glucagon-induced increases in intracellular cAMP were without effect on the rate of cholesterol synthesis.

Nature of the intracellular effector

As the degree of suppression of hepatic cholesterol synthesis changed reciprocally with both increasing amount of cholesterol in the diet (Siperstein, 1970) and increasing length of time on a cholesterol-rich diet (Siperstein, 1965), cholesterol or a metabolite of it was proposed as the inhibitor. But this proposal remains an enigma. In no experiments with subcellular fractions, has cholesterol in any form been able to inhibit its own synthesis. Cholesterol added as a suspension in propylene glycol to microsomal or solubilized HMG-CoA reductase caused no inhibition of activity (Kawachi and Rudney, 1970); mixing of microsomes isolated from normal and cholesterol-fed rats caused no inhibition of HMG-CoA reductase in the normal microsomes (Linn, 1967b; Shapiro and Rodwell, 1969, 1971); lipoproteins isolated from the livers of cholesterol-fed rats had no effect on reductase activity *in vitro* (Shapiro and Rodwell, 1971). Cholesterol and cholesteryl esters, suspended with albumin at very high levels, caused some inhibition of cholesterol synthesis in liver slices, but this was considered non-specific as fatty acid synthesis also was inhibited (Siperstein, 1960).

An inverse relationship between hepatic cholesterol synthesis and the cholesterol content of the liver has been observed many times (Frantz *et al.*, 1954; Dubach *et al.*, 1961; Gould and Swyryd, 1966; Gould *et al.*, 1970; Shapiro and Rodwell, 1971), although Siperstein and Guest (1960) have reported that after cholesterol feeding, a complete suppression of cholesterol synthesis occurs prior to any detectable accumulation of cholesterol in the liver. It is probable

that the cholesterol-induced inhibition of HMG-CoA reductase is the result of an accumulation of effector at a specific intracellular site; the latter observation of Siperstein and Guest (1960) may result from this degree of intracellular localization which can not be detected at early stages by crude measurements in the whole liver. Several of these studies have shown that the increase in hepatic cholesterol content after cholesterol-feeding is predominantly due to an increase in cholesteryl ester rather than free cholesterol. Because HMG-CoA reductase is localized on the endoplasmic reticulum, more recent studies have measured cholesterol content of hepatic microsomes after cholesterol-feeding (Harry *et al.*, 1973; Edwards and Gould, 1974) and after intravenous administration of lipoprotein-cholesterol (Nervi *et al.*, 1975). A suppression of HMG-CoA reductase and an accumulation of cholesteryl ester was observed in each of these studies so cholesteryl ester is therefore a likely effector substance.

Cholesteryl ester is non-polar and most likely occupies the innermost hydrophobic regions of membranes. It would therefore exchange with other membranes very slowly. That mixing experiments have failed to demonstrate an inhibitor substance, may simply reflect lack of transfer of cholesteryl ester.

The exact physical-chemical nature of the circulating cholesterol which triggers the inhibition of HMG-CoA reductase, is uncertain as it has been demonstrated that the level of blood cholesterol *per se* has little influence in this process. Sakakida *et al.* (1963), using chickens, have shown that feeding cholesterol decreased hepatic cholesterol synthesis, but stilbestrol produced

marked hypercholesterolemia which did not inhibit synthesis for at least the first ten days of treatment. Furthermore, the serum of cholesterol-fed chickens, when injected into mice, caused a decrease in synthesis whereas the serum of stilbestrol-treated chickens caused no inhibition of cholesterol synthesis in mice. The stilbestrol has apparently deranged the structure of the plasma cholesterol-lipoprotein complex such that it does not activate the feedback-response. In the first two days following the induction of experimental nephrosis in the rat, the resulting hypercholesterolemia does not cause a decrease in hepatic cholesterol synthesis (Marsh and Drabkin, 1958). It has, therefore, been suggested that only cholesterol absorbed from the intestine is capable of activating the cholesterol feedback mechanism and that endogenous cholesterol is ineffectual.

Recent studies have greatly expanded this topic. Both lecithin-cholesterol and lecithin-cholesteryl ester infusions (IV) increase the cholesterol content of liver and decrease hepatic cholesterogenesis (Jakoi and Quarfordt, 1974). (Unfortunately, these authors have made no distinction between free and esterified cholesterol content of the liver.) These lipid dispersions travel in the blood, presumably without protein and have no difficulty in entering the liver whereas cholesterol of endogenous origin which is surely mobilized as lipoprotein, must be presented to the rat hepatocyte in a specific manner in order to be effective in feedback regulation. In this context, it is noteworthy that Brown and Goldstein (1974) have demonstrated the presence of highly specific low density lipoprotein (LDL) receptors in normal human skin fibroblasts. Goldstein *et al.*

(1974) have further shown that binding of LDL to normal fibroblasts increases intracellular cholesteryl ester formation and decreases cholesterol synthesis. Cells from homozygotes with the autosomal dominant disorder, familial hypercholesterolemia, lack the specific LDL-receptors and neither the intracellular esterification of cholesterol nor the inhibition of cholesterol synthesis are activated by LDL. Free cholesterol suspended in the culture medium (in a non-lipoprotein form) will suppress HMG-CoA reductase activity in the mutant cells (Brown *et al.*, 1974). These experiments give further evidence that, in humans at least, complexed lipoprotein-cholesterol must be correctly presented to the cell in order to initiate a sequence of events leading to suppression of cholesterol synthesis. They also suggest that the intracellular esterification of cholesterol is a *sine qua non* of HMG-CoA reductase suppression.

A similar situation may exist in the rat where Nervi *et al.* (1975) showed that, of the tissues investigated, liver was the only one to remove intravenously injected lipoproteins. (None was removed by stomach, jejunum, colon, kidney, adipose tissue, lung, cardiac muscle, skeletal muscle, skin and brain.) The lipoprotein injection led to an increase in the cholesteryl ester content of the liver (with little change in the content of free cholesterol) and to an inhibition of cholesterol synthesis.

The human fibroblast system and other tissue culture systems have given much information on cellular sterol flux and its control by lipoprotein, but care must be taken when extrapolating from

one system to another. Brown *et al.* (1974) have shown that LDL and very low density lipoprotein (VLDL) from human plasma with a normal cholesterol content, could inhibit human fibroblast HMG-CoA reductase activity. In addition, Bates and Rothblatt (1974) have found that human LDL from normal serum depresses sterol synthesis in a mouse L-cell fibroblast culture. In the rat hepatocyte system (parenchymal cells of adult rat liver), lipoproteins isolated from "normocholesterolemic" rat plasma (including LDL) did not suppress HMG-CoA reductase activity (Breslow *et al.*, 1975a) although lipoproteins (LDL, VLDL, chylomicrons) from hypercholesterolemic rat plasma (animals received cholesterol, cholic acid and propylthiouracil for three months), did suppress reductase activity. These differences in regulation may reflect true differences between liver cells and fibroblasts or may be due to variation between species. Another permutation is that used by Edwards (1975) who reported that normal, human plasma LDL inhibits reductase activity in freshly dispersed rat hepatocytes.

An effect common to each reported system is that of high density lipoprotein (HDL). HDL promotes both sterol efflux and sterol synthesis - or HMG-CoA reductase activity (Bates and Rothblatt, 1974; Breslow *et al.*, 1975a; Edwards, 1975). These effects were also induced by lecithin in the medium where the apparent association of cellular sterol efflux and enhanced HMG-CoA reductase activity was again manifest (Edwards, 1975).

Edwards (1975) has reported that cholesterol in the medium will decrease reductase activity in dispersed rat hepatocytes within 4 hours. This decrease is most likely due to inactivation of

the enzyme but not to suppression of enzyme synthesis (Edwards and Gould, 1974; Higgins and Rudney, 1973). The reductase inhibition does not occur, however, if cellular protein synthesis is prevented by cycloheximide. This implies that protein synthesis is required for the uptake of cholesterol by the cell and/or for its conversion to an inhibitory form. Although it has been implied that cholesteryl ester may be an intracellular repressor of cholesterol synthesis, Kandutsch and Chen (1973) have shown that 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol, all products of the autoxidation of cholesterol, are potent and specific inhibitors of HMG-CoA reductase in mouse L-cell and liver cell cultures in conditions where cholesterol itself is not inhibitory.

In summary, the work with cell cultures has suggested that cholesterol inhibition is mediated by the cellular sterol flux which is influenced by plasma lipoproteins and is dependent upon protein synthesis. Furthermore, intracellular cholesterol esterification is coincidental with inhibition of HMG-CoA reductase by plasma LDL, although the actual inhibitory substance may also be an oxidised metabolite of cholesterol.

2.2.3 Fasting

That fasting and chronic undernutrition decrease hepatic cholesterogenesis, was first reported by Tomkins and Chaikoff (1952). Such dietary treatment is severe, but this may be a natural mode of regulation because the rat is a periodic feeder which consumes the bulk of its diet at night (Kimura *et al.*, 1970). Although Lyon *et al.* (1952) have shown that fasting decreases hepatic fatty acid

synthesis, the effect on cholesterogenesis appears to be of a different nature. Whereas fatty acid synthesis was restored only by glucose (Lyon *et al.*, 1952), the effect on cholesterogenesis is not due to the absence of a specific dietary requirement, because any source of calories (fat, carbohydrate or protein) will promptly restore cholesterol synthesis to normal levels (Tomkins and Chaikoff, 1952). As these dietary substances may be catabolized to intramitochondrial citrate which is a precursor of extra-mitochondrial acetyl-CoA, supply of extra-mitochondrial acetyl-CoA may be an important factor controlled by fasting and contributing to regulation of cholesterol synthesis. Indeed, Foster and Srere (1968) have shown that fasting decreases the level of citrate cleavage enzyme, but they also reported that the level of this enzyme did not influence fatty acid synthesis. Its influence on cholesterol synthesis is unknown. That caloric intake in any form will suffice to restore cholesterol synthesis in fasted rats, is exemplified by the observation by Rao and Ramasarma (1972) that a single IP dose of ATP restores cholesterol synthesis but not fatty acid synthesis.

A major effect of fasting is the decrease in hepatic HMG-CoA reductase activity. This has now been reported many times (Bucher *et al.*, 1960; Linn, 1967b; White and Rudney, 1970). Slakey *et al.* (1972) also observed a decrease in HMG-CoA reductase during fasting and although their data show that the variation in the level of this enzyme under their conditions is sufficient to account for the variation observed in cholesterol synthesis from acetate, they caution that additional regulatory sites are present between mevalonate and squalene, and between squalene and cholesterol.

The presence of a "fasting" effector for HMG-CoA reductase suppression has been suggested. Migicovsky (1964) has reported evidence of a protein fraction in the liver cytosol of starved, but not normal-fed rats, which is inhibitory to cholesterol synthesis *in vitro* and *in vivo*. The data also suggests that this inhibitor may move from mitochondria to the surrounding cytoplasm during starvation.

The mechanism of this hepatic fasting effect may be elucidated by an investigation of cholesterol synthesis in the gastrointestinal tract as liver was the only tissue of seventeen tested, which responded to fasting with reduced cholesterol synthesis (Dietschy and Siperstein, 1967). As the liver and G-I tract together account for 90% of cholesterogenic activity in the whole body (Dietschy and Siperstein, 1967), the highest rate of cholesterogenesis in the fasted rat is found in G-I tract.

2.2.4 Bile Acids

Since cholesterol is the obligatory precursor of the bile acids, changes in the rate of hepatic cholesterol synthesis may affect bile acid production and *vice versa*. Certainly, many data have been interpreted as evidence that bile salts are feedback inhibitors controlling cholesterol synthesis in the liver.

The feeding of bile acids inhibits hepatic cholesterol synthesis (Beher *et al.*, 1959) and HMG-CoA reductase activity (Shefer *et al.*, 1973); and on the other side removing bile acids by way of biliary drainage (Myant and Eder, 1961; Danielsson *et al.*, 1967) and cholestyramine feeding (Back *et al.*, 1969; Huff *et al.*,

1963) both increase hepatic cholesterol synthesis. Acetate incorporation into mevalonate is also inhibited *in vitro* (in rat liver homogenates) by bile salts (Fimognari and Rodwell, 1965). These results have led Fimognari and Rodwell (1965) to conclude that "bile salts may be the cholesterol metabolites responsible for physiological regulation of cholesterol synthesis".

This conclusion has been challenged by several investigators. Siperstein (1960) reported that pure bile salts, used in physiological concentrations, have consistently failed to have an effect on cholesterol synthesis *in vitro*. In fact, Dietschy (1967) and Weis and Dietschy (1969) have emphasized that bile salts have a non-specific toxicity to systems *in vitro* due to their powerful detergent actions. Furthermore, Weis and Dietschy (1969) found that not only biliary drainage, but also biliary obstruction, lead to increases in hepatic cholesterol synthesis. As the former manipulation decreased the bile acid concentration in the liver, and the latter intervention increased bile acid concentration, Weis and Dietschy (1969) concluded that bile acids have no direct function in the control of hepatic cholesterogenesis. They extended this study by demonstrating a restoration of the normal level of cholesterogenesis with the infusion of chylomicron cholesterol after biliary drainage, and therefore concluded that bile acids influence cholesterogenesis indirectly by interfering with the enterohepatic circulation of endogenous cholesterol.

But this latter argument remains unresolved as it has now been shown that hepatic HMG-CoA reductase is reduced by cholic acid feeding of rats with thoracic duct lymph fistulae (Hamprecht *et al.*,

1971) where absorbed cholesterol can not reach the liver to exert a regulatory effect. Further confusion was introduced when Ogilvie and Kaplan (1966) found that cholesterol synthesis *in vitro* is inhibited at a pre-mevalonate reaction by a protein fraction of rat bile, and that only minor inhibitory activity is associated with the dialysate of bile (the bile acids).

2.2.5 Cyclic AMP

This compound, produced within the cell, certainly decreases cholesterol synthesis. Nepokroeff *et al.* (1974) reported that IP injection of dibutyryl cAMP inhibited the diurnal rise of HMG-CoA reductase in normal rat liver. Studies *in vitro* have shown that cAMP inhibits cholesterol synthesis from acetate in liver slices (Bricker and Levey, 1972; Raskin *et al.*, 1974) and inhibits HMG-CoA reductase activity in a washed microsomal system (Beg *et al.*, 1973).

However, a feature of the *in vitro* studies is that the concentrations of cAMP required for inhibitory effects were high (generally 10^{-4} to 10^{-3} M) compared with the known range (10^{-7} to 10^{-5} M) of hepatic cAMP concentrations (Exton *et al.*, 1971). Furthermore, the presence of sufficient glucagon to raise the tissue cAMP level by 50-fold, was without effect on cholesterol synthesis and HMG-CoA reductase activity (Raskin *et al.*, 1974). Raskin *et al.* (1974), therefore, conclude that cholesterol synthesis is independent of changes in the intracellular cAMP concentration over a wide physiological range.

2.2.6 Circulating Hormones

It is difficult to construct a scheme for discrete hormonal effects on HMG-CoA reductase as many of the observations reported are contradictory and it is also apparent that several hormonal systems may act in conjunction. Furthermore, several of the hormones investigated are thought to act in other systems through cAMP, the role of which in HMG-CoA reductase regulation is unclear. Experimental detail will be omitted from the following reports in order that a summary of combined hormonal effects may be more easily constructed.

a. Insulin: HMG-CoA reductase activity in diabetic rats is reduced and arythmic, but slow release insulin therapy restores the level and rhythm of the reductase (Nepokroeff *et al.*, 1974). That hepatic cAMP levels are elevated during diabetes and that insulin restores these levels to normal (Robison *et al.*, 1971), may be important. Furthermore, insulin produces a marked and rapid (maximal at 2 hours) elevation of HMG-CoA reductase levels in normal, and in diabetic rats (Lakshmanan *et al.*, 1973). Since the normal rhythm of HMG-CoA reductase is a result of enzyme synthesis (section 2.2.1), insulin may act by increasing reductase synthesis. Insulin may act in conjunction with a pituitary-controlled hormone, as suggested by the observation that insulin has no effect on HMG-CoA reductase in hypophysectomized rats, whereas the reductase is increased to supra-normal levels when insulin/T_3 is administered (Dugan *et al.*, 1974). However, the insulin-induced response of HMG-CoA reductase is prevented by either glucagon or hydrocortisone (Dugan *et al.*, 1974).

b. Glucagon: Glucagon antagonises the reductase-response of the normal (Lakshmanan *et al.*, 1973), and diabetic rat to insulin (Dugan *et al.*, 1974). Glucagon also inhibits the normal diurnal rise of HMG-CoA reductase (Nepokroeff *et al.*, 1974), although it may be acting solely as an antagonist to insulin which appears to be necessary for the rhythm. It is perhaps important that glucagon raises the intracellular level of hepatic cAMP (Rall and Sutherland, 1958) which, in turn, has been shown to lower the rate of cholesterol synthesis in liver slices (Bricker and Levey, 1972; see section 2.2.5).

c. Thyroid hormone: Hypothyroid rats have a low level of HMG-CoA reductase activity which is restored to normal levels by an injection of L-triiodothyronine (T_3) after a latent period of 30 hours (Guder *et al.*, 1968). In normal rats, T_3 increased HMG-CoA reductase to an activity comparable with the peak level found in control animals (Ness *et al.*, 1973).

Hypophysectomy is reported to abolish the diurnal rhythm and to decrease reductase activity (Edwards, 1973; Ness *et al.*, 1973; Huber *et al.*, 1974). Ness *et al.* (1973) found that administration of T_3 to hypophysectomized rats increased the level of HMG-CoA reductase to 3-4 times that found in normal animals, suggesting that at least one other pituitary-regulated hormone might inhibit the T_3-induced increase in reductase activity. The T_3-stimulation of HMG-CoA reductase in hypophysectomized rats is indeed prevented by hydrocortisone (Ness *et al.*, 1973), glucagon (Dugan *et al.*, 1974) and inhibitors of protein synthesis (Ness *et al.*, 1973).

d. Adrenal hormones:

Cortical - the glucocorticoid, hydrocortisone, has been mentioned in conjunction with insulin and thyroxine. The influence of circulating corticosterone on hepatic cholesterol synthesis has been studied and reported in this thesis and a review of the reported effects of adrenal steroids on cholesterol synthesis is in section 5.

Medullary - epinephrine treatment of intact rats resulted in increased reductase activity within 2 hours, a response which was blocked by cycloheximide (Edwards, 1973). HMG-CoA reductase of adrenalectomized rats did not respond to epinephrine, even in conjunction with cortisone replacement (Edwards, 1973). Like glucagon, epinephrine is known to elevate intracellular levels of cAMP (Exton *et al.*, 1971).

e. Summary:

(i) those elements causing an increase in cholesterogenesis - insulin, thyroid hormone

(ii) those factors permitting the expression of other elements - thyroid hormone permits insulin effect on cholesterogenesis

(iii) those factors antagonising the expression of other elements - glucagon (insulin, thyroid hormone), hydrocortisone (insulin, thyroid hormone).

2.3 β-Hydroxy-β-Methylglutaryl CoA Reductase - Physical Properties

The bulk of the attention given to HMG-CoA reductase has been from a physiological viewpoint, but there has been little investigation of it as an enzymic protein *per se*. Furthermore, the physical and kinetic studies on HMG-CoA reductase have produced many conflicting data concerning its protein nature.

HMG-CoA reductase has a MW around 200,000 (Brown *et al.*, 1973; Heller and Gould, 1973; Higgins *et al.*, 1974; Tormanen *et al.*, 1976), but it may have a trimeric structure of identical subunits with MW 65,000 (Higgins *et al.*, 1974) or it may be a tetramer of identical subunits with MW 47,000 (Tormanen *et al.*, 1976). The estimated K_m of this enzyme for the substrate HMG-CoA, ranges from 8.1×10^{-5} M (Shapiro and Rodwell, 1969) to 3.5×10^{-6} M (Edwards and Gould, 1972).

