THE REGULATION OF HEPATIC 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE

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by

R.A. Easom, B.Sc.

Hannah Research Institute
University of Glasgow
Glasgow

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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(ii) Abbreviations

ACAT	Acyl CoA; cholesterol acyltransferase
ADP	Adenosine - 5′-diphosphate
A.I.S.	Anti-insulin serum
AMP	Adenosine - 5′-monophosphate
ATP	Adenosine - 5′-triphosphate
BSA	Bovine serum albumin
CoA (CoA-SH)	Coenzyme A
Chol 7 -OHase	Cholesterol 7 -hydroxylase
CTP	Cytidine -5′-triphosphate
DCCD	Dicyclohexylcarbodiimide
DEAE	Diethylamino ethyl
DNA	Deoxyribonucleic acid
DTNB	5,5′-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethyleneglycol-bis-(aminoethyl ether)-N-N′-tetra-acetic
GSH	Glutathione (Reduced form)
GSSG	Glutathione (Oxidized form)
G6P	Glucose 6-phosphate
G6PDH	Glucose 6-phosphate dehydrogenase
HDL	High-density lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HRI	Hannah Research Institute
IDL	Intermediate-density lipoprotein
ITP	Inosine-5′-triphosphate
LDL	Low-density lipoprotein
M_r	Molecular weight
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
PAGE	Polyacrylamide gel electrophoresis
PCA	Perchloro-acetic acid
PEP	Phosphoenol pyruvate
PMSF	Phenylmethanesulphonyl fluoride
R_f	
RNA	Ribonucleic acid
mRNA	Messenger RNA
tRNA	Transfer RNA
SDS	Sodium dodecyl sulphate
T_3	Triiodothyronine
TAT	Tyrosine aminotransferase
t.l.c.	Thin-layer chromatography
TRA	Triethanolamine
TRIS	Tris-(hydroxymethyl)-aminomethane
UTP	Uridine-5′-triphosphate
VLDL	Very-low-density lipoprotein

(iii) SUMMARY

The aim of the work presented in this thesis was to assess the physiological significance and role of reversible phosphorylation-dephosphorylation in the short-term regulation of the activity of hepatic HMG-CoA reductase in vivo. Such a mechanism has been well characterized for this enzyme in vitro (see Chapter 1) but, until the present work no demonstration has been made of the influence of this system on the regulation of the enzyme in vivo in response to normal physiological stimuli (see Chapter 1 and Chapter 3). It seemed unlikely that such a complex mechanism of covalent modification would go unused in vivo. The previous work on whole animals was, therefore, reassessed. Particular care was taken in the sampling and homogenization of the tissue to preserve the phosphorylation status of the enzyme that exists in the tissue at the moment of sampling.

Chapter 3 describes a technique, the cold-clamping technique, designed especially for this study. Not only did this technique meet the criterion for the rapid processing of liver tissue (see above) but it also allowed the physical removal of a HMG-CoA lyase activity (which interferes with the assay of HMG-CoA reductase) in whole mitochondria by differential centrifugation of the homogenates. The suitability of this technique for the study of short-term changes in HMG-CoA reductase activity was validated by directly comparing its efficiency in preserving the phosphorylation state of pyruvate kinase, previously established to undergo rapid interconversion between active and inactive forms, with the widely used freeze-clamping technique and the conventional method of mincing tissue prior to homogenization.

Chapter 4 describes the use of the cold-clamping technique to quantitate changes in the fraction of the enzyme in the active, dephosphorylated form in a number of physiological conditions characterized by acute hormonal and/or nutritional changes. It was demonstrated that this parameter of HMG-CoA reductase showed a marked diurnal rhythm; its value was rapidly reduced under conditions of food deprivation for periods ranging from 4 to 24h. It also varied widely in rats during the transition between the gravid and lactating states. These changes appeared to occur independently of large changes in the total enzyme activity, (an indication of the amount of enzyme protein) suggesting that each parameter was controlled either by different hormones by a differential response to the same hormonal or nutritional factor. Significantly, the change in the phosphorylation status of the enzyme in each condition appeared to be the primary mechanism through which the expressed activity (the closest estimate of the actual activity in vivo of HMG-CoA reductase was rapidly modulated in co-ordination with the immediate physiological significance of the animal (see Section 4.5).

In the final two chapters experiments are described which were designed to assess the importance of hormonal (Chapter 5) and/or nutritional (Chapter 6) factors in the short-term regulation of HMG-CoA reductase by reversible phosphorylation in vivo. A significant observation from Chapter 4 indicated that the changes in the fraction of enzyme in the active form in each physiological condition (except lactation) closely reflected the expected changes in the plasma insulin concentration in the animal. In Chapter 5, this suggestion was supported by, for example, the acute and chronic

changes observed in this parameter in animals treated with anit-insulin serum and in severely insulin-dependent diabetic animals respectively. Further investigation into the role of hormones having antagonistic effects to insulin on liver metabolism (e.g. glucagon and epinephrine) revealed that insulin had a dominant role in the regulation of the phosphorylation state of HMG-CoA reductase *in vivo*. The observations are discussed in the light of the response of this parameter to different physiological conditions, in the light of the current knowledge of the regulation of cellular kinases and phosphatases for which HMG-CoA reductase enzyme is a substrate.