At least seven methods have been published for solubilizing the reductase from a microsomal preparation:

A. Incubation with snake venom (phospholipase A) - Brown *et al.* (1973).

B. Incubation with 4 M KCl - Brown *et al.* (1973).

C. Freezing and glycerol extraction - Brown *et al.* (1973).

D. Slow freezing/thawing - Heller and Gould (1973).

E. Lyophilisation and buffer extraction - Ackerman *et al.* (1974).

F. Acetone powder and buffer extraction - Linn (1967a).

G. Treatment with sodium deoxycholate - Kawachi and Rudney (1970).

This plethora of methods produces soluble enzyme preparations which have a wide and controversial range of properties, particularly in

relation to cold response. The soluble reductase produced by methods A, B and C is irreversibly cold-labile but can be protected from cold-inactivation by 4 M KCl (Brown *et al.*, 1973). These authors suggest that sub-unit dissociation occurs at 4° as a result of weakened hydrophobic interactions, a phenomenon associated with low temperatures (Scheraga *et al.*, 1962). Heller and Gould (1974) have reported that HMG-CoA reductase solubilized by slow freezing and thawing of microsomes (method D) is reversibly inactivated by cold temperature and is irreversibly inactivated rather than protected by 4 M KCl (at 0°). Method E yields a preparation showing no evidence of cold lability and which is reversibly destabilized by 4 M KCl. Such a spectrum of observations implies that some of these responses are not an inherent property of the enzyme, but are related to the method of preparation.

One aspect of preparation overlooked by each investigator cited, is the presence or absence of lipid associated with the soluble enzyme produced by each method. That HMG-CoA reductase activity does not sediment with endoplasmic reticulum on centrifugation is the exclusive criterion of solubility used in each investigation cited, but the resultant enzyme solubilized may be a pure protein or a lipoprotein, depending on the preparation.

Treatment of microsomes with glycerol and high salt concentrations leave the lipid bilayer intact (Brown *et al.*, 1973) and the HMG-CoA reductase solubilized by these methods is probably pure protein. The enzyme extracted from an acetone powder would also be depleted of lipids which remain in the acetone phase (Penefsky and Tzagoloff, 1971). Slow freezing/thawing and lyophilization, processes which crudely disrupt the membrane bilayer, may result in a soluble reductase which still has bound lipid.

For a membrane-bound enzyme, consideration of its lipid interactions is essential in studying its protein behaviour. Certainly, the pH response curves and the substrate saturation curves are similar for soluble and microsomal reductase. Solubilization does not affect the substrate affinities of the active sites (Brown *et al.*, 1973; Heller and Gould, 1974). But Linn, who first demonstrated solubilization of this enzyme, briefly noted that she could solubilize an amount of HMG-CoA reductase greater than that thought to be present in her microsomal preparations. She stated that "the enzyme activity of the particulate material behaved as though only a fraction of its capability was being exhibited" (Linn, 1967a). This single observation is a strong reason for the acknowledgement of lipid-reductase effects. Furthermore, HMG-CoA reductase is the sole membrane-bound enzyme between acetate and squalene in the cholesterol biosynthetic pathway (section 2.1), another fact which compels conjecture on the effects and functions of membrane, and in particular lipid, interactions with this enzyme.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Animals

Adult, male rats (hooded Wistar), approximately three months old, were used in all experiments. Water and Charlick's M164 mouse cubes were available *ad libitum*. Animals were housed in windowless, but well-ventilated rooms and, except where stated, lights were on at 1700 hours, off at 0500 hours. Animals were killed *ca.* 1100 hours.

3.2 Reagents

HMG acid, N,N'-dicyclohexylcarbodiimide, coenzyme A (Li salt), NADP, G-6-PO_4, and G-6-PO_4 dehydrogenase (Type XI) - Sigma Chemical Company, St. Louis, Mo., U.S.A.

Amberlite resin (IRA 410) - B.D.H., Poole, England.

Activated charcoal ("Norit A") - Matheson, Coleman and Bell, Los Angeles, Calif., U.S.A.

Sodium [1-^{14}C]acetate, [1,2-^{3}H]cortisol - The Radiochemical Centre, Amersham, Buckinghamshire, England.

[3-^{14}C]HMG acid - New England Nuclear, Boston, Mass., U.S.A.

Benzene and acetone were redistilled with sodium before use.

6-Doxyl stearic acid was a gift from Dr. John Raison, Plant Physiology Unit, C.S.I.R.O., North Ryde, Australia 2113.

3.3 Measurement of hepatic cholesterogenesis

Cholesterol synthesis *in vitro* was measured by incubating liver slices with [1-^{14}C]acetate and subsequently isolating and counting ^{14}C-labelled cholesterol as total digitonin-precipitable sterol, basically as described by Sabine *et al.* (1967).

Following sacrifice of the animal by decapitation, the liver was rapidly excised and placed in chilled Krebs-Henseleit bicarbonate buffer (pH 7.3). All incubations were performed in triplicate using 200 mg of tissue slices, approximately 0.4 mm thick (McIlwain-Buddle tissue chopper). These were placed in flasks containing 4 μmol of sodium[1-^{14}C]acetate (0.15 μCi) in 2 ml of buffer, gassed with an O_2:CO_2 (95:5) mixture, sealed and shaken mechanically at 37°C. Tissue was inactivated after 2 hours with 0.2 N H_2SO_4 and sterols extracted and counted (Sabine *et al.*, 1967). Under these conditions, ^{14}C-cholesterol production was linear for at least 2 hours.

3.4 Measurement of hepatic HMG-CoA reductase activity

Enzyme activity in unwashed microsomal preparations was measured essentially by the method of Goldfarb and Pitot (1971). DL-[3-^{14}C]HMG-CoA, 12.5 nmol, was added to 1 ml of incubation mixture which contained an NADP reducing system (Goldfarb and Pitot, 1971), and incubated for 30 min with 0.7 to 2.5 mg of microsomal protein which is in the linear range of the assay. The reaction was terminated with 10 N NaOH which also lyses free coenzyme A from unreacted [3-^{14}C]HMG-CoA. It was then acidified to lactonize the [3-^{14}C]mevalonic acid, saturated with Na_2SO_4, and extracted three times with 10 ml of ethyl ether.

Although [5-^{3}H]mevalonic acid was not used to determine the extraction efficiency in each sample (Goldfarb and Pitot, 1971), the method of extraction gave a repeatable 65-70% recovery of mevalonate. All tubes were shaken together for 3 min on a mechanical shaker and then liquid nitrogen was used to snap-freeze the bottom aqueous layer, allowing the ether extract to be decanted. [3-^{14}C]mevalonolactone was isolated using the thin-layer chromatographic technique of Shapiro *et al.* (1969).

Two control systems were tested, one which had no cofactor, and one which used boiled enzyme.

Table 3.1 Effect of cofactor and microsomal preparation on HMG-CoA reductase.

	HMG-CoA reductase activity (nmol MVA/mg/h)	
	Unwashed microsomes	Washed microsomes
+ NADPH	33.2	17.3
- NADPH	8.2	-
- NADPH, boiled	-	-

Table 3.1 indicates that MVA was formed in the absence of added cofactor when unwashed microsomes were used. This implies the presence of endogenous NADPH which is removed by washing the microsomes since no product is formed in the latter situation. Washed microsomes,

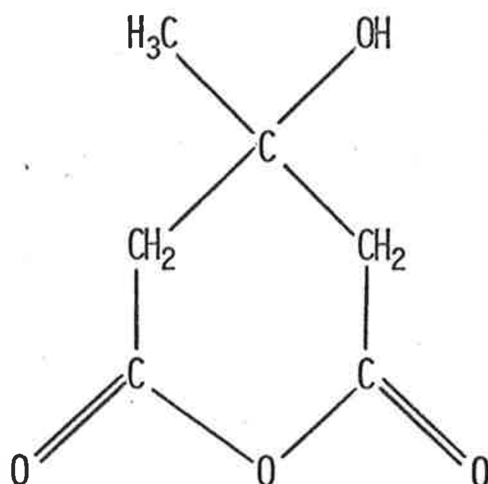
assayed in the absence of added NADPH, are therefore adequate controls, but Table 3.1 reveals a decrease in HMG-CoA reductase activity after washing. Boiled enzyme (2 min) was therefore used in control tubes and unwashed microsomes were used in all incubations.

3.5 Synthesis of [3-^{14}C]HMG-CoA

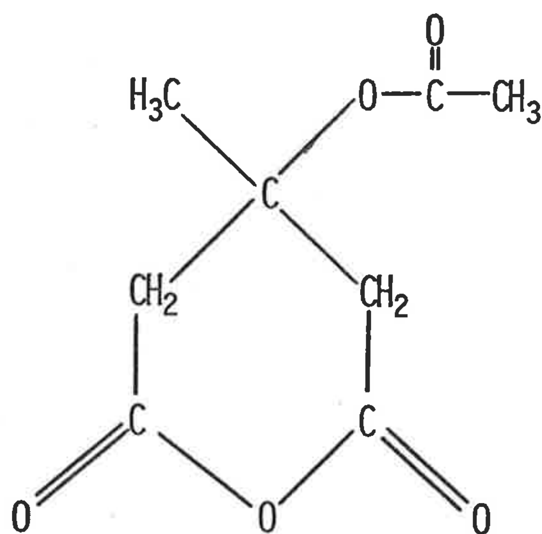
The synthesis of HMG-CoA from HMG acid is best achieved by conversion of the acid first to HMG anhydride (Figure 3.1) which is then reacted with reduced coenzyme A to form the thioester. HMG anhydride was originally synthesized by refluxing HMG acid and acetic anhydride with boiling benzene for 1 hour (Hilz *et al.*, 1958). However, Louw *et al.* (1969) found this method unreliable as it results not in the hydroxylated form, but in an acetylated derivative, 3-acetoxy-3-methylglutaric anhydride (Figure 3.1). Louw *et al.* (1969) have, therefore, published an "improved" method of synthesis which incorporates acetic acid in the reaction mixture. They report that after removal of solvents and reagents by vacuum distillation, fine crystalline needles of HMG anhydride remain.

Many attempts were made in this laboratory to prepare HMG anhydride according to the method of Louw *et al.* (1969), but instead of fine needles in the distilling flask, a syrup resulted which yielded a "waxy" solid after crystallization from anhydrous benzene. Although not identified further, it was probably 3-acetoxy-3-methylglutaric anhydride according to the description by Louw *et al.* (1969) of syrup formation by this compound. Goldfarb and Pitot (1971) had also found

Fig.3.1



3-hydroxy-3-methylglutaric(HMG) anhydride



3-acetoxy-3-methylglutaric anhydride

this method unsatisfactory and described another synthetic method whereby a carboxyl group of HMG acid is allowed to undergo nucleophilic attack on N,N'-dicyclohexylcarbodiimide, forming soluble HMG anhydride and insoluble dicyclohexylurea within several minutes.

This method (Goldfarb and Pitot, 1971) has been successful in this laboratory on many occasions and, after evaporating the solvent and recrystallizing from anhydrous benzene, crystalline needles form. These have a m.p. of 100-102° and the infrared absorption spectrum (1 mg/200 mg KBr) from a Perkin-Elmer 237 spectrophotometer showed a hydroxyl band at 3500 cm^{-1} and two anhydride bands, one at 1805 cm^{-1} and one at 1750 cm^{-1}, which conforms to the description by Louw *et al.* (1969) of HMG anhydride. Reaction of the anhydride with coenzyme A was allowed in aqueous solution (pH 7.5) according to the method of Louw *et al.* (1969).

Descending paper chromatography (Whatman No. 3 MM) with the solvent system n-butanol-acetic acid-water (5:2:3), followed by radioactivity analysis showed for one preparation: principle spot R_f 0.32 ([3-^{14}C]HMG-CoA), 66%; R_f 0.77 ([3-^{14}C]HMG acid), 32%; traces R_f 0.87, 2%. Similar yields were obtained for all preparations. The identification of compounds is based on the R_f values reported by Louw *et al.* (1969). The spot at R_f 0.87 may be [3-^{14}C]3-acetoxy-3-methylglutaric acid which formed during chromatography.

This preparation was used without further purification for the assay system described in section 3.4. For the HMG-CoA reductase assay system described in section 4, purified substrate was used; this was prepared as follows. After separation from

[3-^{14}C]HMG acid, [3-^{14}C]3-acetoxy-3-methylglutaric acid, and unreacted coenzyme A, the strip containing [3-^{14}C]HMG-CoA was cut from the chromatogram and the compound eluted with distilled water adjusted to pH 5.5 (HCl) because the thioester bond is most stable in acid conditions. Chromatography of the eluate resulted in a radioactive spot with R_f 0.32, and no other detectable radioactivity, i.e. pure [3-^{14}C]HMG-CoA which was stable for at least 6 months.

The specific activity in all preparations was 200 μCi/mmol.

3.6 Measurement of blood corticosterone level

Corticosterone was determined basically according to the method described by Bassett and Hinks (1969), using the steroid-binding properties of corticosteroid-binding globulin (CBG), as recognized by Murphy *et al.* (1963). Canine CBG solution, which binds both cortisol and corticosterone, consisted of 10 ml dog plasma (eluted from a Sephadex column to remove most of the free corticosteroids) in 200 ml of 0.12 M phosphate buffer (pH 7.3), plus 1 ml ethanol and 8 μCi (180 pmol) [1,2-^{3}H]cortisol.

Briefly, 70 μl of blood were collected from the tail vein of rats, deproteinized and extracted in 2 ml of ethanol, centrifuged and stored at -15°. Aliquots (0.05 ml) of corticosterone standards and unknowns were assayed in duplicate as described by Bassett and Hinks (1969), using Sephadex (G-25) to separate protein-bound [1,2-^{3}H]cortisol from free [1,2-^{3}H]cortisol. Samples were in the range 20-125 ng/ml corticosterone.

4. SIMPLIFIED ASSAY FOR HMG-CoA REDUCTASE

4. SIMPLIFIED ASSAY FOR HMG-CoA REDUCTASE

4.1 Introduction

HMG-CoA reductase has been assayed in animal tissue by many methods which although accurate, are generally time-consuming. For radiotracer assays, separation of the product mevalonic acid, from the substrate HMG-CoA, has been variously achieved by Celite column chromatography (Regen *et al.*, 1966), gas-liquid chromatography (Siperstein *et al.*, 1966) and thin-layer chromatography (Shapiro *et al.*, 1969). A spectrophotometric assay which measures free coenzyme A released during the reaction, has been described (Hulcher and Oleson, 1973) but colourimetry is generally less sensitive than radiotracer methods.

Huber *et al.* (1973a) have described a "simple and rapid" radiochemical assay for HMG-CoA reductase. After extraction of ^{14}C-HMG acid and ^{14}C-mevalonolactone with ether, these reactants and products are separated on small columns of anion exchange resin, with the non-polar lactone eluted and the ^{14}C-HMG acid retained. Huber *et al.* (1973a) do not report whether the columns of Biorad 1x8, packed in Pasteur pipettes, can be successfully regenerated for further assays, or whether new columns must be prepared for each assay.

Equivalent columns of IRA 410, Cl^- form, were tested in this laboratory and, of 120 nmol HMG acid loaded onto the columns, only 0.5% was eluted. However, after regenerating the columns of resin with 25 ml NaCl (4%) and thoroughly rinsing, 10.1% of a load of 120 nmol HMG acid was eluted. The columns can not therefore be

successfully regenerated and would need repacking for each assay. Furthermore, this assay does not obviate the need for several ether extractions, procedures which inevitably introduce variation. If the non-extracted reaction mixture is added directly to the column, buffer salts interfere with the ionic sequestering of HMG acid.

In order to waive the extraction procedure, a system which does not use ionic binding was developed. Charcoal was found to bind HMG-CoA successfully in buffer solution, presumably by a hydrophobic interaction between the large, planar coenzyme A moiety and the planar carbon arrays of charcoal.

4.2 Method

Activated charcoal ("Norit A") was boiled in 1 N HCl (to remove cations), filtered, washed and thoroughly dried.

Incubations were performed as described (section 3.4), excepting the following details: (a) only purified ^{14}C-HMG-CoA which had no detectable free ^{14}C-HMG acid (section 3.5), was used, and (b) incubation volume was 0.5 ml and incubation time 10 min. The reaction was stopped by the addition of 1.0 ml of 5 N formic acid. 0.5 ml slurry of charcoal (100 mg) in BSA (2%) was added and allowed to stand for 10 min to allow absorption of unreacted ^{14}C-HMG-CoA by the charcoal. BSA permits the charcoal to remain in suspension and when centrifuged, the charcoal forms a tight pellet and does not adhere to the walls of the incubation tube (polystyrene). The mixture was centrifuged (30 min, 4000 g). 1 ml of supernatant was removed, added to 10 ml Dioxan/PPO (5 g/l) and radioactivity determined. The supernatant was shown to

contain only ^{14}C-mevalonolactone (Silica gel G TLC, acetone:benzene, 1:1).

4.3 Results and Discussion

In this system, MVA formation is proportional to the amount of microsomal protein within a range of 0-4.2 mg protein/incubation (Figure 4.1). The amount of mevalonolactone not bound by charcoal was 27 ± 0.8%, whereas only 1.2 ± 0.1% of substrate was left unbound. The amount of binding was unaffected by protein concentration.

The reaction rate is linear with time for at least 20 min with 3.4 mg protein/incubation, but for only 9 min with 6.8 mg protein/incubation (Figure 4.2). The thin-layer chromatographic assay of Goldfarb and Pitot (1971) (section 3.4) was compared with this "charcoal" assay (Figure 4.2), and although the absolute values obtained from one assay deviate from those of the other assay, the trend of each curve is identical. At the low protein level (Figure 4.2a), both assays indicate a direct proportionality of MVA formation with time for 20 min, whereas at the high protein level (Figure 4.2b) both curves deviate from linearity after 9 min. The difference between assays in calculated MVA formation may be an inherent problem of the correction for quenching of radioactivity since both assays require very different scintillation systems to determine radioactivity. Using a Packard tri-carb Model 3375 liquid scintillation spectrometer,

Figure 4.1

MVA formation was measured by the "charcoal" assay described in section 4.2. Each point represents the mean of triplicate determinations and vertical lines represent the standard error of the mean.

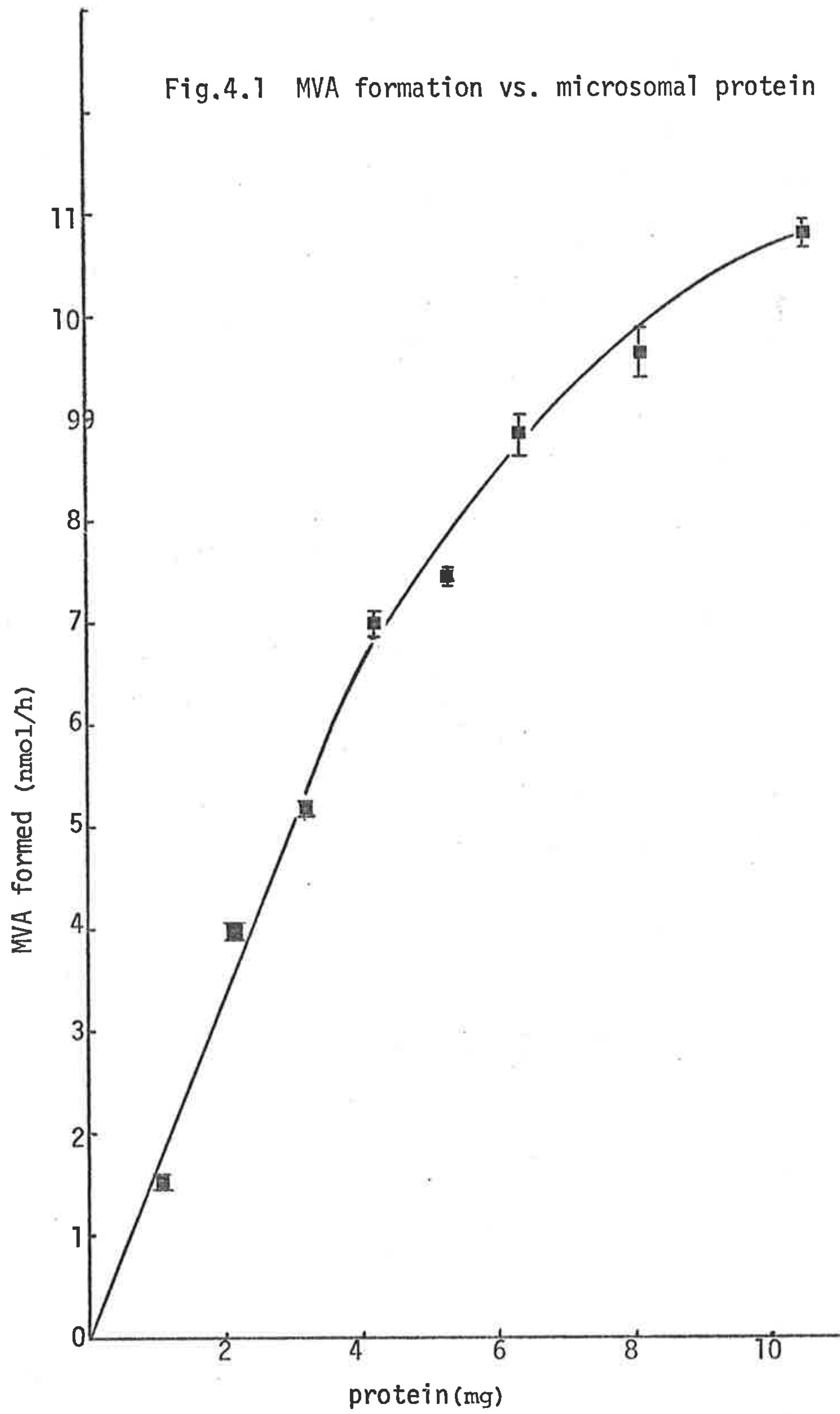
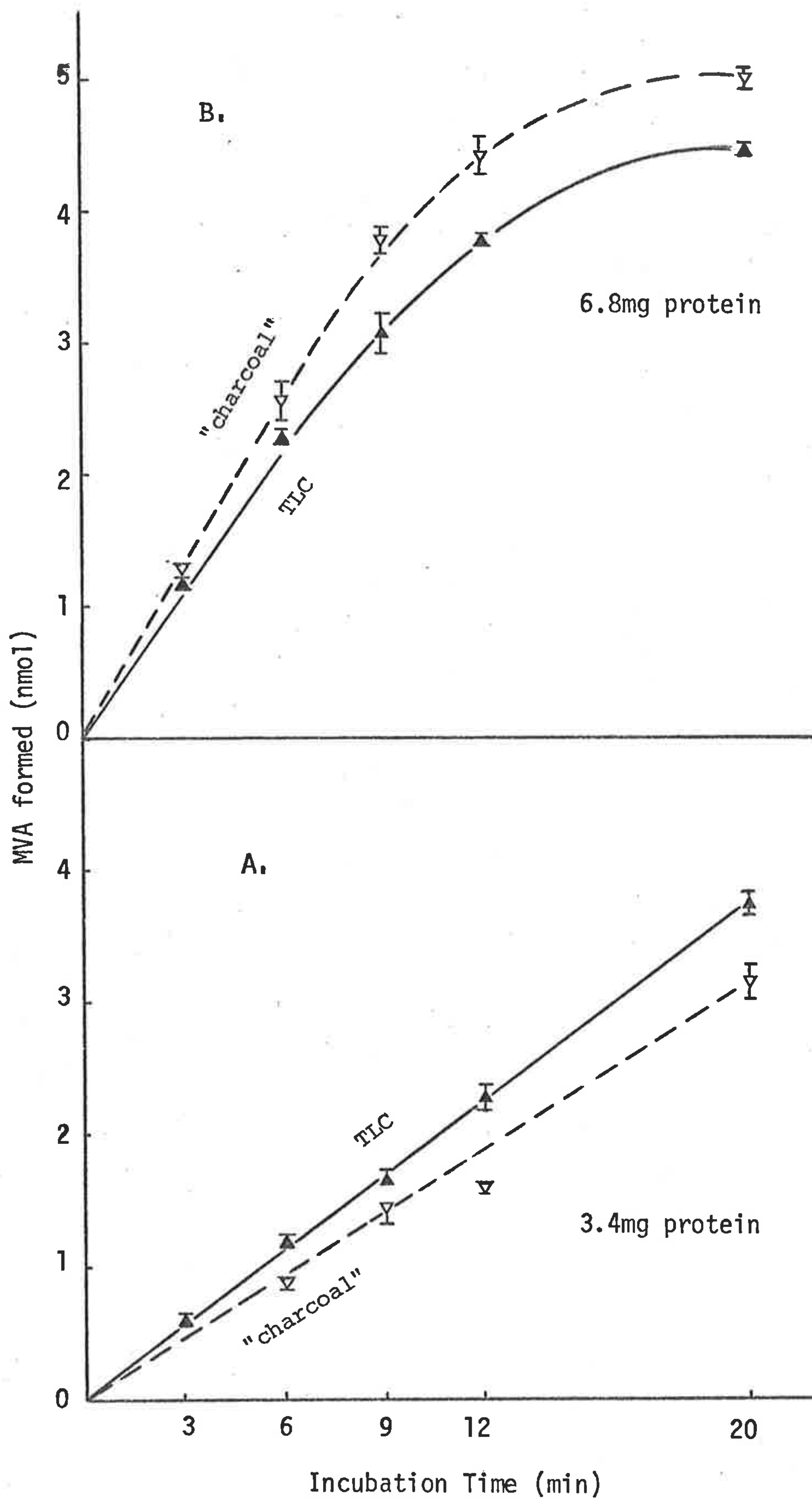


Fig.4.1 MVA formation vs. microsomal protein
MVA formed (nmol/h)
protein (mg)
0
1
2
3
4
5
6
7
8
9
10
11
2
4
6
8
10

Figure 4.2

MVA formation was measured at two protein concentrations by the "charcoal" assay described in section 4.2 (▽------▽), and the thin-layer chromatographic assay described in section 3.4 (▲———▲). Each point represents the mean of triplicate determinations and vertical lines represent the standard error of the mean.

Fig.4.2 MVA formation vs. incubation time



a method of channel ratios indicated the "charcoal" system (1 ml water + 10 ml Dioxan/PPO 5 g/l) had a counting efficiency *ca.* 50% and the TLC system (silica gel + 5 ml toluene/PPO 5 g/l) was counted with an efficiency *ca.* 80%.

Although the data obtained by each method are similar, the use of charcoal adsorbent has several advantages:

(a) an extraction procedure which introduces much variation unless precisely controlled, is not necessary, and

(b) spreading TLC plates, loading compounds and removing gel for radioactivity determinations, is time-consuming by comparison with the "charcoal" method of separation.

The main disadvantage is the low recovery (27%) of mevalonolactone.

Two new and rapid procedures have recently been described, one using benzene extraction (Goodwin and Margolis, 1976) and the other using a single chloroform extraction (Tormanen *et al.*, 1976). Neither of these methods requires chromatography and that described by Tormanen *et al.* (1976) uses phase separating paper to remove water from the extractant. It is therefore a simple method which may have greater recovery of mevalonolactone than does the "charcoal" assay, although recovery rate was not reported by Tormanen *et al.* (1976).

5. CORTICOSTERONE AND THE CONTROL HEPATIC CHOLESTEROL SYNTHESIS

5. CORTICOSTERONE AND THE CONTROL OF HEPATIC CHOLESTEROL SYNTHESIS

5.1 Introduction

In the rat, the liver accounts for the major fraction of cholesterol synthesized in the body. This hepatic synthesis is subject to a number of separate but interacting controls, the balance of which determines the actual rate at which cholesterol is being synthesized at any time: (i) cholesterol continuously absorbed from the gut exerts a feedback inhibition; (ii) there is a feeding/fasting response, and (iii) there is a diurnal rhythm of synthesis. There is now considerable evidence also to suggest that the reaction catalysed by HMG-CoA reductase is normally the main rate-controlling step in each of the above situations (section 2.2).

Hickman *et al.* (1972) reported that adrenalectomy abolishes the diurnal rhythm of cholesterol synthesis (as measured *in vitro* from [1-^{14}C]acetate) and causes synthesis to remain at a uniformly high level. Furthermore, these results were in accord with the rate-controlling function proposed for HMG-CoA reductase (section 2.2), in that synthesis from ^{14}C-mevalonate was unaffected by adrenalectomy. These observations (Hickman *et al.*, 1972) led to the suggestion that corticosterone, which is the predominant adrenal steroid present in rat plasma (Zenker and Bernstein, 1958) and which also undergoes a diurnal rhythm (David-Nelson and Brodish, 1969), may play an essential role in the diurnal rhythm of cholesterogenesis.

Since there is much confusion regarding the function of glucocorticoids in cholesterogenic regulation (see Discussion - 5.4), the relation of corticosterone to hepatic cholesterol synthesis has now been further investigated by: (i) measuring plasma corticosterone levels in normal animals and in those subject to either continuous light or continuous darkness, conditions which may alter the daily rhythm of plasma corticosterone, and (ii) relating these levels to subsequent measures of both hepatic cholesterol synthesis *in vitro* and hepatic HMG-CoA reductase activity.

5.2 Methods

Reagents and assay conditions are described in section 3. The lighting regimes under which animals were housed, were different from those described in section 3. Four groups of 14 animals were housed in pairs, for one month, in windowless but well ventilated rooms under either continuous light, continuous darkness or control conditions (lights on at 0600, off at 1800 hours). In each experiment, both a control group and a light-adjusted group were used simultaneously. For corticosterone determinations, blood samples were taken from all rats at 4 hour intervals over a 24 hour period. After a further 24 hours the animals were sacrificed, 2 pairs at a time, at 4 hour intervals for measurement *in vitro* of hepatic cholesterol synthesis and HMG-CoA reductase activity. In the continuous dark situation an infra-red lamp was used for inspecting food and water.

5.3 Results

5.3.1 Normal diurnal rhythms

Figure 5.1a shows the diurnal variation of blood corticosterone levels in rats sampled from the tail vein at 4 hour intervals over a 24 hour period. A rhythm is obvious, with the peak and nadir occurring at approximately 1800 and 0600 hours respectively, confirming many reports of this phenomenon (Cheifetz *et al.*, 1968; David-Nelson and Brodish, 1969).

Also confirming the findings of many others were the observed diurnal fluctuations in cholesterol synthesis (Back *et al.*, 1969; Bortz and Steele, 1973; Hickman *et al.*, 1972) and HMG-CoA reductase activity (Edwards and Gould, 1972; Hamprecht *et al.*, 1969; Huber *et al.*, 1973b; Shapiro and Rodwell, 1972), with maxima around midnight and minima around midday (Figure 5.1b).

5.3.2 Effect of continuous darkness

Holmquest *et al.* (1966) and Cheiftez *et al.* (1968) have shown that the environmental lighting regime is a potent "zeitgeber" for the adrenocortical rhythm in the rat. The effect of continuous darkness on blood corticosterone fluctuation and concomitant alterations in the rhythms of both hepatic cholesterol synthesis and HMG-CoA reductase activity were therefore examined.

Figure 5.2a illustrates that 4 weeks of continuous darkness altered the rhythm of blood corticosterone from that found under controlled lighting (Figure 5.1a). A rhythm persisted, but appeared to advance in phase by approximately 4 hours.

Figure 5.1

Diurnal variations in a: blood corticosterone levels, and b: HMG-CoA reductase activity and cholesterol synthesis (expressed as % incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols) in animals housed under controlled light (12L:12D). Each point of 5.1a represents the average of data obtained with 14 rats, while each point of 5.1b represents the average of data obtained with 2 rats. Mean values ± SEM are shown for two experiments. The reductase activity in experiment II was considerably lower than that in experiment I but the reasons for this are unknown. The substrate preparation used in experiment II may have been contaminated.

Fig.5.1 normal light

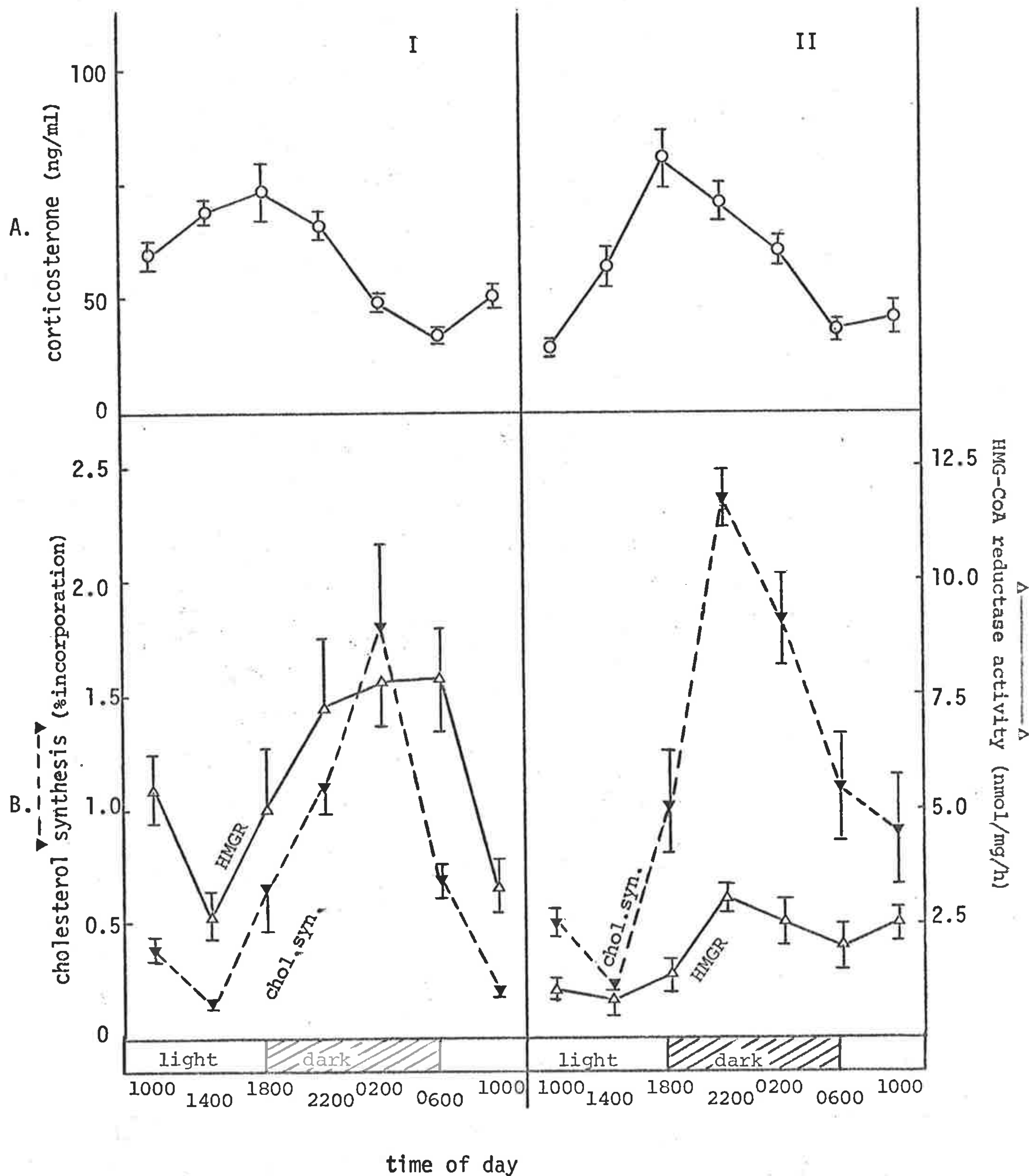
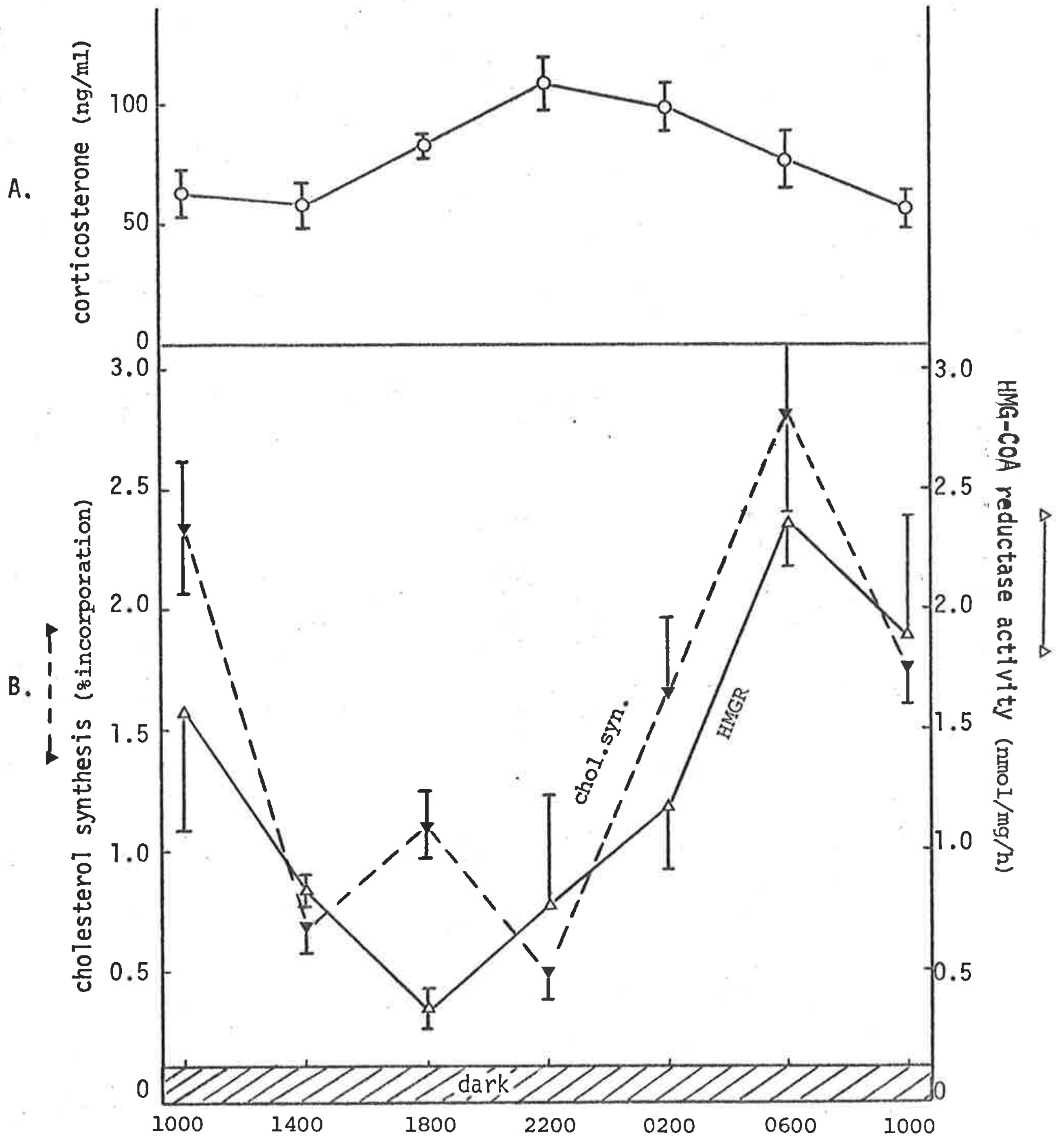


Figure 5.2

Diurnal variations in a: blood corticosterone levels, and b: HMG-CoA reductase activity and cholesterol synthesis (expressed as % incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols) in animals after 4-week exposure to continuous darkness. Each point of 5.2a represents the average of data obtained with 14 rats, while each point of 5.2b represents the average of data obtained with 2 rats. Vertical lines represent the standard error of the mean.

Fig.5.2 continuous dark



The effect of continuous darkness on cholesterol synthesis and reductase activity (Figure 5.2b) appears similar to that described above for corticosterone, there being an advanced phase change of 4-8 hours in the persistent rhythms of both variables.

5.3.3 Effect of continuous light

Cheifetz *et al.* (1968) concluded from their results that an 8-week exposure to continuous light abolishes the diurnal rhythm of blood corticosterone in rats. Figure 5.3a suggests that a 4-week exposure to continuous light did not completely abolish the diurnal rhythm of blood corticosterone in these animals, although the curve is flatter than that under normal lighting (Figure 5.1a).

Figure 5.3a, however, represents the average diurnal variation of 14 animals. The results from the individual animals, some of which are illustrated in Figure 5.4, suggest that continuous light does not actually abolish the rhythm but rather alters its phase between individuals, in a seemingly random fashion. A graph of average results, if flattened, may thus indicate an abolition of corticosterone rhythm as Cheifetz *et al.* (1968) have suggested, but it may also reflect a desynchrony of the rhythm among individual animals.

Figure 5.3b illustrates the effect of continuous light on cholesterol synthesis and HMG-CoA reductase. It appears that cholesterol synthesis retains a cyclic pattern, although the maximum rate occurs at 1000 hours and the minimum at 1400 hours. However, the variation in reductase activity was altered from the variation in

Figure 5.3

Diurnal variations in a: blood corticosterone levels, and b: HMG-CoA reductase activity and cholesterol synthesis (expressed as % incorporation of [1-^{14}C]acetate into digitonin-precipitable sterols) in animals after 4-week exposure to continuous light. Each point of 5.3a represents the average of data obtained with 14 rats, while each point of 5.3b represents the average of data obtained with 2 rats. Vertical lines represent the standard error of the mean.