In Chapter 6, the previously published effects of mevalonolactone on the phosphorylation state of HMG-CoA reductase both *in vivo* and *in vitro* were confirmed using the cold-clamping technique on the livers of animals intubated with mevalonolactone. Most significantly, however, this treatment produced a reversal of the dominant effect of insulin during the early hours of the dark period. The mechanism by which this could be achieved is discussed. This chapter also describes the apparently little effects of various dried milk fractions (with the exception of buttermilk) administered to animals by stomach intubation on the phosphorylation state of HMG-CoA reductase.

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CHAPTER 1

INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase; (E.C. 1.1.1.34) catalyzes a major regulatory step in cellular cholesterol synthesis (see e.g. Rodwell et al., 1976; Fig. 1.1) as well as the synthesis of other compounds essential for cell proliferation and maintenance. Under most physiological conditions the activity of HMG-CoA reductase determines the flux through the cholesterogenic pathway (Fig. 1.1) from cytosolic acetyl-CoA (Shapiro & Rodwell, 1971; Brown et al., 1979). The enzyme catalyzes the reduction of HMG-CoA (formed from acetyl-CoA by the successive actions of acetoacetyl-CoA thiolase (E.C. 2.3.1.9) and HMG-CoA synthase (E.C. 4.1.3.5) in the cytosol) to mevalonate (Fig. 1.2). The latter is known to serve at least two functions in cell proliferation and maintenance: (i) It is a precursor of cholesterol which is primarily required for membranogenesis (see Section 1.1) and (ii) it is the precursor of a number of non-sterol products required for cell growth (for reviews see Brown & Goldstein, 1980; Siperstein, 1984). These products include dolichol (which is required for the synthesis of many cell surface and secreted glycoproteins), ubiquinone (a component of the mitochondrial electron transport chain) and isopentenyladenine (which subsequently forms an important component of tRNA) (see Fears, 1981; Fig.1.1). Furthermore, recent evidence suggests that mevalonate serves as a rapid and essential initiator of DNA replication during the S-phase of the cell cycle

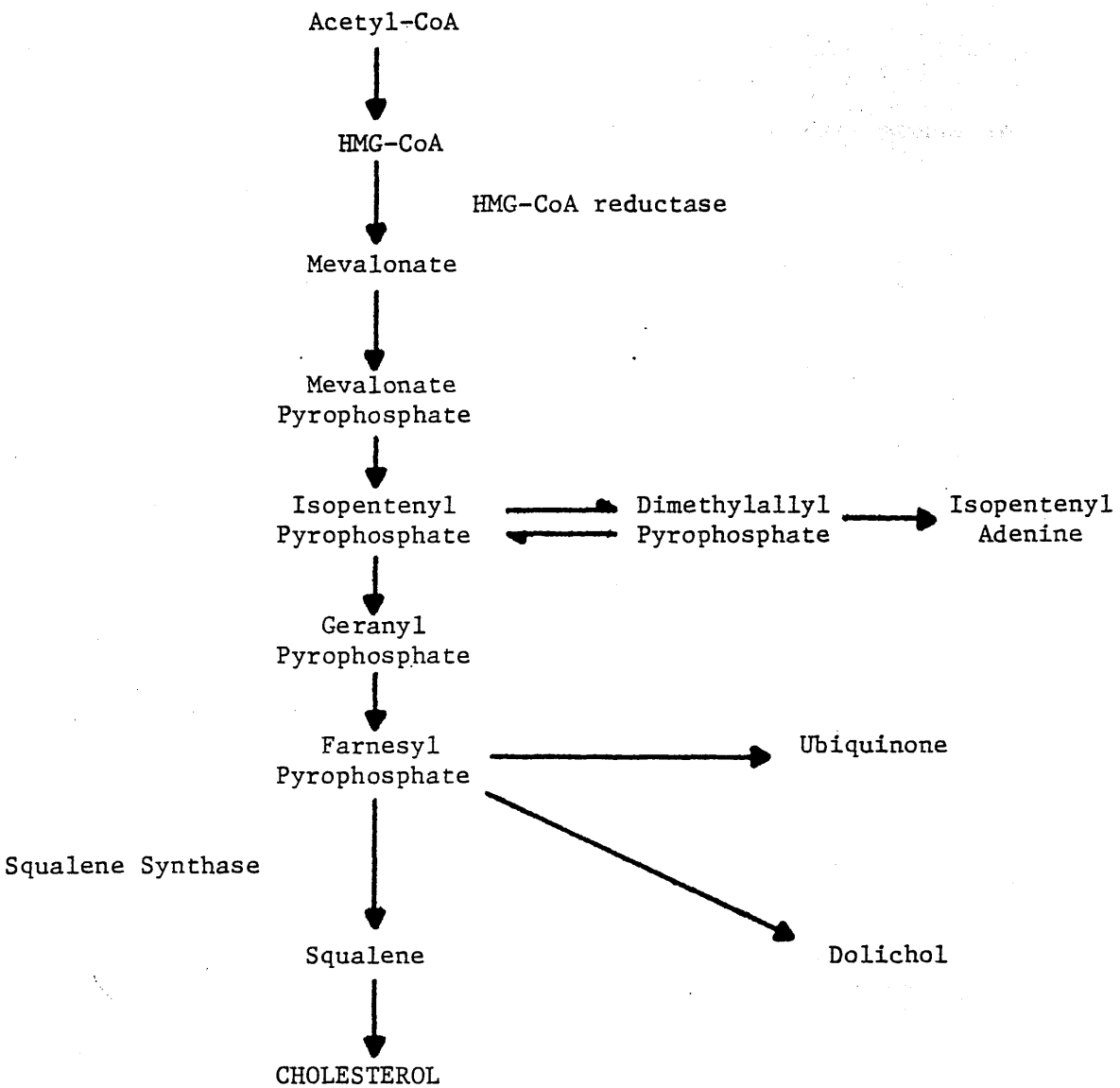


Fig. 1.1 Biosynthetic pathway of cholesterogenesis from acetyl-CoA

Figure also demonstrates the branched pathway of mevalonate metabolism.