Fig.5.3 continuous light

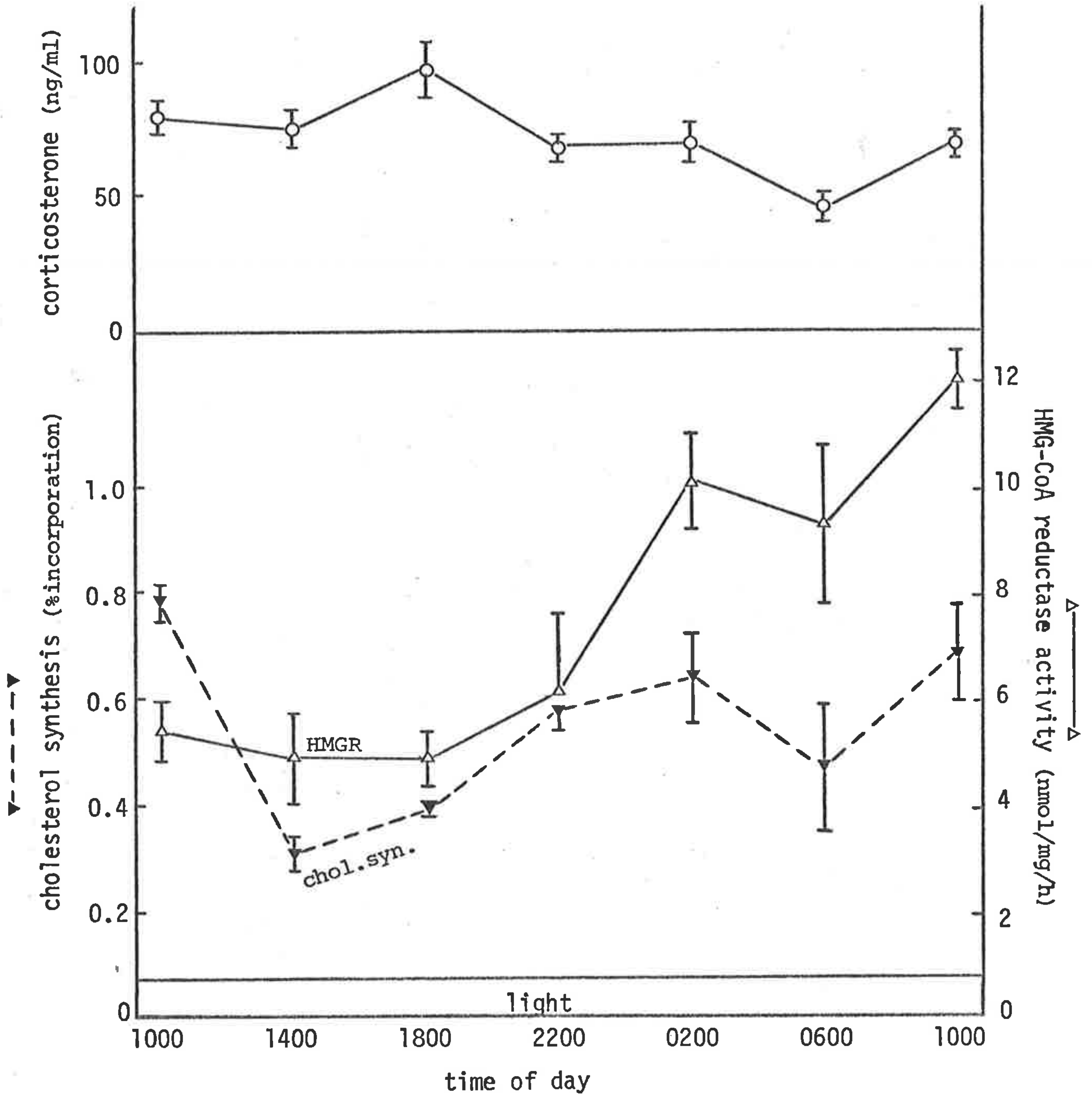
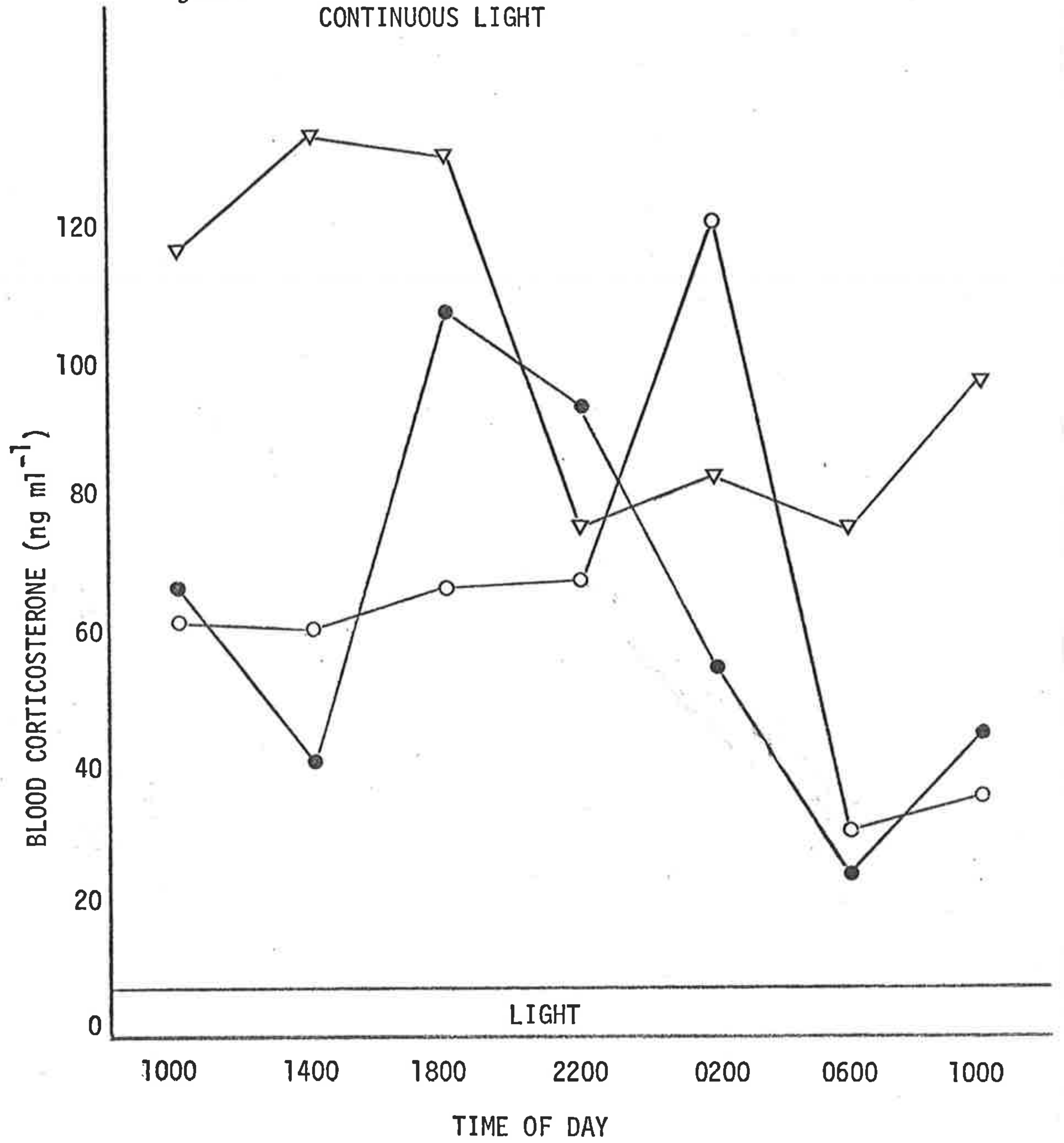


Figure 5.4

Diurnal blood corticosterone variations in three individual animals after a 4-week exposure to continuous light.

Fig.5.4



cholesterol synthesis. This suggests that the conversion of HMG-CoA to mevalonate by HMG-CoA reductase is not a major rate-controlling reaction of the cholesterol biosynthetic pathway in rats exposed to continuous light (see section 5.3.4).

5.3.4 Relationship between HMG-CoA reductase activity and cholesterol synthesis

This relationship was investigated by using regression analysis of the rate of cholesterol synthesis upon reductase activity for each experimental lighting situation. There was a significant regression ($p < 0.05$) of cholesterol synthesis upon reductase activity in controlled lighting and continuous darkness, but not in continuous light (Figure 5.5). In this context it is interesting that other enzyme systems, both hepatic and neural, have been shown to adopt, on exposure to continuous light or darkness, different levels of activity from those under normal lighting (Nair and Casper, 1969; Wurtman *et al.*, 1963).

5.3.5 Relationship between blood corticosterone and cholesterol synthesis/HMG-CoA reductase activity

In an attempt to demonstrate any significant relationship between these variables, regression analysis was used to determine whether the rate of synthesis of cholesterol or HMG-CoA reductase activity correlated with the level of blood corticosterone at the time of sacrifice or at any previous time point. Since a measure of blood corticosterone variation over 24 hours and a subsequent measure

Figure 5.5

Regression analysis of the rate of cholesterol synthesis on HMG-CoA reductase activity in continuous darkness, continuous light, and controlled light. The results used for analysis are those represented in Figures 5.1b, 5.2b, 5.3b. r = correlation coefficient, 12 degrees of freedom.

Fig.5.5 regression analysis

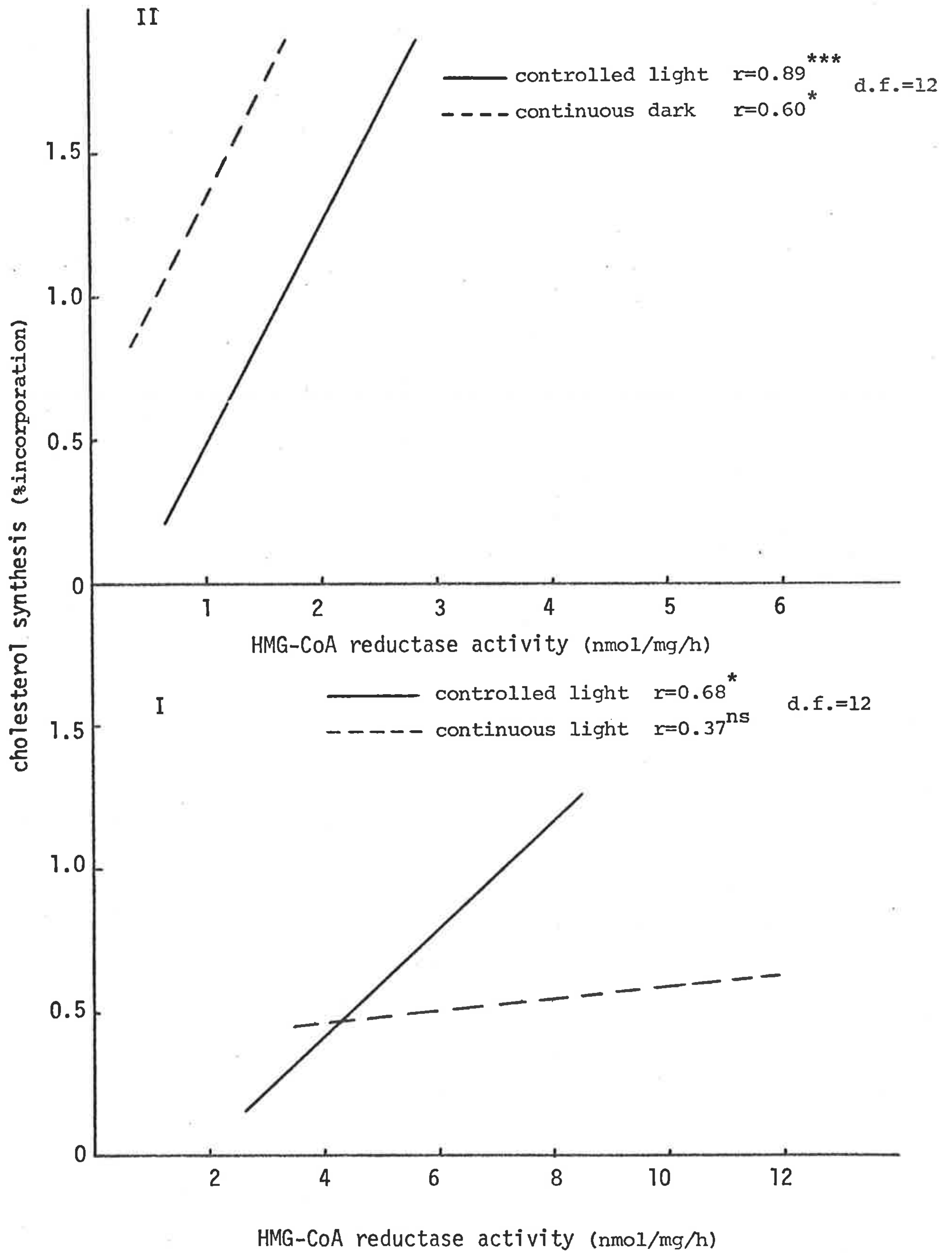


Table 5.1

a Results only from animals housed under controlled light are represented. In both continuous light and continuous darkness there was no significant regression of cholesterol synthesis or HMG-CoA reductase activity on blood corticosterone level at any time prior to killing.

b Correlation coefficient. The calculation of each r value is based on data from 14 animals.

c F value tests whether the regression coefficient is significantly different from zero.

d Cholesterol synthesis.

e HMG-CoA reductase activity.

of the rate of cholesterol synthesis and HMG-CoA reductase activity over 24 hours was available for each animal, variation between individual animals could be effectively eliminated.

The results of this analysis (Table 5.1) indicate that, in controlled lighting, blood corticosterone level was negatively correlated with the rate of hepatic cholesterol synthesis and hepatic HMG-CoA reductase activity 16-20 hours later. This correlation was observed in the controlled lighting situations only, there being no significant regression of corticosterone level on cholesterol synthesis or reductase activity under continuous light or darkness.

Table 5.1 Regression of cholesterol synthesis and HMG-CoA reductase activity on blood corticosterone level[a].

Hours before killing		Experiment I		Experiment II	
		r[b]	F[c]	r	F
24	Cholesterol[d]	0	0	0	1.136
	HMGR[e]	-0.061	0.041	0.045	0.025
20	Cholesterol	-0.568	5.250*	-0.184	0.421
	HMGR	-0.533	4.363*	-0.240	0.736
16	Cholesterol	-0.208	0.498	-0.762	16.586**
	HMGR	-0.015	0.003	-0.671	9.847**
12	Cholesterol	0.075	0.062	-0.241	0.742
	HMGR	0.067	0.050	-0.006	0.001
8	Cholesterol	0.252	0.748	0.288	1.088
	HMGR	0.530	4.298*	0.340	1.573
4	Cholesterol	0.403	2.140	0.448	3.024
	HMGR	0.242	0.687	0.275	0.984
0	Cholesterol	-0.409	2.215	-0.281	1.031
	HMGR	-0.449	2.777	-0.497	3.948

* $P < 0.05$

** $P < 0.01$

5.4 Discussion

A peripheral but important point of this study has been the observation that, while there is a significant correlation between HMG-CoA reductase activity and cholesterol synthesis (measured as the incorporation of ^{14}C from acetate into liver sterol) in controlled light and in continuous darkness, this relationship does not hold in continuous light (Figure 5.5). This argument is based on the assumption that the rate of synthesis of cholesterol is always proportional to [1-^{14}C]acetate incorporation, although it is acknowledged that the specific activity of the acetyl-CoA pool, from which cholesterol is synthesized, could vary in different conditions. This potential problem could be circumvented by measuring the incorporation of ^{3}H from $^{3}H_2O$ into cholesterol.

The results depicted in Figure 5.5 have three points of interest: first, they confirm the conclusion reached by many workers (section 2.2) that, at least in controlled lighting, HMG-CoA reductase is the major rate-controlling enzyme of the cholesterol biosynthetic pathway; second, they show that this relationship also exists in animals subjected to continuous darkness; third, they reveal that in continuous light, changes in HMG-CoA reductase activity have a relatively minor effect on cholesterol synthesis from acetate, implying that the kinetic properties of either this enzyme or other enzymes in the pathway have been altered by continuous lighting. It is therefore interesting that Ramasarma (1972) has implied that, because the increase in cholesterol synthetic rate at midnight compared to noon was less than the increase in HMG-CoA reductase activity, then the

reductase may not be rate-limiting at midnight. Furthermore, Dugan *et al.* (1972) have presented strong evidence of a nutritional state (namely, fasting after controlled feeding) in which HMG-CoA reduction was not rate-limiting for cholesterol synthesis from acetate. It thus seems likely that the reductase enzyme is not rate-limiting under all conditions of lighting and nutrition.

The literature relating adrenal steroids and the control of hepatic cholesterol synthesis has been particularly confusing. Several workers have investigated the effect of adrenalectomy on hepatic cholesterogenesis measured at a single point in time: Perry and Bowen (1956) and Gould *et al.* (1970) claim that adrenalectomy decreases, whilst Willmer and Foster (1960) claim that it increases hepatic cholesterogenesis. Unfortunately, no mention of the time of death or description of lighting schedules was given in any of these studies and it is therefore difficult to assess the meaning of "increase" or "decrease" relative to the diurnally varying rate of hepatic cholesterogenesis of control rats. Reports of the effect of adrenalectomy on the diurnal rhythm of hepatic cholesterogenesis are equally unresolved. Edwards (1973) reported that adrenalectomy abolishes the circadian rhythm of HMG-CoA reductase in rat liver and a constant, low level of activity results; Hickman *et al.* (1972) and Sabine *et al.* (1972) report an abolition of rhythm and a resultant high rate of cholesterol synthesis; Huber *et al.* (1972) claimed that the circadian rhythm of the reductase is unaffected by adrenalectomy. Steroid replacement experiments have likewise resulted in both elevation (Willmer and Foster, 1960) and depression (Nejad and Chaikoff, 1964) of hepatic cholesterogenesis.

While it is agreed that adrenalectomy and steroid replacement experiments are useful for investigating corticosteroid involvement in cholesterogenic regulation, the approach used in this study of measuring inherent fluctuations in blood corticosterone levels and relating these to the rate of cholesterogenesis is relatively free from the complicating variables introduced by pharmacological and surgical manipulations.

The evidence presented in Table 5.1 lends credence to the suggestion by Hickman *et al.* (1972) that, if removing the adrenal glands removes an inhibition on cholesterol synthesis, then the peak of plasma corticosterone at 1800 hours may be responsible for, or at least correlated with, the nadir of cholesterol synthesis at 1200 hours, 18 hours later. These results further suggest that although corticosterone is an important factor in modulating the rate of cholesterol synthesis in controlled lighting, other factors (such as nutrition) override this under continuous light and darkness. The extensive lag phase (16-20 hours) of corticosterone action implies that, rather than directly affecting HMG-CoA reductase, it is probably influencing the rate of cholesterol synthesis by an indirect route, through an action on other endocrine glands or perhaps by affecting the rate of metabolism of cholesterol and thereby adjusting the "feedback" response.

Fregly (1960) has demonstrated that thyroid weight is increased in the rat upon adrenalectomy and Fletcher and Myant (1958) have shown that thyroxine treatment of rats elevates cholesterol

synthesis from acetate. This resulted in the suggestion of Willmer and Foster (1960) that the elevation of hepatic cholesterogenesis seen after adrenalectomy in their experiments may have been an indirect effect due to an elevated basal metabolic rate mediated through the thyroid gland. More explicit is the evidence given by Ness *et al.* (1973) that L-triiodothyroxine increases the synthesis of HMG-CoA reductase (after 30 hours) but that hydrocortisone prevents this increase.

Lakshmanan *et al.* (1973) have shown that HMG-CoA reductase is markedly stimulated by insulin within 2 hours and, since blood insulin level has a diurnal rhythm similar in pattern to that of HMG-CoA reductase (Kaul and Berdanier, 1972), this hormone may be responsible for the diurnal rhythm of hepatic cholesterol synthesis. In support of this is the restoration of the reductase rhythm in diabetic rats by slow-release insulin therapy (Nepokroeff *et al.*, 1974). But the insulin-induced stimulation of the reductase is also blocked by hydrocortisone (Dugan *et al.*, 1974) as is the T_3-response.

The data herein presented, combined with related results from other workers, suggests that glucocorticoids do influence the rhythm and level of cholesterol synthesis. They may act directly in doing this but most likely are modifiers of other hormonal effectors. It is clear that several relatively independent controls are concomitantly functioning to determine the overall rate of cholesterol synthesis, and it is therefore a difficult exercise to determine the discrete influence of any one control differentiated from the total mosaic.

An experimental parameter that could have received more consideration was the food intake of the rats. The arguments centred on food vs. light as the principle agents determining the rhythms of HMG-CoA reductase and cholesterol synthesis, have been outlined in section 2.2.1. Dugan *et al.* (1972) report that controlled feeding can override the light:dark cycle to alter these rhythms, which conversely have been shown to persist in fasted animals (Hickman *et al.*, 1972; Bortz and Steele, 1973). Nevertheless, food intake is a potent influence on reductase activity and cholesterol synthesis, and food intake is clearly influenced by light cycling. Huber *et al.* (1973b) reported that their rats ingest 79% of their food during the dark period. Huber *et al.* (1973b) also reported that an 8-day exposure to continuous light or continuous darkness has little influence on the feeding pattern, although the rats used in this experiment were exposed for 1 month to their respective lighting schedules.

Nevertheless, the experimental design whereby blood corticosterone levels are compared with cholesterol biosynthetic activity is still valid for investigating the relationship between these two variates. Lighting schedules may trigger an internal "zeitgeber" which regulates both feeding and corticosterone rhythms. In the "free-running", light-adjusted situations, the internal "zeitgeber" may be continually stimulated or suppressed, leading to aberrant feed-intake which may dominate corticosterone effects.

Since cholesterol-feeding overrides and prevents the stimulation of HMG-CoA reductase by insulin (Lakshmanan *et al.*, 1975), hormones can perhaps be viewed as "fine-tuners" of the more basic

mechanisms controlled by diet. Although cholesterol feeding does not prevent the T_3-induced stimulation of HMG-CoA reductase, Lakshmanan *et al.* (1975) consider thyroid hormone indirectly stimulates the reductase by lowering serum and liver cholesterol concentrations thereby deleting the "feed-back" response. The amount of cholesterol in the diet is obviously a powerful factor in determining the ultimate rate of hepatic cholesterol synthesis, and the mechanism of action of dietary cholesterol is therefore considered in section 6.

6. DIETARY FEEDBACK CONTROL OF HEPATIC CHOLESTEROL SYNTHESIS

— INTRACELLULAR MECHANISM

6. DIETARY FEEDBACK CONTROL OF HEPATIC CHOLESTEROL SYNTHESIS - INTRACELLULAR MECHANISM

6.1 Introduction

That hepatic cholesterol synthesis in many higher animals is suppressed by dietary cholesterol has been demonstrated many times (Siperstein, 1970; Sabine, 1977) since the original observations of Gould (1951). Furthermore, both indirect and direct evidence supports the hypothesis that the primary site of the cholesterol-feedback response is at the reaction catalysed by HMG-CoA reductase (section 2.2.2) which is the sole membrane-bound enzyme in the biosynthetic pathway between acetate and squalene (section 2.1). Considerable effort has been spent in elucidating the nature of the intracellular suppressing mechanism of cholesterol synthesis, but neither the inhibitor nor its action is clearly resolved in spite of many accumulated data.

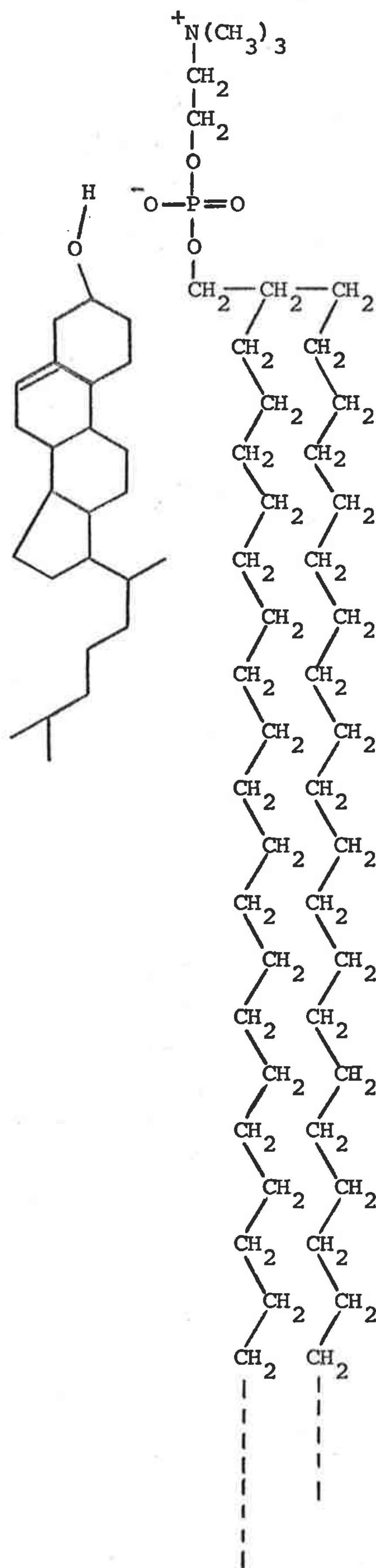
Cholesterol-feeding effectively represses the synthesis of HMG-CoA reductase after 18 hours, but this effect follows 14 hours after an earlier inhibition of activity (4 hours after the commencement of cholesterol-feeding) (Higgins and Rudney, 1973). However, the K_m values for the enzyme from the livers of normal-fed and cholesterol-fed rats, do not differ significantly (Shapiro and Rodwell, 1969), which suggests that this initial feedback phenomenon is not due to classical allosteric inhibition.

The membrane-association of HMG-CoA reductase has been represented in sections 2.1 and 2.3 as being anomalous in the context of the entire biosynthetic pathway, but this feature becomes relevant when identified with a function. It is proposed that the initial

inhibition of HMG-CoA reductase activity after cholesterol-feeding can be explained by examining the structure/function relationship existing between the enzyme and its surrounding membrane lipids. Since the initial suggestion by Raison *et al.* (1971) that activation energies (E_a) of some membrane-bound enzymes are responsive to the physical state (fluidity in particular) of their membrane lipids, this relationship has been demonstrated many times (Raison, 1973a). For instance, Raison and McMurchie (1974) have used electron spin resonance (ESR) to demonstrate temperature-induced phase changes (abrupt changes in molecular ordering) in the lipid components of mitochondrial membranes from sheep liver at 29° and 17°, and from rat liver at 24° and 8°. Furthermore, they report that the E_a of succinate oxidase changes dramatically at each of the phase change temperatures for the respective systems, indicating that conformational changes in membrane proteins can be induced by their lipid environments.

While temperature-induced membrane fluidity changes are not expected to be a feature of homeothermic systems, membrane fluidity can also be manipulated by the extent of unsaturation of the phospholipid acyl chains (Raison, 1973b), phospholipid chain length, extent of hydration, presence of cholesterol, and several other factors such as drugs (Chapman, 1973). Cholesterol in particular, is a powerful modulator of phospholipid chain motion and hence fluidity of phospholipid membranes. Darke *et al.* (1972) have demonstrated that when added to lecithin liposomes, the cholesterol hydroxyl group becomes located adjacent to the lecithin phosphate group with the remainder of the steroid molecule reaching into the phospholipid bilayer (Figure 6.1).

Fig.6.1 LECITHIN-FREE CHOLESTEROL INTERACTIONS



This results in motional restriction of the lecithin hydrocarbon chain to about the tenth carbon atom whereas the remainder of the chain is more mobile (Darke *et al.*, 1972). Kroes *et al.* (1972) have confirmed that cholesterol also increases the viscosity of biological membranes (guinea pig erythrocytes) when incorporated *in vivo* by the animal during cholesterol-feeding.

Not only free cholesterol, but also cholesteryl ester can alter membrane fluidity (Janiak *et al.*, 1974), although the effects of the esterified forms of cholesterol have not been investigated as extensively as those of free cholesterol. Janiak *et al.* (1974) have shown that cholesteryl linolenate decreases the bilayer width of an egg lecithin system which suggests that it decreases ordering of the phospholipid acyl chains. But in this system, the effect is strongly influenced by the extent of hydration.

The basis of the following hypothesis lies in the observations that (i) hepatic HMG-CoA reductase is bound to the endoplasmic reticulum (sections 2.1 and 2.3), (ii) cholesterol-feeding increases the cholesterol (in particular cholesteryl ester) content of the hepatic endoplasmic reticulum (section 2.2.2), (iii) variation in cholesterol content alters the physical characteristics of artificial (Darke *et al.*, 1972; Janiak *et al.*, 1974) and biological (Kroes *et al.*, 1972) membranes, in particular altering membrane fluidity, and (iv) membrane fluidity can regulate the conformation and activation energy of membrane-bound enzymes (Raison, 1973a, 1973b). It is, therefore, proposed that after cholesterol-feeding, the increased cholesterol content of the hepatic endoplasmic reticulum alters the fluidity of

certain membrane lipids, thereby increasing the E_a (and decreasing the activity) of HMG-CoA reductase. Such a mechanism does not imply an altered K_m value.

The E_a of HMG-CoA reductase was measured in microsomes isolated from the livers of normal-fed, and cholesterol-fed, rats. Studies were also begun on the physical properties of these hepatic microsomes, using the technique of electron spin resonance (ESR).

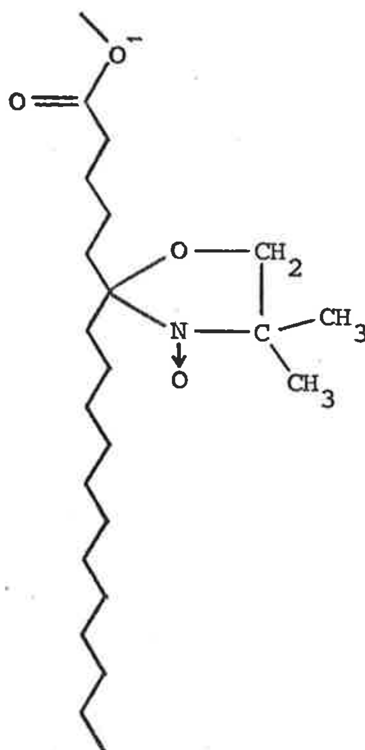
6.2 Methods

HMG-CoA reductase was assayed as described in section 3.4.

Electron spin resonance (ESR)

ESR was used to determine the effect of temperature and cholesterol on the rotational motion of a "spin-label" incorporated into the hydrophobic region of hepatic endoplasmic reticulum membranes. Spin-labels generally contain a stable nitroxide radical which confers paramagnetic properties making them suitable for resonance detection when exposed to a changing magnetic field. In this instance, the paramagnetic reporter group is linked to stearic acid at the sixth carbon, providing a spin-label (6NS) (Figure 6.2) which can be introduced into membranes. The carboxyl group remains bound at the polar region of the bilayer while the alkyl chain of stearic acid containing the paramagnetic reporter group, extends into the hydrophobic region of the membrane (Seelig and Hasselbach, 1971).

Fig.6.2



6-doxylstearic acid (6NS)

(4',4'-dimethyloxazolidine-N-oxyl-6-stearic acid)

The ESR spectrum is sensitive to the spatial orientation and motional characteristics of the spin-label which in turn reflects the extent of movement of surrounding molecules, in this case the phospholipid acyl chains (Raison *et al.*, 1971). The motion parameter calculated from ESR spectra according to Raison *et al.* (1971), and used in this study, is the rotational correlation time (τ_c) which can be roughly considered as the time required for a molecule to tumble through an arc of 90° (Raison *et al.*, 1971).

Hepatic endoplasmic reticulum was isolated as for HMG-CoA reductase assay (section 3.4) and the microsomal pellet was suspended in 1 ml Hepes (pH 7.4), 0.25 M sucrose buffer. The 6NS spin-label, dissolved in ethanol, was added (1.5 μl) to 0.15 ml aliquot of membrane suspension to give a spin-label concentration approximately 10^{-2} M. Ferricyanide was added (10^{-1} M final concentration).

Spectra were recorded with a Varian (model E-4) spectrometer, the temperature of the sample being varied from $0\text{-}44^o$ with a Varian temperature control accessory (V-4557). The scan-width was 100 G with a mid-point at 3240 G and 4 G modulation.

6.3 Results

Temperature response of HMG-CoA reductase

Initial attempts to measure the E_a of HMG-CoA reductase in freshly prepared microsomes were unsuccessful. An example of these attempts is depicted in Arrhenius form in Figure 6.3 which indicates a higher enzyme activity at 33° than that measured at 37°. The data represented in Figure 6.4a confirm this effect for incubation times

Figure 6.3

Enzyme was assayed in freshly prepared microsomes as described (section 3.4). Each value represents the mean of triplicate determinations at each temperature.

Fig.6.3 Arrhenius plot

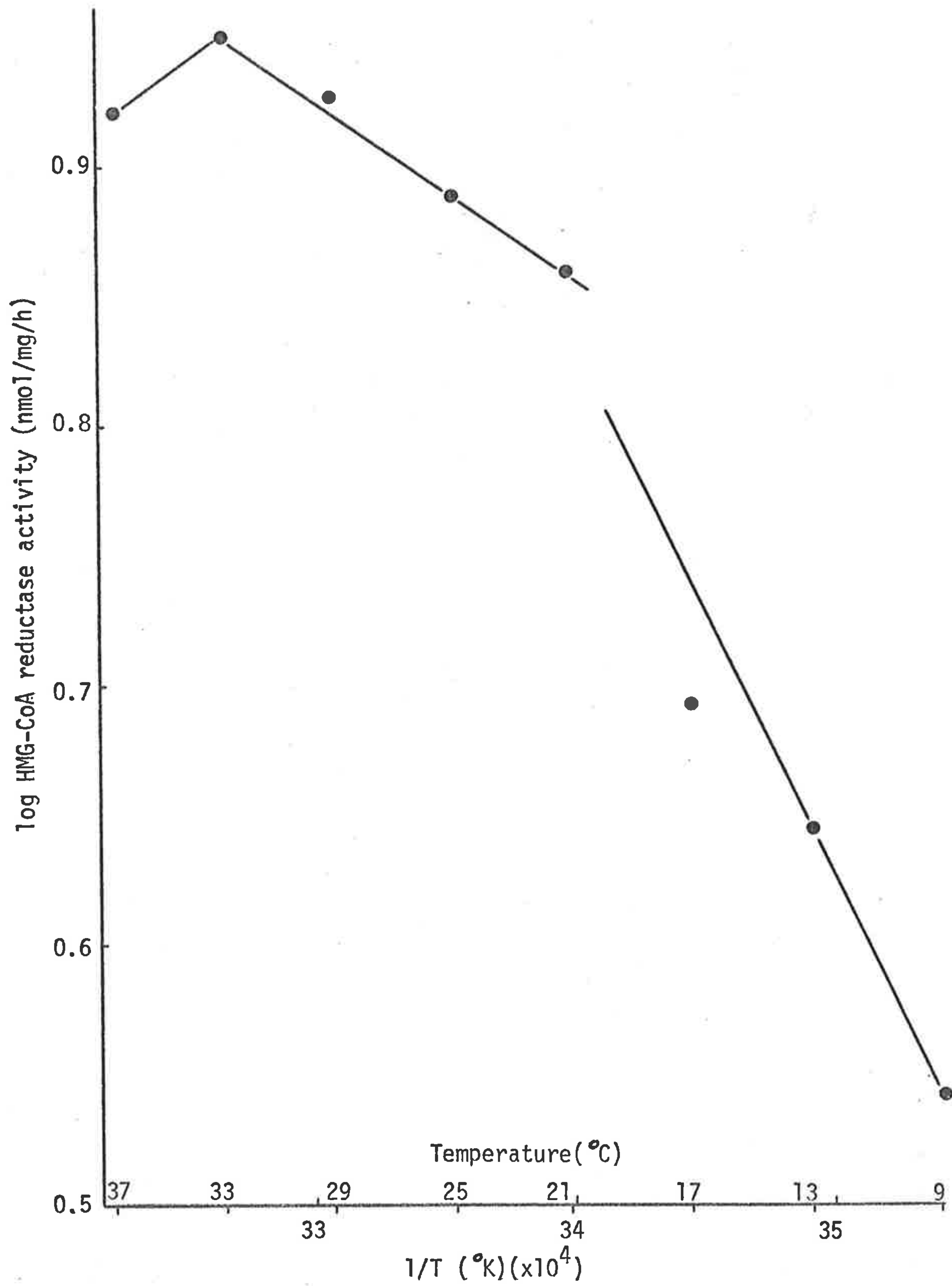


Table 6.1

HMG-CoA reductase was assayed as described (section 3.4). All values (mean ± S.E.M.) are the results of triplicate determinations, using aliquots from a pooled suspension of microsomes isolated from the livers of 4 rats on each diet and stored at -4°C for 3 days.

longer than 30 min in this instance. However, this phenomenon was not evident with microsomal HMG-CoA reductase stored at -4° for 3 days before assay (Figure 6.4b). This freezing procedure therefore allowed E_a determination of HMG-CoA reductase.

Effect of diet and assay temperature on HMG-CoA reductase

The effect of assay temperature on the activity of HMG-CoA reductase from normal-fed and cholesterol-fed rats is shown in Table 6.1.

Table 6.1 Effect of diet and assay temperature on the activity of hepatic microsomal HMG-CoA reductase.

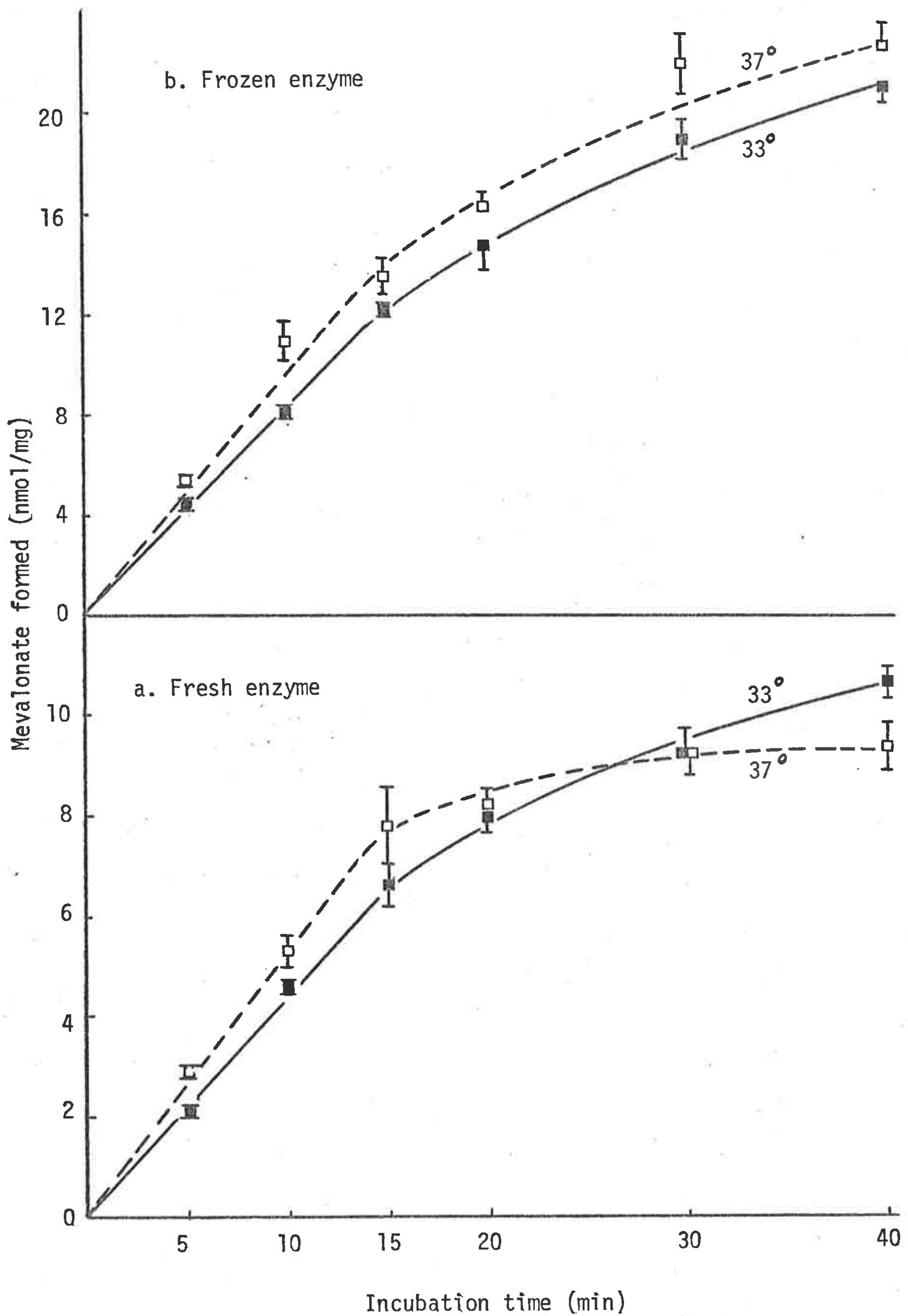
Temp. (^{o}C)	Specific activity of HMG-CoA reductase (nmol/mg/h)	
	Normal diet	Cholesterol-rich diet (5% for 2 days)
23	0.92 ± 0.22	-
25	1.51 ± 0.03	-
28	2.78 ± 0.37	-
31	3.43 ± 0.22	0.70 ± 0.12
34	4.39 ± 0.12	1.23 ± 0.08
37	6.36 ± 0.40	2.59 ± 0.12

Although HMG-CoA reductase activity in the hepatic microsomes from cholesterol-fed rats was not detectable below 31°, it can be seen that the response of the reductase to assay temperature is

Figure 6.4

After preparation of the pooled microsomes from 2 rats, HMG-CoA reductase was assayed as described in section 3.4, a: in the fresh preparation, and b: after storage at -4°C for three days. Each value represents the mean ± SEM of triplicate determinations.