(Redrawn from Brown & Goldstein, 1980)

possibly through the formation of isopentenyl adenine (Fig. 1.3); for a review see Siperstein (1984).

1.1 Overview of Cholesterol Homeostasis

Cholesterol (see Fig. 1.1) is the most abundant sterol in vertebrates and is essential for body function (Myant, 1981). It is a major component of cell membranes where it plays an important role in their structural and functional integrity (Chen, 1984; Sabine, 1977, 1983). Thus every cell has a universal requirement for cholesterol and this is reflected by the fact that every mammalian cell-type investigated, except for the enuclear erythrocyte, has the capacity to synthesize cholesterol (Sabine, 1977).

Cholesterol is also the major precursor for the biosynthesis of steroid hormones in steroidogenic tissues (Gwynne & Strauss, 1982), bile acids (Myant, 1981) and vitamin D (Lehninger, 1982) in the liver. In addition, cholesterol is a major constituent of the plasma lipoproteins.

1.1.2 The Role of Liver in the Control of Body Cholesterol Homeostasis

In the rat, approximately 50% of the total body sterol synthesis, (determined by the rate of $[^3H]$-H_2O incorporation into digitonin-precipitable sterols, see Jeske & Dietschy, 1980) occurs in the liver. The majority of the outstanding synthesis occurs in the intestine, skin and carcass (primarily striated muscle and bone marrow) (Turley et al., 1981). This situation is, however, apparently unique to the rat since similar analyses conducted in

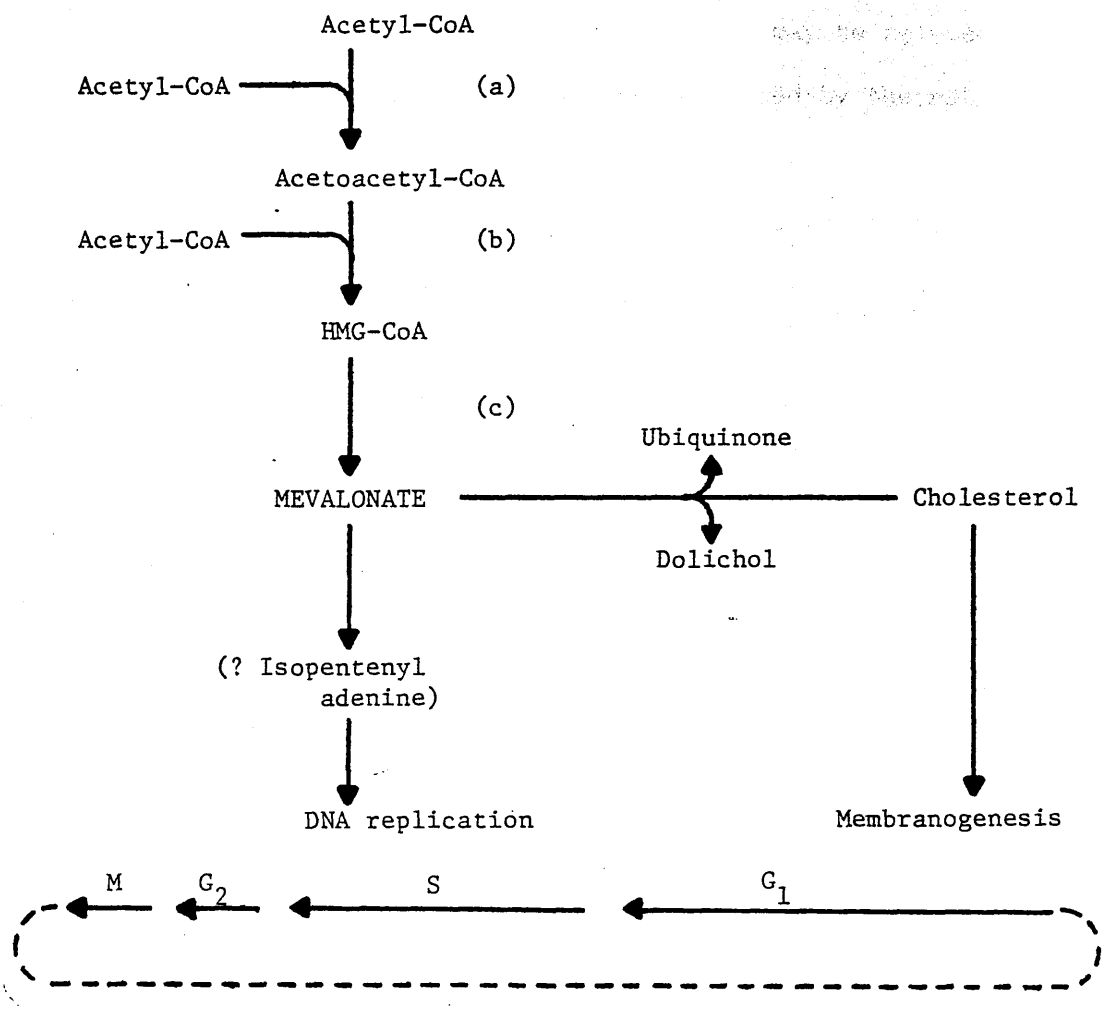


Fig. 1.2 Relation of HMG-CoA reductase to the pathways of formation of intermediates required for cell growth and proliferation