Fig.6.4 Effect of storage and assay temperature on the time curve of HMG-CoA reductase



markedly affected by the diet. The feed-back response is evident in that hepatic reductase activity of cholesterol-fed rats was only 40% that of normal-fed rats when measured at 37°. This value, however, decreased to 28% at 34° and 20% at 31°.

This effect can be more easily seen in the Arrhenius plots (Figure 6.5) of the data in Table 6.1, the slopes of which represent the activation energies of HMG-CoA reductase for each diet. Calculating E_a (in the range 37-31°) from the slope of the Arrhenius plot (Raison, 1973b) yields a value of 19.5 Kcal/mole/^{o}K for the reductase from normal-fed rats, and 41.2 Kcal/mol/^{o}K for that from cholesterol-fed animals. Cholesterol-feeding has therefore produced a large increase in E_a of hepatic HMG-CoA reductase, which increase is consistent with a reduced rate of cholesterol synthesis.

For the enzyme from normal-fed animals, there appears to be a discontinuity in slope between 25° and 28° (Figure 6.5). The slope in the lower temperature range represents an activation energy of 45.2 Kcal/mol/^{o}K, a value similar to that from cholesterol-fed animals (41.2 Kcal/mol/^{o}K) in the higher temperature range.

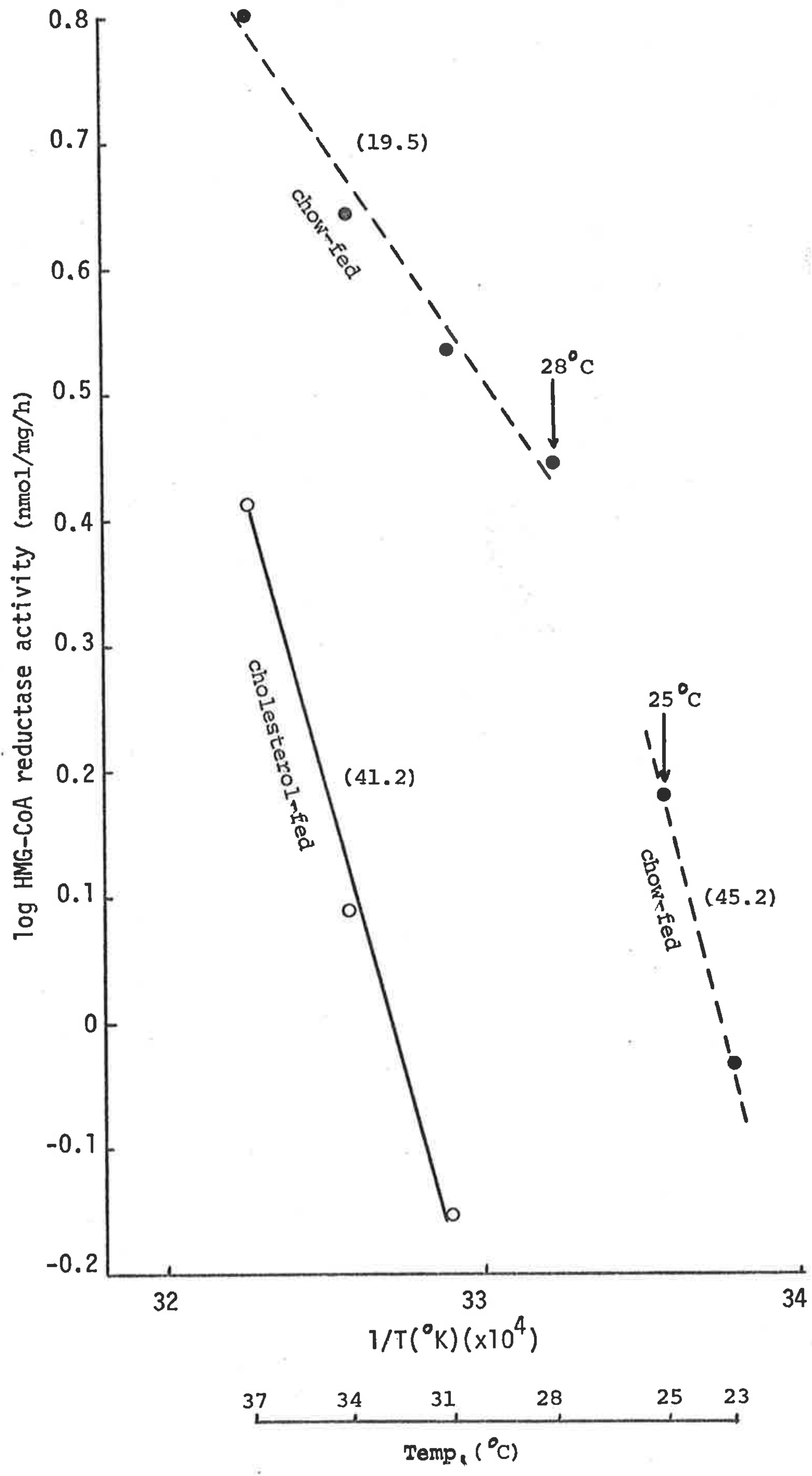
6.4 Discussion

The difference in temperature response of frozen HMG-CoA reductase from fresh reductase (Figure 6.4) is an empirical observation which has been used in this investigation without further enquiry. Fresh enzyme is apparently denaturing faster at 37° than at 33° and the progressive loss of total activity is sufficient to overcome the increased specific activity due to temperature. The effect of storage

Figure 6.5

Arrhenius plot of the data in Table 6.1. Activation energies (Kcal/mol/^{o}K) corresponding to each slope and calculated according to Raison (1973b), are shown.

Fig.6.5 Arrhenius plot-effect of feeding cholesterol



at -4° for 3 days, after which the above phenomenon is absent (Figure 6.4b), may be to allow the degradation of the labile fraction of the total population of enzyme molecules. The remaining molecules are sufficiently stable for the temperature response of activity to greatly exceed the protein denaturation due to temperature and therefore allow expression of the direct relationship between the logarithm of activity and the inverse of absolute temperature (Figure 6.5) predicted from the Arrhenius equation (Raison, 1973b). For the reductase from normal-fed animals however, the direct relationship is evident only within fixed temperature ranges, there being a change in slope around 25° (Figure 6.5).

The discontinuity, a response not predicted by Arrhenius' original theory (Johnson *et al.*, 1954), has been observed in many other biological systems and can be explained by the occurrence of a phase change in the lipid component of the membrane, particularly in the lipid micro-environment of the actual enzyme (Lyons, 1972; Raison, 1973b). Furthermore, a lipid phase change in this temperature region (*ca.* 25°) has been observed many times in mammalian membranes by ESR spectroscopy (Raison, 1973a, 1973b) and is probably the transition from a liquid-crystalline phase to the initiation of gel formation. A gross decrease in fluidity of the membrane phospholipid acyl chains in the gel phase seems the most probable factor responsible for the large increase in E_a of HMG-CoA reductase from normal-fed animals which was observed in the lower temperature region.

In this experiment, the livers of the cholesterol-fed animals contained an average of 4.82 mg cholesterol/g wet weight, compared to 2.31 mg for the control animals. Although the hepatic

microsomal cholesterol content was not measured in this experiment, it has been established that an increase in total hepatic cholesterol content following cholesterol-feeding is coincident with an increase in microsomal cholesterol content (Harry *et al.*, 1973). Furthermore, the introduction of free cholesterol into artificial (Darke *et al.*, 1972) and natural (Kroes *et al.*, 1972) membranes inhibits the motion of the phospholipid acyl chains. As mentioned in section 6.1, Janiak *et al.* (1974) have reported that cholesteryl linolenate also perturbs lecithin membranes, increasing their fluidity in this case, although the effect is influenced by the extent of hydration. The effects of cholesteryl esters in general on membranes have not been investigated as extensively as those of free cholesterol.

The increase in E_a of HMG-CoA reductase with increased cholesterol content can perhaps be considered analogous to the E_a increase with lowered temperature in that both increases are due to restraint of the acyl chains (decreased fluidity). Strengthening this concept is the demonstration that cholesterol acts as a modulator of phospholipid fluidity to alter the activity of various ATPase enzymes *in vitro* (Kimelberg and Papahadjopoulos, 1974) and *in vivo* (Farias *et al.*, 1975), although cholesterol may not be acting as a normal physiological regulator in these instances.

A physical investigation of the hepatic endoplasmic reticulum membranes from normal-fed and cholesterol-fed rats would be complementary to this enzyme study. ESR spectroscopy was used to begin preliminary studies of the fluidity of the membrane phospholipid acyl

chains. The 6NS spin-label anchored by its carboxyl group at the polar phospholipid head-group region, was used. It monitors molecular motion superficially in the hydrophobic region of the membrane. The precise position of the paramagnetic reporter group, the nitroxide radical, in the microsomal membrane is not known since this is pH dependent and the relationship has been elucidated only in homogeneous lecithin dispersions (Barratt and Laggner, 1974). Nevertheless free cholesterol has been shown to have maximal effect on the fatty acyl chains between carbons 2 to 8 (Darke *et al.*, 1972), and thus the 6NS spin-label could be expected to detect any alteration in fluidity due to free cholesterol.

Whereas free cholesterol is anchored by its hydroxyl group at the phospholipid head-group and extends only into the superficial hydrophobic region of a membrane (Darke *et al.*, 1972), esterified cholesterol could be either entirely within the hydrophobic region or could likewise be anchored at the polar head-group region by its ester bonding (Loomis *et al.*, 1974; Janiak *et al.*, 1974). Figure 6.6 illustrates some positional opportunities for cholesteryl ester in a phospholipid bilayer. The 6NS label may therefore be suitable also for detecting pertubations in the membrane due to cholesteryl ester anchored at the polar head-group region.

The preliminary study has suggested that cholesterol-feeding in fact does not alter the fluidity of the hepatic endoplasmic reticulum in the superficial hydrophobic region monitored by 6NS (Figure 6.7). Figure 6.7 shows that in the region monitored by 6NS, the variation of membrane fluidity with temperature is not altered by

Fig.6.6

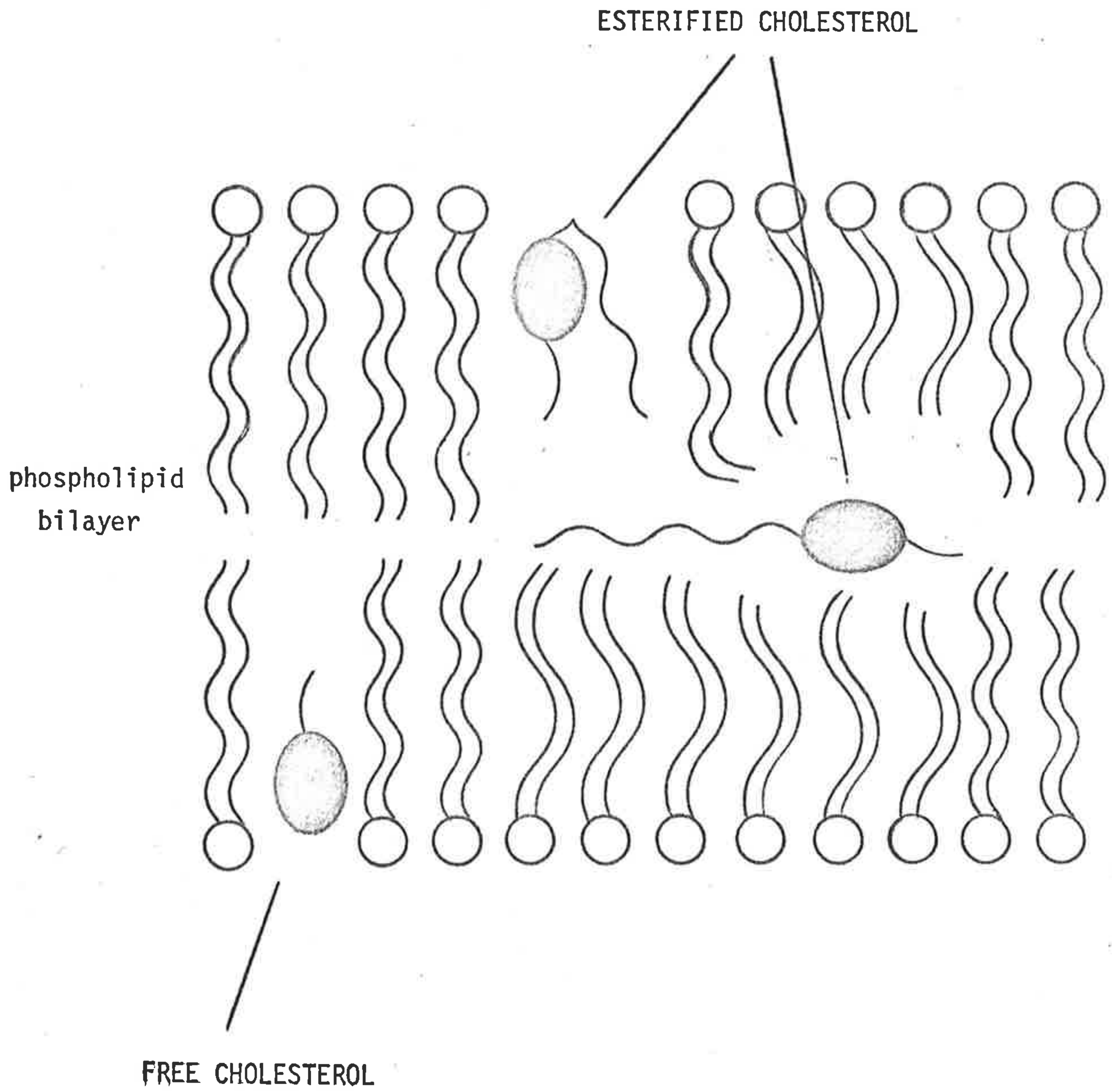


Figure 6.7

Arrhenius plots of the rotational correlation time (τ_c) of the 6NS spin label in hepatic microsomes from normal-fed and cholesterol-fed (5% for 2 days) rats. τ_c is a parameter of molecular motion and is described in section 6.2.

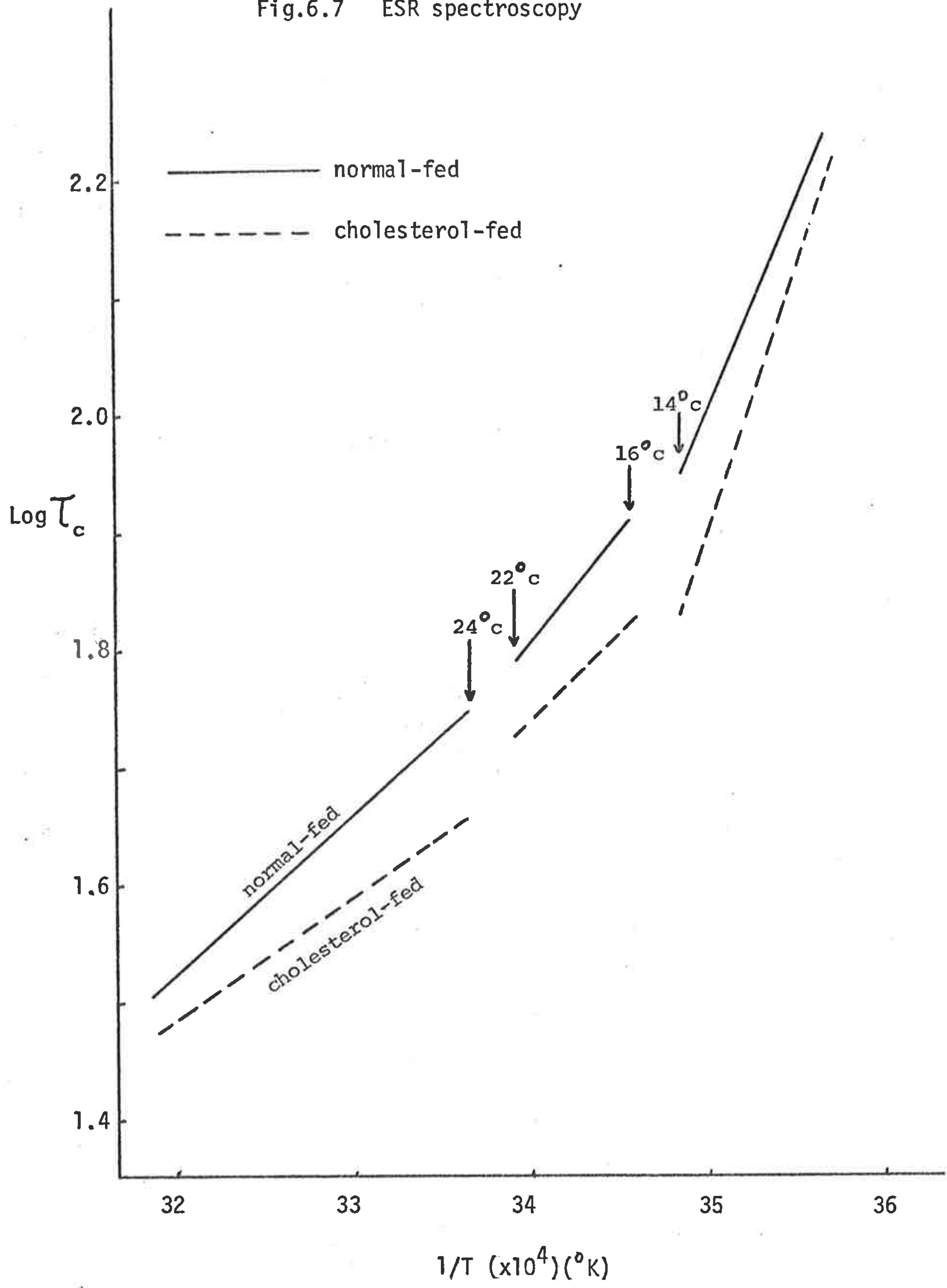


Fig.6.7 ESR spectroscopy
normal-fed
cholesterol-fed
2.2
2.0
1.8
1.6
1.4
Log τ_c
14°c
16°c
22°c
24°c
normal-fed
cholesterol-fed
32
33
34
35
36
1/T (x10⁴)(°K)

feeding cholesterol to the rats. The "breaks" (Figure 6.7) indicate phase changes in the membrane, the one at the highest temperature occurring in the region of that detected by the enzymic study (Figure 6.5).

Although the ESR results do not support the proposal that cholesterol-feeding alters the fluidity of hepatic endoplasmic reticulum, they do not refute the proposal. That the concentration of *esterified* cholesterol in the endoplasmic reticulum increases after cholesterol feeding could be particularly important in the eventual localization of dietary cholesterol in this hepatic membrane. The positional opportunities available to cholesteryl ester (Figure 6.6) must be considered in physical studies of the effect of cholesterol-feeding on hepatic endoplasmic reticulum. In this case, the 6NS label would not be adequate for detecting fluidity changes due to cholesteryl ester in the deep hydrophobic regions of the membrane. Furthermore, it has been shown that cholesterol is not necessarily distributed homogeneously in phospholipid bilayers, but may interact preferentially with a single phospholipid species (Verkleij *et al.*, 1974). It is therefore possible that cholesterol may be localized around HMG-CoA reductase and not cause gross fluidity changes in the entire membrane.

Cholesteryl ester involvement in dietary feedback regulation has been discussed in section 2.2.2. Briefly, not only do cholesteryl ester levels in hepatic endoplasmic reticulum increase after cholesterol feeding, but Goldstein *et al.* (1974) have also shown that the enzymic esterification of cholesterol in human fibroblasts is increased in response to low density lipoprotein (LDL) which also decreases cholesterol synthesis. In fibroblasts from homozygotes with

familial hypercholesterolemia, LDL neither suppresses cholesterol synthesis nor stimulates cholesterol esterification. These processes appear inseparable. Furthermore, exogenous cholesterol contained in low density lipoproteins, rather than endogenously synthesized sterol, appears to be the substrate for esterification (Goldstein *et al.*, 1974) and therefore provides a system for intracellular regulation of sterol synthesis by dietary cholesterol via cholesteryl ester.

The idea that cholesteryl ester, being the intracellular effector, could explain the lack of success in demonstrating inhibition *in vitro* of cholesterol synthesis and HMG-CoA reductase activity, was also introduced in section 2.2.2. Cholesterol added in any mixture to subcellular fractions has not been able to inhibit its own synthesis. Furthermore, microsomes isolated from cholesterol-fed rats (presumably with elevated cholesteryl ester concentrations) cause no inhibition of HMG-CoA reductase in normal microsomes (section 2.2.2). These observations on systems not capable of protein synthesis were considered to be evidence that the feedback phenomenon is due to repression of enzyme synthesis in the intact cell rather than inhibition of enzyme activity which could not be demonstrated. But cholesteryl ester would exchange between membranes very slowly since it is completely apolar and probably within the hydrophobic interior of a membrane. Goodman and Lequire (1975) have observed the relative slowness with which cholesteryl esters are exchanged between lipoproteins, compared with the ready exchangeability of free cholesterol. Lack of effector (inhibitor) transfer alone may explain the failure to observe an inhibition *in vitro* in mixing experiments. In this context, Siperstein (1965) has reported

an inhibition of mevalonate synthesis *in vitro* by addition of microsomal membranes from cholesterol-fed rats, but no experimental details such as length of preincubation, were reported.

In spite of the increase in hepatic cholesteryl ester after cholesterol-feeding, free cholesterol may still be the effective molecular inhibitor. Mayer and Hummel (1976) have reported that microsomal HMG-CoA reductase is inhibited *in vitro* after preincubation with cholesterolesterase enzyme, which generates free cholesterol. The formation of cholesteryl ester in response to cholesterol-feeding may therefore be a mechanism of storing and localizing effector near HMG-CoA reductase, but the ultimate inhibition may be due to the release *in situ* of free cholesterol.

The hypothesis, therefore, that dietary cholesterol exerts its rapid inhibition on hepatic, membrane-bound HMG-CoA reductase by modifying (with free or esterified cholesterol) the fluidity of the lipid environment of this enzyme and thereby altering configuration and activation energy, is not disproved. This initial effect is followed by the eventual repression of enzyme synthesis (Higgins and Rudney, 1973). This scheme allows for both short-, and long-term, control over hepatic cholesterol synthesis by dietary cholesterol. It is also a scheme which accounts for observations previously unexplained. These are (i) the K_m's of the reductase from normal-fed and cholesterol-fed rats are the same (Shapiro and Rodwell, 1969), yet there is a rapid inhibition of reductase activity independent of that due to repression of enzyme synthesis, (ii) soluble cytosolic HMG-CoA reductase is either not affected or is inhibited to a lesser degree and less consistently

by cholesterol-feeding, than is microsomal-bound reductase (Siperstein, 1965, 1970; Siperstein and Fagan, 1966), and (iii) removal of lipid by Triton 1339 from inhibited enzyme already solubilized with deoxycholate, completely restores activity (Siperstein, 1966). The apparent inhibition of the reductase by its native membrane alone has been noticed by Linn (1967a) and discussed in section 2.3. Furthermore, HMG-CoA reductase is the sole membrane-bound enzyme between acetate and squalene (section 2.1), and yet the membrane association is not necessary for its activity (section 2.3).

The concept presented here can be pursued in several experimental directions. Further ESR studies could employ spin labelled esters of stearic acid which may be able to monitor the regions of membrane containing cholesteryl ester. ESR spectroscopy with partially purified HMG-CoA reductase may allow subtle localized membrane changes to be detected. Furthermore, many derivatives of cholesterol have been shown to be more potent inhibitors of cholesterol synthesis and HMG-CoA reductase in tissue culture systems, than cholesterol itself (Kandutsch and Chen, 1973, 1974; Breslow *et al.*, 1975b). A list of predictions and measurements of the effect of these compounds on membrane fluidity could be compared with a list of their inhibitory potentials for sterol synthesis.

Kinetic studies of alteration to the enzymic reaction itself are also indicated. Farias *et al.* (1975) have demonstrated that lipid fluidity can alter the Hill coefficients of several membrane-bound enzymes (mostly ATPase enzymes). Measuring the Hill coefficients for HMG-CoA binding to HMG-CoA reductase from normal-fed and cholesterol-fed rats can give information regarding the substrate binding processes

of this enzyme, whether it is a cooperative process and whether the cooperativity is influenced by cholesterol-feeding. The Hill coefficients could also be measured at different temperatures, a manipulation which is known to alter membrane fluidity.

A possible extension of this proposal is that membrane fluidity changes induced by cholesterol-feeding lead not only to the initial inhibition of HMG-CoA reductase activity, but also to its following repression of synthesis. Pronczuk and Fillios (1968) reported that increased cholesterol concentration in hepatic endoplasmic reticulum measured after cholesterol-feeding, results in a disaggregation of the membrane-bound polysomes and a consequent increase in monosomes. This provides a mechanism whereby hepatic protein synthesis could be inhibited by cholesterol-feeding, thus completing an entire scheme of the molecular mechanism responsible for the inhibition of hepatic sterol synthesis by dietary cholesterol.

7. CONCLUDING REMARKS

7. CONCLUDING REMARKS

Although cholesterol is synthesized in the rat by all tissues so far investigated, the liver is the major source of endogenous cholesterol. Furthermore, the rate of hepatic cholesterol synthesis is tightly regulated by the network of agents described in section 2.2.

The importance of corticosterone in the regulation of hepatic cholesterol synthesis has been investigated in this study by comparing inherent fluctuations in both variates. The level of blood corticosterone was significantly correlated with the rate of cholesterogenesis 16-20 hours later and the possibility of corticosterone being a modulator of other hormones which also affect cholesterol synthesis, was discussed (section 5). In this instance, corticosterone may function as an antagonist to the action of insulin on hepatic HMG-CoA reductase.

Although the particular integrated pattern of hormonal secretions found in the rat is peculiar to the vertebrate phylum (Barrington, 1964), even yeasts possess a mechanism for regulation of cholesterol synthesis by exogenous cholesterol (McNamara and Rodwell, 1972). In the rat, therefore, hormones can perhaps be viewed as secondary controls imposed on the more fundamental regulation by dietary cholesterol which has indeed been shown to override hormonal influence (Lakshmanan *et al.*, 1975).

Although the suppression of hepatic cholesterol synthesis by dietary cholesterol has been demonstrated many times, its mechanism of action has not been fully elucidated. An hypothesis for the mechanism of action of dietary cholesterol based on reported observations, was therefore presented and tested. It is proposed that dietary

cholesterol regulates the activity of the rate-controlling enzyme of cholesterol synthesis, HMG-CoA reductase, by modifying the fluidity of the lipid microenvironment of this microsomal enzyme. The increase in HMG-CoA reductase activation energy caused by dietary cholesterol is related to alterations in the membrane phospholipid environment, which supports this hypothesis (section 6). Furthermore, it has been shown that the hypothesis and its ramifications can account for many reported observations previously not integrated into a single scheme, particularly those related to aspects of the membrane association of HMG-CoA reductase (section 6).

Since control of cholesterol synthesis by dietary cholesterol is a basic phenomenon in both experimental and evolutionary terms, the proposal for its mode of action presented here makes further testing and modification important.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ackerman, M.E., W.L. Redd, and T.J. Scallen (1974). Solubilization of 3-hydroxy-3-methylglutaryl coenzyme A reductase from lyophilized rat liver microsomes: lack of evidence for cold lability in this soluble enzyme preparation. *Biochem. Biophys. Res. Comm. 56,* 29-35.

Back, P., B. Hamprecht, and F. Lynen (1969). Regulation of cholesterol biosynthesis in rat liver: diurnal changes of activity and influence of bile acids. *Arch. Biochem. Biophys. 133,* 11-21.

Ballard, F.J. (1972). Supply and utilization of acetate in mammals. *Am. J. Clin. Nutr. 25,* 773-779.

Barratt, M.D., and P. Laggner (1974). The pH-dependence of ESR spectra from nitroxide probes in lecithin dispersions. *Biochim. Biophys. Acta 363,* 127-133.

Barrington, E.J.W. (1964). Hormones and evolution, English Universities Press, London.

Bassett, J.M., and N.T. Hinks (1969). Micro-determination of cortico-steroids in ovine peripheral plasma: effects of venipuncture, corticotrophin, insulin and glucose. *J. Endocr. 44,* 387-403.

Bates, S.R., and G.H. Rothblat (1974). Regulation of cellular sterol flux and synthesis by human serum lipoproteins. *Biochim. Biophys. Acta 360,* 38-55.

Beg, Z.H., D.W. Allmann, and D.M. Gibson (1973). Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and with protein fractions of rat liver cytosol. *Biochem. Biophys. Res. Comm. 54,* 1362-1369.

Beher, W.T., G.D. Baker, and W.L. Anthony (1959). Effect of dietary cholic acid on *in vivo* cholesterol metabolism. *Proc. Soc. Exp. Biol. Med. 100,* 3-6.

Bloch, K. (1965). The biological synthesis of cholesterol. *Science 150,* 19-28.

Bloch, K., and D. Rittenberg (1942). On the utilization of acetic acid for cholesterol formation. *J. Biol. Chem. 145,* 625-636.

Bloch, K., E. Borek, and D. Rittenberg (1946). Synthesis of cholesterol in surviving liver. *J. Biol. Chem. 162,* 441-449.

Bortz, W.M., and L.A. Steele (1973). Synchronization of hepatic cholesterol synthesis, cholesterol and bile acid content, fatty acid synthesis and plasma free fatty acid levels in the fed and fasted rat. *Biochim. Biophys. Acta 306,* 85-94.

Breslow, J.L., D.R. Spaulding, D.A. Lothrop, and A.W. Clowes (1975a). Effect of lipoprotein on 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity in rat liver cell culture: special suppressant effect of a lipoprotein isolated from hypercholesterolemic rat plasma. *Biochem. Biophys. Res. Comm. 67*, 119-125.

Breslow, J.L., D.A. Lothrup, D.R. Spaulding, and A.A. Kandutsch (1975b). Cholesterol, 7-ketocholesterol and 25-hydroxycholesterol uptake studies and effect on 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts. *Biochim. Biophys. Acta 398*, 10-17.

Bricker, L.A., and G.S. Levey (1972). Evidence for regulation of cholesterol and fatty acid synthesis in liver by cyclic adenosine 3',5'-monophosphate. *J. Biol. Chem. 247*, 4914-4915.

Brown, M.S., and J.L. Goldstein (1974). Familial hypercholesterolemia: defective binding of lipoproteins to cultured fibroblasts associated with impaired regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity. *Proc. Nat. Acad. Sci. U.S.A. 71*, 788-792.

Brown, M.S., S.E. Dana, J.M. Dietschy, and M.D. Siperstein (1973). 3-Hydroxy-3-methylglutaryl coenzyme A reductase solubilization and purification of a cold-sensitive microsomal enzyme. *J. Biol. Chem. 248*, 4731-4738.

Brown, M.S., S.E. Dana, and J.L. Goldstein (1974). Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in cultured human fibroblasts. *J. Biol. Chem. 249*, 789-796.

Bucher, N.L.R., K. McGarrahan, E. Gould, and A.V. Loud (1959). Cholesterol biosynthesis in preparations of liver from normal, fasting, X-irradiated, cholesterol-fed, triton or Δ^4-cholest-3-one-treated rats. *J. Biol. Chem. 234*, 262-267.

Bucher, N.L.R., P. Overath, and F. Lynen (1960). β-hydroxy-β-methyl-glutaryl coenzyme A reductase, cleavage and condensing enzymes in relation to cholesterol formation in rat liver. *Biochim. Biophys. Acta 40*, 491-501.

Channon, H.J. (1925). Cholesterol synthesis in the animal body. *Biochem. J. 19*, 424-432.

Chapman, D. (1973). In "Biological Membranes" (D. Chapman and D.F.H. Wallach, eds.), pp. 91-144. Academic Press, New York.

Cheifetz, P., N. Gaffud, and J.F. Dingman (1968). Effects of bilateral adrenalectomy and continuous light on the circadian rhythm of corticotropin in female rats. *Endocrinology 82*, 1117-1124.

Cornforth, J.W., R.H. Cornforth, C. Donninger, and G. Popják (1965). Studies on the biosynthesis of cholesterol. *Proc. Roy. Soc. (London) B, 163*, 492-514.

Danielsson, H., K. Einarsson, and G. Johansson (1967). Effect of biliary drainage on individual reactions in the conversion of cholesterol to taurocholic acid. *Europ. J. Biochem.* *2*, 44-49.

Darke, A., E.G. Finer, A.G. Flook, and M.C. Phillips (1972). Nuclear magnetic resonance study of lecithin-cholesterol interactions. *J. Mol. Biol.* *63*, 265-279.

David-Nelson, M.A., and A. Brodish (1969). Evidence for a diurnal rhythm of corticotrophin-releasing factor (CRF) in the hypothalamus. *Endocrinology* *85*, 861-866.

Dietschy, J.M. (1967). Effects of bile salts on intermediate metabolism of the intestinal mucosa. *Fed. Proc.* *26*, 1589-1598.

Dietschy, J.M., and M.D. Siperstein (1967). Effect of cholesterol feeding and fasting on sterol synthesis in seventeen tissues of the rat. *J. Lipid Res.* *8*, 97-104.

Dubach, U., L. Recant, E. Hatch, and M.B. Koch (1961). Negative feedback mechanism of cholesterol synthesis in experimental nephrosis. *Proc. Soc. Exp. Biol. Med.* *106*, 136-139.

Dugan, R.E., L.L. Slakey, A.V. Briedis, and J.W. Porter (1972). Factors affecting the diurnal variation in the level of β-hydroxy-β-methylglutaryl coenzyme A reductase and cholesterol-synthesizing activity in rat liver. *Arch. Biochem. Biophys.* *152*, 21-27.

Dugan, R.E., G.C. Ness, M.R. Lakshmanan, C.M. Nepokroeff, and J.W. Porter (1974). Regulation of hepatic β-hydroxy-β-methylglutaryl coenzyme A reductase by the interplay of hormones. *Arch. Biochem. Biophys.* *161*, 499-504.

Dvornik, D., M. Kraml, J. Dubuc, M. Givner, and R. Gaudry (1963). A novel mode of inhibition of cholesterol biosynthesis. *J. Am. Chem. Soc.* *85*, 3309.

Edwards, P.A. (1973). Effect of adrenalectomy and hypophysectomy on the circadian rhythm of β-hydroxy-β-methylglutaryl coenzyme A reductase activity in rat liver. *J. Biol. Chem.* *248*, 2912-2917.

Edwards, P.A. (1975). Effect of plasma lipoproteins and lecithin-cholesterol dispersions on the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase of isolated rat hepatocytes. *Biochim. Biophys. Acta* *409*, 39-50.

Edwards, P.A., and R.G. Gould (1972). Turnover rate of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase as determined by use of cycloheximide. *J. Biol. Chem.* *247*, 1520-1524.

Edwards, P.A., and R.G. Gould (1974). Dependence of the circadian rhythm of hepatic β-hydroxy-β-methylglutaryl coenzyme A on ribonucleic acid synthesis. *J. Biol. Chem.* *249*, 2891-2896.

Erickson, S.K., and R.G. Gould (1974). Correlation of chromatin bound free and esterified cholesterol with HMG-CoA reductase activity in rat liver. *Fed. Proc. 33*, 1549.

Exton, J.H., G.A. Robison, E.W. Sutherland, and C.R. Park (1971). Studies on the role of adenosine 3',5'-monophosphate in the hepatic actions of glucagon and catecholamines. *J. Biol. Chem. 246*, 6166-6177.

Farias, R.N., B. Bloj, R.D. Morero, F. Sineriz, and R.E. Trucco (1975). Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. *Biochim. Biophys. Acta 415*, 231-251.

Fimognari, G., and V.W. Rodwell (1965). Cholesterol biosynthesis: mevalonate synthesis inhibited by bile salts. *Science 147*, 1038.

Fletcher, K., and N.B. Myant (1958). Influence of the thyroid on the synthesis of cholesterol by liver and skin *in vitro*. *J. Physiol. 144*, 361-372.

Foster, D.W., and P.A. Srere (1968). Citrate cleavage enzyme and fatty acid synthesis. *J. Biol. Chem. 243*, 1926-1930.

Frantz, I.D., H.S. Schneider, and B.T. Hinkelman (1954). Suppression of hepatic cholesterol synthesis in the rat by cholesterol feeding. *J. Biol. Chem. 206*, 465-469.

Fregly, M.J. (1960). Production of hypertension in adrenalectomized rats given hypertonic salt solution to drink. *Endocrinology 66*, 240-254.

Gardner, J.A., and P.E. Landner (1914). The origin and destiny of cholesterol in the animal organism. Part XI - The cholesterol content of growing chickens under different diets. *Proc. Roy. Soc. Lond. B 87*, 229-236.

Gaylor, J.L. (1974). "Enzymes of sterol biosynthesis" in Biochemistry of Lipids, ed. T.W. Goodwin (Butterworths, Lond.). (MTP International Reviews of Science *4*, 1-37).

Goldfarb, S., and H.C. Pitot (1971). Improved assay of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res. 12*, 512-515.

Goldstein, J.L., S.E. Dana, and M.S. Brown (1974). Esterification of low density lipoprotein cholesterol in human fibroblasts and its absence in homozygous familial hypercholesterolemia. *Proc. Nat. Acad. Sci. U.S.A. 71*, 4288-4292.

Goodman, Z.D., and V.S. Lequire (1975). Transfer of esterified cholesterol from serum lipoproteins to the liver. *Biochim. Biophys. Acta 398*, 325-336.

Goodwin, C.D., and S. Margolis (1976). Improved methods for the study of hepatic HMG CoA reductase: one-step isolation of mevalonolactone and rapid preparation of endoplasmic reticulum. *J. Lipid Res. 17*, 297-303.

Gould, R.G. (1951). Lipid metabolism and atherosclerosis. *Amer. J. Med. 11*, 209-227.

Gould, R.G., and E.A. Swyryd (1966). Sites of control of hepatic cholesterol biosynthesis. *J. Lipid Res. 7*, 698-707.

Gould, R.G., V.B. Kojola, and E.A. Swyryd (1970). Effects of hypophysectomy, adrenalectomy, cholesterol feeding, and puromycin on the radiation-induced increase in hepatic cholesterol biosynthesis in rats. *Radiat. Res. 41*, 57-69.

Guder, W., I. Nolte, and O. Wieland (1968). The influence of thyroid hormones on β-hydroxy-β-methylglutaryl coenzyme A reductase of rat liver. *Eur. J. Biochem. 4*, 273-278.

Hamprecht, B., C. Nüssler, and F. Lynen (1969). Rhythmic changes of hydroxymethylglutaryl coenzyme A reductase activity in livers of fed and fasted rats. *FEBS Lett. 4*, 117-121.

Hamprecht, B., R. Roscher, G. Waltinger, and C. Nüssler (1971). Influence of bile acids on the activity of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase. 2. Effect of cholic acid in lymph fistula rats. *Eur. J. Biochem. 18*, 15-19.

Harry, D.S., M. Dini, and N. McIntyre (1973). Effect of cholesterol feeding and biliary obstruction on hepatic cholesterol biosynthesis in the rat. *Biochim. Biophys. Acta 296*, 209-220.

Heller, R.A., and R.G. Gould (1973). Solubilization and partial purification of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys. Res. Comm. 50*, 859-865.

Heller, R.A., and R.G. Gould (1974). Reversible cold inactivation of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase from rat liver. *J. Biol. Chem. 249*, 5254-5260.

Hickman, P.E., B.J. Horton, and J.R. Sabine (1972). Effect of adrenalectomy on the diurnal variation of hepatic cholesterogenesis in the rat. *J. Lipid Res. 13*, 17-22.