(Redrawn from Siperstein, 1984)

other animal species reveal that, in general, the liver contributes less than 50% and, in some cases i.e. in rabbit and guinea-pig, only 16-18% to the total body sterol synthesis (Spady et al., 1983). This predominance of hepatic synthesis in the rat may be related to the large absolute amount of cholesterol synthesized by the rat (100mg/day per kg body wt.; Turley & Dietschy, 1982). For example, it is possible that in man (where total body synthesis amounts only to 9mg of cholesterol/day per kg body wt.; (Turley & Dietschy, 1982) the liver may account for a lower percentage contribution to the total body sterol formation (see Dietschy, 1984).

The important functions served by cell cholesterol necessitate that the amount and distribution of the body (and cellular) pools of cholesterol are strictly controlled (Spector et al., 1979). Since mammals do not possess enzymes that are capable of degrading the sterol nucleus, the rate at which the body acquires cholesterol (from the diet or from de novo synthesis) must be balanced by the rate at which the body can excrete the sterol molecule (through the formation of bile or the excretion of steroids in the urine) (see Turley & Dietschy, 1982; Dietschy, 1984). This concept is schematically represented in Fig. 1.3.

This critically important balance of cholesterol across the body is maintained by an elaborate series of regulatory and transport mechanisms in which the liver plays a central role (Fig. 1.4). Cholesterol is absorbed from the diet by the epithelial cells of the small intestine, a process facilitated by the detergent action of bile salts secreted into the intestine (Westergaard & Dietschy, 1976; Thomson & Dietschy, 1981). The cholesterol thus absorbed and that

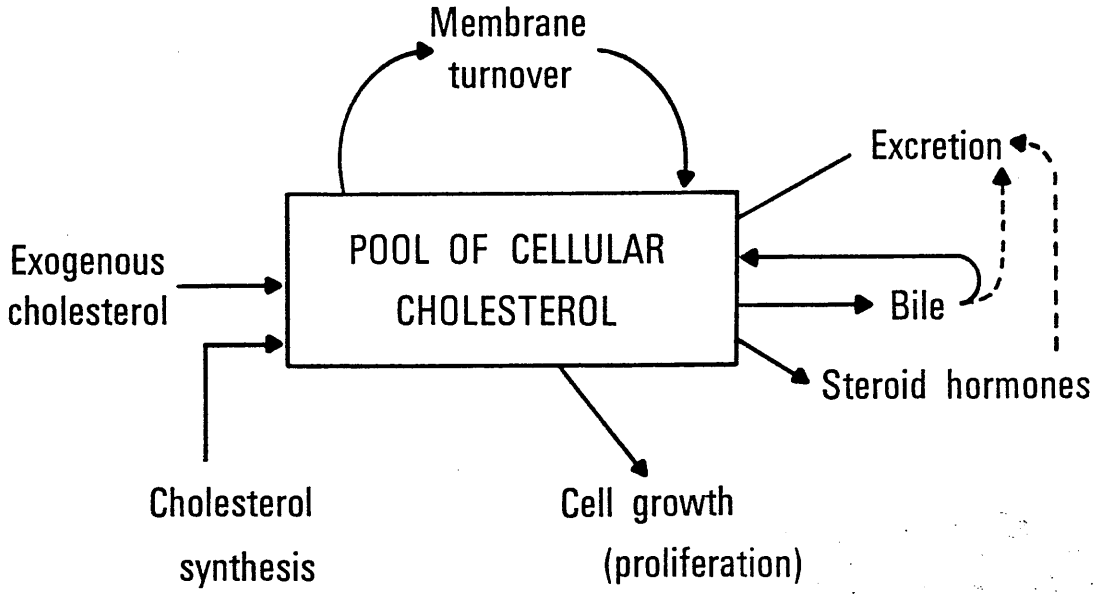


Fig. 1.3 The principal determinants of cholesterol homeostasis
The body pool of cholesterol remains relatively constant in the adult animal. The rate of cholesterol excretion through the skin and gastrointestinal tract ("excretion") and conversion to bile acids and steroid hormones must equal the rate of cholesterol acquisition. (----) signifies eventual excretion.

synthesized de novo from acetyl-CoA in these cells is incorporated into chylomicrons (Fig. 1.4). These particles are secreted into the lymphatic system and thence into the peripheral bloodstream. Here they are depleted of triglyceride by the action of lipoprotein lipase situated on the endothelial surface of the capillaries of peripheral tissue like adipose tissue and muscle (Fielding, 1978; Cryer, 1981). The residual chylomicron remnants which still contain nearly all the absorbed cholesterol (in both free and esterified forms) are cleared almost quantitatively by the liver via receptor-mediated endocytosis after binding by specific receptors (Hui et al., 1981; Kita et al., 1982; Cooper et al., 1982). The liver also receives cholesterol from remnants of very-low-density-lipoprotein (VLDL) (i.e. intermediate-density-lipoprotein (IDL) and low-density-lipoprotein (LDL) (Goldstein et al., 1983) and from high-density lipoprotein (HDL) (Mahley, 1983; see Fig. 1.4). However, the cholesterol in these particles is primarily of endogenous origin.

The liver secretes cholesterol- and triglyceride-rich VLDL into the plasma. These particles are also metabolized in peripheral muscle and adipose tissues by lipoprotein lipase and the resultant cholesterol-rich particle (IDL) binds with high affinity to hepatic LDL-receptors. These particles are rapidly cleared from the plasma by this route (Eisenberg & Levy, 1975). The LDL particles, which have a high cholesterol/apoprotein ratio, are then cleared from the plasma relatively slowly by binding to the high-affinity LDL-receptors in both the liver and peripheral tissues (Pittman et al., 1979; Kita et al., 1982; Brown & Goldstein, 1983). The accumulation of cholesterol within the peripheral tissues is

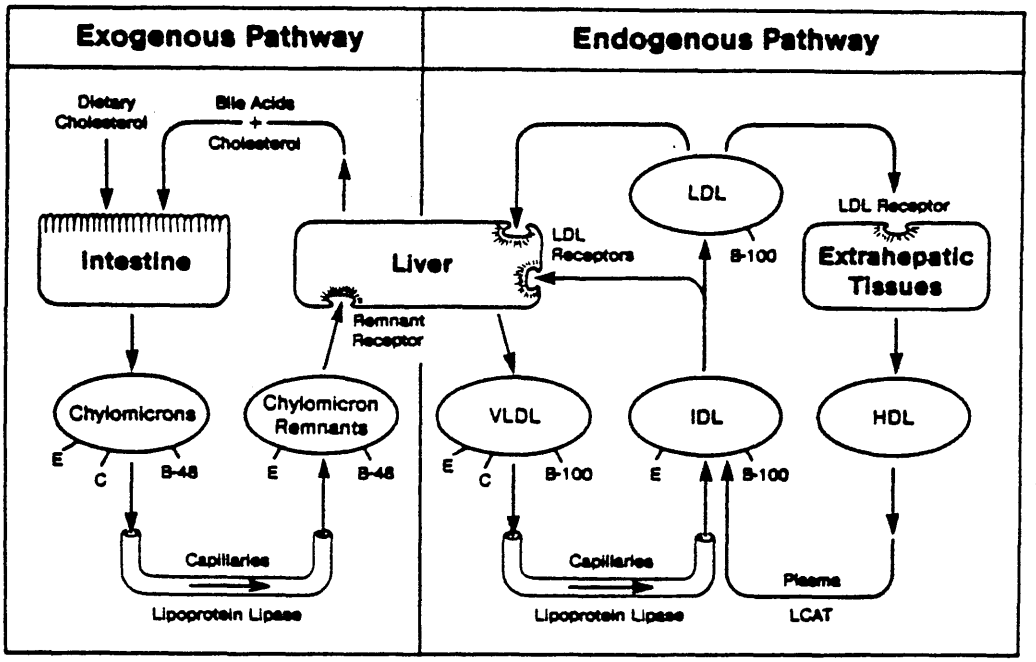


Fig. 1.4 Separate pathways for receptor-mediated Metabolism of lipoproteins carrying endogenous and exogenous cholesterol.

The distinction between exogenous and endogenous cholesterol applies to the immediate source of the cholesterol in the plasma lipoproteins. The role of each lipoprotein in cholesterol homeostasis is discussed in the text. The 'labels' to the lipoproteins in the diagram signify the prominent apoproteins associated with the binding of that lipoprotein with its receptors.

VLDL: Very Low Density Lipoprotein
LDL: Low Density Lipoprotein
HDL: High Density Lipoprotein
LCAT: Lecithin Cholesterol Acyltransferase

(from: Goldstein et al. 1983)

prevented by its abstraction from these tissues by HDL (Tall & Small, 1979; Nicoll et al., 1979; Daniels et al., 1981; Mahley, 1983) and the cholesterol is then either transferred to IDL via a mechanism involving LCAT (see Fig. 1.4; Owen & McIntyre, 1982) or directly to the liver (Brown et al., 1981; Hui et al., 1981).

In view of the constant delivery of cholesterol to the liver by these various mechanisms, it is necessary that the intracellular concentration of free cholesterol of this organ be carefully controlled. The liver has three ways of maintaining an optimal cholesterol concentration; namely by the co-ordinate regulation of (i) uptake, (ii) excretion/storage and (iii) synthesis.

(i) Uptake: In some species (e.g man, hamster) the rate of uptake of LDL is decreased under conditions of increased dietary cholesterol delivery to the liver. This is achieved by reducing the number of its specific receptors on the surface of the liver (see Goldstein & Brown, 1984). In man (Applebaum-Bowden et al., 1984) or rabbits (Kovanen et al., 1981) this phenomenon results because, contrary to the LDL receptors, chylomicron remnant receptors are not regulated (Brown & Goldstein, 1983; Mahley & Innerarity, 1983). In the rat, however, neither of these receptors appear to be regulated (Wade et al., 1984).