Higgins, M., and H. Rudney (1973). Regulation of rat liver β-hydroxy-β-methylglutaryl-CoA reductase activity by cholesterol. *Nature New Biol. 246*, 60-61.

Higgins, M., T. Kawachi, and H. Rudney (1971). The mechanism of the diurnal variation of hepatic HMG-CoA reductase activity in the rat. *Biochem. Biophys. Res. Comm. 45*, 138-144.

Higgins, M.J.P., D. Brady, and H. Rudney (1974). Rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase: a comparison and immunological study of purified solubilized preparations, and alteration of enzyme levels by cholestyramine feeding. *Arch. Biochem. Biophys. 163,* 271-282.

Hilz, H., J. Knappe, E. Ringelmann, and F. Lynen (1958). Methylglutaconase, ein neue Hydratase die am Stoffwechsel verzweigter Carbonsäuren beteiligt ist. *Biochem. Z. 329,* 476-489.

Holmquest, D.L., K. Retiene, and H.S. Lipscomb (1966). Circadian rhythms in rats: effects of random lighting. *Science 152,* 662-666.

Huber, J., and B. Hamprecht (1972). Tageszeitlicher Rhythmus der Hydroxymethylglutaryl-CoA-Reduktase in der Rattenleber, I. *Hoppe-Seyler's Z. Physiol. Chem. 353,* 307-312.

Huber, J., B. Hamprecht, O. Müller, and W. Guder (1972). Tageszeitlicher Rhythmus der Hydroxymethylglutaryl-CoA-Reduktase in der Rattenleber, II. *Hoppe-Seyler's Z. Physiol. Chem. 353,* 313-317.

Huber, J., S. Latzin, and B. Hamprecht (1973a). A simple and rapid radiochemical assay for 3-hydroxy-3-methylglutaryl-Coenzyme A reductase. *Hoppe-Seyler's Z. Physiol. Chem. 354,* 1645-1647.

Huber, J., S. Latzin, O. Langguth, B. Brauser, V.P. Gabel, and B. Hamprecht (1973b). The influence of bilateral superior cervical ganglionectomy, continuous light and continuous darkness on the diurnal rhythm of hydroxymethylglutaryl-coenzyme A reductase in rat liver. *FEBS Lett. 31,* 261-265.

Huber, J., W. Guder, O.A. Müller, S. Latzin, H. Ganser, and B. Hamprecht (1974). Influence of hypophysectomy, thyroid hormone and insulin on the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in rat liver. *Hoppe-Seyler's Z. Physiol. Chem. 355,* 669-674.

Huff, J.W., J.L. Gilffillan, and V.M. Hunt (1963). Effect of cholestyramine, a bile acid binding polymer, on plasma cholesterol and fecal bile acid excretion in the rat. *Proc. Soc. Exp. Biol. Med. 114,* 352-355.

Hulcher, F.H., and W.H. Oleson (1973). Simplified spectrophotometric assay for microsomal 3-hydroxy-3-methylglutaryl CoA reductase by measurement of coenzyme A. *J. Lipid Res. 14,* 625-631.

Jakoi, L., and S.H. Quarfordt (1974). The induction of hepatic cholesterol synthesis in the rat by lecithin mesophase infusions. *J. Biol. Chem. 249,* 5840-5844.

Janiak, M.J., C.R. Loomis, G.G. Shipley, and D.M. Small (1974). The ternary phase diagram of lecithin, cholesteryl linoleate and water: phase behaviour and structure. *J. Mol. Biol. 86,* 325-339.

Johnson, F.H., H. Eyring, and M.J. Polissar (1954). "The kinetic basis of molecular biology", John Wiley and Sons, Inc., New York.

Kaul, L., and C.D. Berdanier (1972). Diurnal rhythms of serum insulin and hepatic NADP-linked dehydrogenases in meal-fed rats. *Fed. Proc. 31*, 669.

Kandutsch, A.A., and H.W. Chen (1973). Inhibition of sterol synthesis in cultured mouse cells by 7α-hydroxycholesterol, 7β-hydroxycholesterol, and 7-ketocholesterol. *J. Biol. Chem. 248*, 8408-8417.

Kandutsch, A.A., and H.W. Chen (1974). Inhibition of sterol synthesis in cultured mouse cells by cholesterol derivatives oxygenated in the side chain. *J. Biol. Chem. 249*, 6057-6061.

Kandutsch, A.A., and S.E. Saucier (1969). Prevention of cyclic and triton-induced increases in hydroxymethyl glutaryl coenzyme A reductase and sterol synthesis by puromycin. *J. Biol. Chem. 244*, 2299-2305.

Kawachi, T., and H. Rudney (1970). Solubilization and purification of β-hydroxy-β-methylglutaryl coenzyme A reductase from rat liver. *Biochemistry 9*, 1700-1705.

Kimelberg, H.K., and D. Papahadjopoulos (1974). Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^+ + K^+)-stimulated adenosine triphosphatase. *J. Biol. Chem. 249*, 1071-1080.

Kimura, T.K., T. Maji, and K. Ashida (1970). Periodicity of food intake and lipogenesis in rats subjected to two different feeding plans. *J. Nutrit. 100*, 691-697.

Krebs, H.A., and K. Henseleit (1932). Untersuchungen über die Harnstoffbildung im Tierkörper. *Z. Physiol. Chem. 210*, 33-66.

Kroes, J., R. Ostwald, and A. Keith (1972). Erythrocyte membranes - compression of lipid phases by increased cholesterol content. *Biochim. Biophys. Acta 274*, 71-74.

Lakshmanan, M.R., C.M. Nepokroeff, G.C. Ness, R.E. Dugan, and J.W. Porter (1973). Stimulation by insulin of rat liver β-hydroxy-β-methyl-glutaryl coenzyme A reductase and cholesterol-synthesizing activities. *Biochem. Biophys. Res. Comm. 50*, 704-710.

Lakshmanan, M.R., R.E. Dugan, C.M. Nepokroeff, G.C. Ness, and J.W. Porter (1975). Regulation of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase activity and cholesterol levels of serum and liver in various dietary and hormonal states. *Arch. Biochem. Biophys. 168*, 89-95.

Landner, P.E. (1915). IX. On the cholesterol content of the tissues of growing rats when under various diets. *Biochem. J. 9*, 78-96.

Linn, T.C. (1967a). The demonstration and solubilization of β-hydroxy-β-methylglutaryl coenzyme A reductase from rat liver microsomes. *J. Biol. Chem. 242*, 984-989.

Linn, T.C. (1967b). The effect of cholesterol feeding and fasting upon β-hydroxy-β-methylglutaryl coenzyme A reductase. *J. Biol. Chem. 242*, 990-993.

Loomis, C.R., M.J. Janiak, D.M. Small, and G.G. Shipley (1974). The binary phase diagram of lecithin and cholesteryl linolenate. *J. Mol. Biol. 86*, 309-324.

Louw, A.I., I. Bekersky, and E.H. Mosbach (1969). Improved synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). *J. Lipid Res. 10*, 683-686.

Lowenstein, J.M. (1968). Citrate and the conversion of carbohydrate into fat. *Biochem. Soc. Symp. 27*, 61-86.

Lyon, I., M.S. Masri, and I.L. Chaikoff (1952). Fasting and hepatic lipogenesis from C^{14}-acetate. *J. Biol. Chem. 196*, 25-32.

Lyons, J.M. (1972). Phase transitions and control of cellular metabolism at low temperatures. *Cryobiology 9*, 341-350.

Marsh, J.B., and D.L. Drabkin (1958). Metabolic channeling in experimental nephrosis. V. Lipide metabolism in the early stages of the disease. *J. Biol. Chem. 230*, 1083-1091.

Martin, D.B., and P.R. Vagelos (1962). Mechanism of tricarboxylic acid cycle regulation of fatty acid synthesis. *Biochem. Biophys. Res. Comm. 7*, 101-106.

Mayer, D., and W. Hummel (1976). Cholesterol, as an *in vitro* inhibitor of HMG-CoA reductase in microsomes. *Proc. 19th Internat. Conf. Biochem. Lipids.*

Migicovsky, B.B. (1964). Further studies with the mitochondrial inhibitor of cholesterol synthesis. *Canad. J. Biochem. 42*, 105-110.

Miziorko, H.M., K.D. Clinkenbeard, W.D. Reed, and M.D. Lane (1975). 3-Hydroxy-3-methylglutaryl Coenzyme A synthase. *J. Biol. Chem. 250*, 5768-5773.

Murphy, B.E.P., W. Engelberg, and C.J. Pattee (1963). Simple method for the determination of plasma corticoids. *J. Clin. Endocr. Metab. 23*, 293-300.

Myant, N.B., and H.A. Eder (1961). The effect of biliary drainage upon the synthesis of cholesterol in the liver. *J. Lipid Res. 2*, 363-368.

McNamara, D.J., and V.W. Rodwell (1972). Regulation of "Active Isoprene" biosynthesis. In Biochemical Regulatory Mechanisms in Eukaryotic Cells. E. Kun and S. Grisolia, eds. J. Wiley & Sons.

Nair, V., and R. Casper (1969). The influence of light on daily rhythm in hepatic drug metabolizing enzymes in rat. *Life Sci. 8*, 1291-1298.

Nejad, N.S., and I.L. Chaikoff (1964). Effect of glucocorticoids and L-thyroxine on acetate carbon incorporation into cholesterol by livers of hypophysectomized rats. *Endocrinology 75*, 396-400.

Nepokroeff, C.M., M.R. Lakshmanan, G.C. Ness, R.E. Dugan, and J.W. Porter (1974). Regulation of the diurnal rhythm of rat liver β-hydroxy-β-methylglutaryl coenzyme A reductase activity by insulin, glucagon, cyclic AMP and hydrocortisone. *Arch. Biochem. Biophys. 160*, 387-393.

Nervi, F.O., H.J. Weis, and J.M. Dietschy (1975). The kinetic characteristics of inhibition of hepatic cholesterogenesis by lipoproteins of intestinal origin. *J. Biol. Chem. 250*, 4145-4151.

Ness, G.C., R.E. Dugan, M.R. Lakshmanan, C.M. Nepokroeff, and J.W. Porter (1973). Stimulation of hepatic β-hydroxy-β-methylglutaryl coenzyme A reductase activity in hypophysectomized rats by L-triiodothyronine. *Proc. Nat. Acad. Sci. U.S.A. 70*, 3839-3842.

Ogilvie, J.W., and B.H. Kaplan (1966). The inhibition of sterol biosynthesis in rat liver homogenates by bile. *J. Biol. Chem. 241*, 4722-4730.

Penefsky, H.S., and A. Tzagoloff (1971). Extraction of water-soluble enzymes and proteins from membranes. *Methods Enzymol. 22*, 205-219.

Perry, W.F., and H.F. Bowen (1956). Effect of adrenalectomy on the incorporation of acetate into fatty acids, cholesterol and acetoacetic acid by rat liver slices. *Am. J. Physiol. 184*, 59-62.

Popják, G. (1970). Conversion of mevalonic acid into prenyl hydrocarbons as exemplified by the synthesis of squalene. *Biochem. Soc. Symp. 29*, 17-33.

Popják, G., and J.W. Cornforth (1960). The biosynthesis of cholesterol. *Adv. Enzymol. 22*, 281-335.

Prończuk, A., and L.C. Fillios (1968). Changes in cholesterol concentrations in rough and smooth endoplasmic reticulum and polysomal profiles in rats fed cholesterol. *J. Nutrit. 96*, 46-52.

Raison, J.K. (1973a). Temperature-induced phase changes in membrane lipids and their influence on metabolic regulation. *Soc. Exp. Biol. Symp. XXVII*, 485-512.

Raison, J.K. (1973b). The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated systems. *J. Bioener. 4*, 357-381.

Raison, J.K., and E.J. McMurchie (1974). Two temperature-induced changes in mitochondrial membranes detected by spin labelling and enzyme kinetics. *Biochim. Biophys. Acta 363*, 135-140.

Raison, J.K., J.M. Lyons, R.J. Mehlhorn, and A.D. Keith (1971). Temperature-induced phase changes in mitochondrial membranes detected by spin labelling. *J. Biol. Chem. 246*, 4036-4040.

Rall, J.W., and E.W. Sutherland (1958). Formation of a cyclic adenine ribonucleotide by tissue particles. *J. Biol. Chem. 232*, 1065-1076.

Ramasarma, T. (1972). Control of biogenesis of isoprenoid compounds in animals. *Curr. Top. Cell Reg. 6*, 169-207.

Rao, G.S., and T. Ramasarma (1972). ATP in the regulation of cholesterol biosynthesis - a supra-energetic role. *Biochem. Biophys. Res. Comm. 49*, 225-229.

Raskin, P., J.D. McGarry, and D.W. Foster (1974). Independence of cholesterol and fatty acid biosynthesis from cyclic adenosine monophosphate concentration in perfused rat liver. *J. Biol. Chem. 249*, 6029-6032.

Regen, D., C. Riepertinger, B. Hamprecht, and F. Lynen (1966). The measurement of β-Hydroxy-β-methyl-glutaryl-CoA reductase in rat liver; effects of fasting and refeeding. *Biochem. Z. 346*, 78-84.

Ritter, M.C., and M.E. Dempsey (1973). Squalene and sterol carrier protein: structural properties, lipid-binding, and function in cholesterol biosynthesis. *Proc. Nat. Acad. Sci. U.S.A. 70*, 265-269.

Ritter, M.C., M.E. Dempsey, and I.D. Frantz Jr. (1972). Control of pathways of cholesterol biosynthesis is by a squalene and sterol carrier protein (SCP). *Fed. Proc. 31*, 430.

Robison, G.A., R.W. Butcher, and E.W. Sutherland (1971). "Cyclic AMP" (Academic Press, New York).

Rodwell, V.W. (1972). Cholesterol regulation of hepatic HMG-CoA reductase. *Biochem. Soc. Symp. 35*, 295-301.

Rodwell, V.W., D.J. McNamara, and D.J. Shapiro (1973). Regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Adv. Enzymol. 38*, 373-412.

Sabine, J.R. (1977). "Cholesterol" (Marcel-Dekker, New York).

Sabine, J.R., S. Abraham, and I.L. Chaikoff (1967). Control of lipid metabolism in hepatomas: insensitivity of rate of fatty acid and cholesterol synthesis by mouse hepatoma BW7756 to fasting and to feedback control. *Cancer Res. 27*, 793-799.

Sabine, J.R., B.J. Horton, and P.E. Hickman (1972). Control of cholesterol synthesis in hepatomas: absence of diurnal rhythm in hepatomas 7794A and 9618A. *Eur. J. Cancer 8*, 29-32.

Sakakida, H., C.C. Shediac, and M.D. Siperstein (1963). Effect of endogenous and exogenous cholesterol on the feedback control of cholesterol synthesis. *J. Clin. Invest. 42*, 1521-1528.

Scallen, T.J., M.W. Schuster, and A.K. Dhar (1971). Evidence for a noncatalytic carrier protein in cholesterol biosynthesis. *J. Biol. Chem. 246*, 224-230.

Scheraga, H.A., G.N. Némethy, and I.Z. Steinberg (1962). The contribution of hydrophobic bonds to the thermal stability of protein conformations. *J. Biol. Chem. 237*, 2506-2508.

Seelig, J., and W. Hasselbach (1971). A spin label study of sarcoplasmic vesicles. *Eur. J. Biochem. 21*, 17-21.

Shapiro, D.J., and V.W. Rodwell (1969). Diurnal variation and cholesterol regulation of hepatic HMG-CoA reductase activity. *Biochem. Biophys. Res. Comm. 37*, 867-872.

Shapiro, D.J., and V.W. Rodwell (1971). Regulation of hepatic 3-hydroxy-3-methyl glutaryl coenzyme A reductase and cholesterol synthesis. *J. Biol. Chem. 246*, 3210-3216.

Shapiro, D.J., and V.W. Rodwell (1972). Fine structure of the cyclic rhythm of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Differential effects of cholesterol feeding and fasting. *Biochemistry 11*, 1042-1045.

Shapiro, D.J., R.L. Imblum, and V.W. Rodwell (1969). Thin layer chromatographic assay for HMG-CoA reductase and mevalonic acid. *Anal. Biochem. 31*, 383-390.

Shefer, S., S. Hauser, V. Lapar, and E.H. Mosbach (1973). Regulatory effects of sterols and bile acids on hepatic 3-hydroxy-3-methyl-glutaryl CoA reductase and cholesterol 7α-hydroxylase in the rat. *J. Lipid Res. 14*, 573-580.

Siperstein, M.D. (1960). The homeostatic control of cholesterol synthesis in liver. *Amer. J. Clin. Nutr. 8*, 645-650.

Siperstein, M.D. (1965). "Developmental and metabolic control mechanisms and neoplasia." pp. 427-451. Williams & Wilkins, Baltimore, Maryland, 1965.

Siperstein, M.D. (1966). Feedback control of cholesterol synthesis in normal, malignant and premalignant tissues. *Proc. Can. Cancer Res. Conf. 7*, 152-162.

Siperstein, M.D. (1970). Regulation of cholesterol biosynthesis in normal and malignant tissues. *Curr. Top. Cell Reg. 2,* 65-100.

Siperstein, M.D., and V.M. Fagan (1964). Studies on the feedback regulation of cholesterol synthesis. *Adv. Enz. Reg. 2,* 249-264.

Siperstein, M.D., and V.M. Fagan (1966). Feedback control of mevalonate synthesis by dietary cholesterol. *J. Biol. Chem. 241,* 602-609.

Siperstein, M.D., and M.J. Guest (1959). Studies on the homeostatic control of cholesterol synthesis. *J. Clin. Invest. 38,* 1043.

Siperstein, M.D., and M.J. Guest (1960). Studies on the site of the feedback control of cholesterol synthesis. *J. Clin. Invest. 39,* 642-652.

Siperstein, M.D., V.M. Fagan, and J.M. Dietschy (1966). A gas-liquid chromatographic procedure for the measurement of mevalonic acid synthesis. *J. Biol. Chem. 241,* 597-601.

Slakey, L.L., M.C. Craig, E. Beytia, A. Briedis, D.H. Feldbruegge, R.E. Dugan, A.A. Qureshi, C. Subbarayan, and J.W. Porter (1972). The effects of fasting, refeeding and time of day on the levels of enzymes effecting the conversion of β-hydroxy-β-methylglutaryl coenzyme A to squalene. *J. Biol. Chem. 247,* 3014-3022.

Spencer, A., L. Corman, and J.M. Lowenstein (1964). Citrate and the conversion of carbohydrate into fat. *Biochem. J. 93,* 378-388.

Steinberg, D., and J. Avigan (1960). Studies of cholesterol biosynthesis. II. The role of desmosterol in the biosynthesis of cholesterol. *J. Biol. Chem. 235,* 3127-3129.

Tomkins, G.M., and I.L. Chaikoff (1952). Cholesterol synthesis by liver. I. Influence of fasting and of diet. *J. Biol. Chem. 196,* 569-573.

Tormanen, C.D., W.L. Redd, M.V. Srikantaiah, and T.J. Scallen (1976). Purification of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Biochem. Biophys. Res. Comm. 68,* 754-762.

Verkleij, A.J., P.H.J.Th. Ververgaert, B. de Kruyff, and L.L.M. Van Deenen (1974). The distribution of cholesterol in bilayers of phosphatidylcholines as visualized by freeze fracturing. *Biochim. Biophys. Acta 373,* 495-501.

Weis, H.J., and J.M. Dietschy (1969). Failure of bile acids to control hepatic cholesterogenesis: evidence for endogenous cholesterol feedback. *J. Clin. Invest. 48,* 2398-2408.

White, L.W., and H. Rudney (1970). Regulation of 3-hydroxy-3-methylglutarate and mevalonate biosynthesis by rat liver homogenates. Effects of fasting, cholesterol feeding, and Triton administration. *Biochemistry 9,* 2725-2731.

Willmer, J.S., and T.S. Foster (1960). The influence of adrenalectomy and individual steroid hormones upon the metabolism of acetate-1-^{14}C by rat liver slices. II. Incorporation into cholesterol. *Can. J. Biochem. Physiol. 38,* 1393-1397.

Wurtman, R.J., J. Axelrod, and L.S. Phillips (1963). Melatonin synthesis in the pineal gland: control by light. *Science 142,* 1071-1073.

Yamamoto, S., and K. Bloch (1970). Enzymatic studies on the oxidative cyclizations of squalene. *Biochem. Soc. Symp. 29,* 35-43.

Zenker, N., and D.E. Bernstein (1958). The estimation of small amounts of corticosterone in rat plasma. *J. Biol. Chem. 231,* 695-701.