(ii) Excretion/Storage: In all mammalian species the formation of bile acids is the major mechanism for the excretion of cholesterol of both endogenous and exogenous origin from the liver (Myant, 1981). Consistent with this function, dietary cholesterol increases the rate of bile acid synthesis which is achieved through the activation of the first enzyme in the pathway, cholesterol

7α-hydroxylase, (Chol 7α-OHase; E.C. 1.14.13.17; Myant & Mitropoulos, 1977). The main function of bile acids synthesis is to ensure an optimal supply of bile salts for the digestion and absorption of lipid from the small intestine. Consequently, the flux through this pathway is controlled by 'feedback' inhibition by both bile acids synthesized for secretion (Shefer et al., 1969) and bile acids delivered back to the liver after reabsorption from the intestine as part of the enterohepatic cycle (EHC; Grundy, 1978). Dietary cholesterol increases the rate of bile acid synthesis through the activation of Chol 7α-OHase (Myant & Mitropoulos, 1977; Mitropoulos et al., 1978); the increase in bile acid synthesis is also accompanied by an increased secretion of free cholesterol in the bile.

In conditions of increased delivery of cholesterol to the liver, free-cholesterol homeostasis is further maintained through increased formation of cholesterol esters which can be stored without detrimental effects to the cell. Under these conditions the activity of acyl CoA:cholesterol acyltransferase (ACAT; E.C. 2.3.1.26) is stimulated (Spector et al., 1979; Mitropoulos et al., 1978; Erickson et al., 1980).

In view of the common roles of ACAT and Chol 7α-OHase together with HMG-CoA reductase (see below) in cholesterol homeostasis, it would be expected that the enzymes are co-ordinately regulated. It is noteworthy that each of these enzymes is regulated by reversible phosphorylation (see Scallen & Sanghvi, 1983). However, whereas increased phosphorylation of HMG-CoA reductase results in its inhibition (see Section 1.4) phosphorylation of ACAT and Chol

7α-OHase results in their activation. Furthermore, all three enzymes are associated with the endoplasmic reticulum (Scallen & Sanghvi, 1983). This common localization may facilitate the co-ordinate regulation of the enzymes by factors which affect the membrane environment of the enzymes e.g. cholesterol itself.

(iii) Synthesis: Modulation of the rate of cholesterol synthesis is potentially a very complex process because (a) the number of steps involved is large (see Fig. 1.1), (b) the cholesterogenic pathway competes for substrate with fatty acid synthesis (Gibbons et al., 1982) and (c) cholesterol is only one of the several products formed from a common intermediate, farnesyl pyrophosphate (see Section 1.1; Fig. 1.1). However, one enzyme in particular, HMG-CoA reductase, is thought to be most closely linked to the regulation of cholesterol synthesis. Thus under a wide range of physiological conditions varying from fasting to cholestyramine feeding, the large (approximately 50-fold) changes in the rate of cholesterogenesis are closely paralleled by changes in HMG-CoA reductase activity (Shapiro & Rodwell, 1971; Brown et al., 1979). However, several groups of workers have suggested that other regulatory sites in the cholesterol pathway may also become important under certain conditions, including cholesterol feeding (White & Rudney, 1970; Clinkenbeard et al., 1975a), glucocorticoid administration (Ramachandran et al., 1978 and fasting (Rodwell et al., 1976). These alternative sites include HMG-CoA synthase (E.C. 4.1.3.5; White & Rudney, 1970; Clinkenbeard et al., 1975b; Ramachandran et al., 1978; Balasubramaniam et al., 1977) and

squalene synthase (E.C. 2.5.1.21; Rodwell et al., 1976; Slakey et al., 1972; Faust et al., 1979).

The work reported in the present thesis was concerned exclusively with the regulation of HMG-CoA reductase activity in vivo. In the following section, a detailed discussion of the mechanisms involved in the regulation of HMG-CoA reductase activity is presented with an emphasis on the involvement of reversible phosphorylation-dephosphorylation.

1.2 Hepatic HMG-CoA Reductase

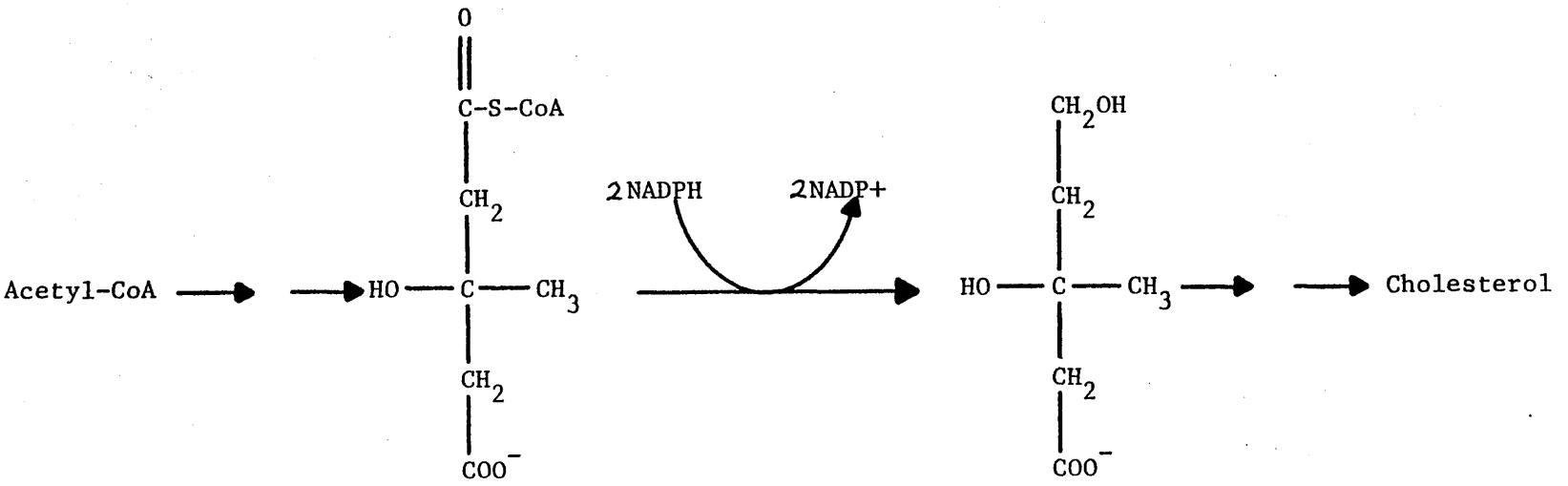
1.2.1 The reaction

HMG-CoA reductase catalyzes the reduction of HMG-CoA (from acetyl-CoA, see Section 1. 2) to mevalonate in a 2-stage mechanism which is essentially irreversible and requires NADPH as co-factor (Fig. 1.5).

When assayed in vitro in microsomal preparations of rat liver, HMG-CoA reductase has a pH optimum of 7.3-7.7 (Shapiro & Rodwell, 1969). The K_m values for HMG-CoA and NADPH have been reported to be within the ranges of 0.5-2.6μM and 30.0-73.0μM respectively (see Rogers et al., 1983).

1.2.2 Topology

The development of a cell line (UT-1) which massively over-produces (100- to 1000-fold) HMG-CoA reductase protein when adapted to grow in the presence of a competitive inhibitor of the enzyme, compactin (Chin et al., 1982a; Luskey et al., 1983), has enabled the topographical study of HMG-CoA reductase. These studies



3-Hydroxy-3-methyl glutaryl-CoA

Mevalonate

Fig. 1.5 The 3-hydroxy-3-methylglutaryl-CoA reductase reaction

have revealed that HMG-CoA reductase is a 97kDa transmembrane glycoprotein (Chin et al., 1982, 1984; Liscum et al., 1983a; Brown & Simoni, 1984) consisting of 887 amino acids (Chin et al., 1984) and comprised of two domains (Liscum et al., 1985; Fig. 1.6). The NH_2-terminal domain (35kDa; 339 amino acids) is extremely hydrophobic and believed to cross the endoplasmic reticular membranes seven times (Liscum et al., 1985). It is glycosylated by a N-linked 'high mannose' type oligosaccharide (Liscum et al., 1983a). This domain is inserted into the membrane co-translationally, although apparently without a hydrophobic signal sequence (Brown & Simoni, 1984; see Goldstein & Brown, 1984). The second hydrophilic domain (62kDa) projects out into the cytoplasm and contains the catalytic site (Liscum et al., 1983b, 1985; Phillips & Ness, 1984; see Fig. 1.6). The reason for this complex structure of HMG-CoA reductase is not known but it is possible that the membrane domain serves as a receptor for LDL-derived cholesterol (see Section 1.1.2 & 1.3.1(i); Liscum et al., 1985). The hydrophilic catalytic domain can be separated from the hydrophobic membrane domain by treatment with a Ca^{2+}-dependent, leupeptin-and calpastatin-sensitive protease calpain-II (Parker et al., 1984; Liscum et al., 1983b; see Fig. 1.6). This treatment yields a membrane-bound (62kDa; termed '62k') and soluble (53kDa; termed '53k') catalytically active fragments (Parker et al., 1984). The '62k' fragment is possibly a precursor of the '53k' soluble fragment although the '53k' species may also be released directly from the native 97kDa enzyme (termed '97k') (Parker et al., 1985).

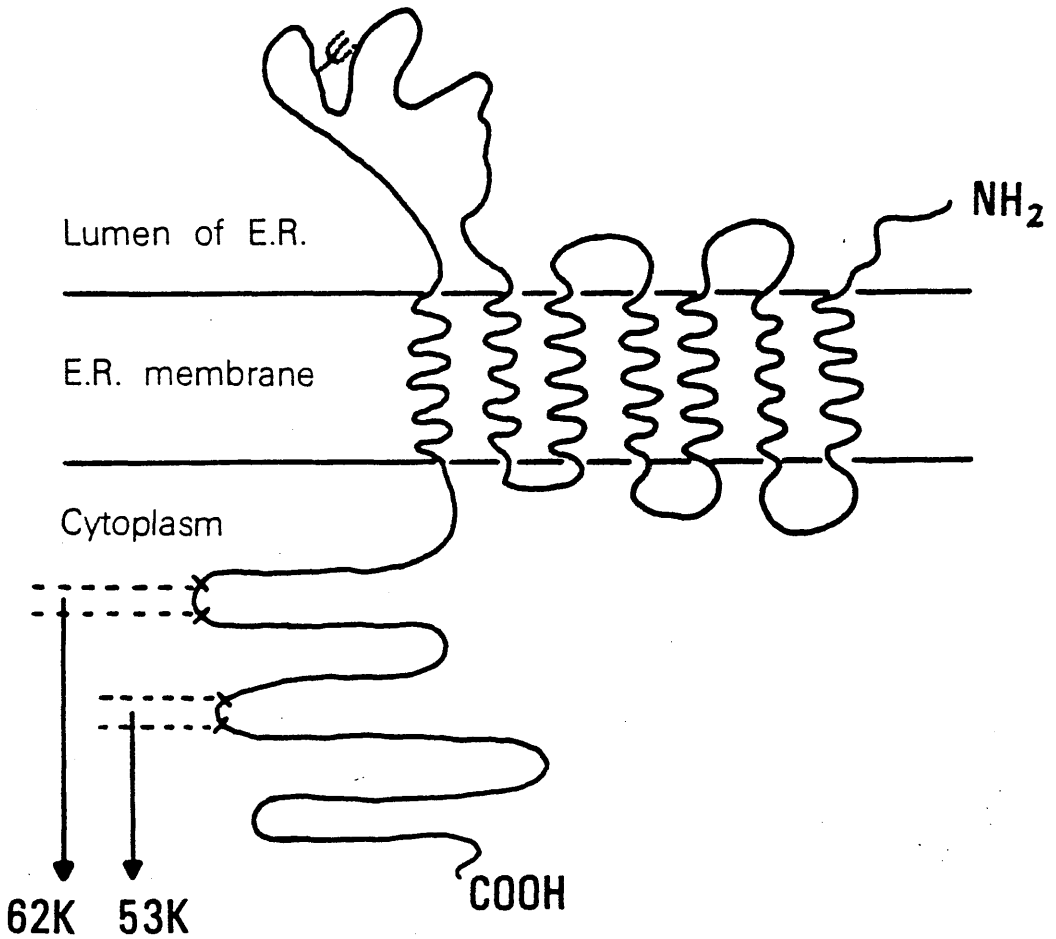


Fig. 1.6 Schematic model representing the topology of HMG-CoA reductase in the endoplasmic reticulum (E.R.) membrane

Seven hydrophobic regions, indicated by the waved lines, span the E.R. membrane. The cytosolic domain (C-terminus) contains two sites of proteolytic cleavage yielding the loosely bound 62K and soluble 53K species of HMG-CoA reductase respectively. The N-linked carbohydrate chain is indicated between the sixth and seventh membrane spanning region.

(Redrawn from Liscum et al. 1985)

1.3 Multivalent Control of HMG-CoA Reductase

As would be expected from the fact that the expressed activity of HMG-CoA reductase is the principal determinant of the rate of cholesterol biosynthesis, the activity of the enzyme changes in response to alterations in cellular and body cholesterol homeostasis (Section 1.1). The effective activity of HMG-CoA reductase *in vivo* is determined by two parameters: (a) the quantity or concentration of enzyme protein (i.e. the number of enzyme molecules as a gene product) in the tissue. This depends on the relative rates of enzyme synthesis and degradation. (b) The specific activity of the individual enzyme molecules. This is known to be modulated by covalent modification and the state of the membrane in which reductase resides. Although the mechanisms through which these two parameters control the activity of HMG-CoA reductase are distinct, a number of effectors of HMG-CoA reductase e.g. cholesterol, oxysterols or insulin/glucagon (Ingebritsen, 1983) operate via both mechanisms (see below) and as will be described later covalent modification of the enzyme may affect its rate of degradation.

1.3.1 Regulation of HMG-CoA Reductase by Modulation of Hepatic Enzyme Concentration: Factors that Influence Enzyme Synthesis and Degradation

1.3.1 (i) Lipoprotein cholesterol

As implied in the previous section, the increased delivery of cholesterol to the liver either by chylomicron remnants (exogenous cholesterol) or LDL (endogenous cholesterol) produces a feedback inhibition of cholesterol biosynthesis and a parallel decrease in the

acitivity of HMG-CoA reductase (Nervi et al., 1975; Nervi & Dietschy, 1975; Rodwell et al., 1976). Although changes in the intracellular levels of cholesterol acutely modulate the catalytic activity of existing enzyme (see Chapter 6), studies on cells in culture have shown that the synthesis and degradation of HMG-CoA reductase protein are also affected by the inclusion of LDL-cholesterol in the culture medium (Brown et al., 1974; Faust et al., 1982; Hardgrave et al., 1979). Thus, for example, the addition of LDL to the medium of UT-1 cells suppresses the synthesis of immunoprecipitable HMG-CoA reductase protein by more than 98% (Faust et al., 1982) and antagonizes the over-production of enzyme characteristic of these cells. In addition, a 3-fold enhancement of the rate of degradation of HMG-CoA reductase was observed in these cells which was accompanied by the regression of the extensive proliferation of the endoplasmic reticulum in these cells (Orci et al., 1984; Anderson et al., 1983). By contrast, fibroblasts genetically deficient in LDL-receptors exhibit elevated HMG-CoA reductase activity (Brown et al., 1981). Similarly, the removal of cholesterol from the plasma membranes of cells with HDL results in the increased synthesis and decreased degradation of HMG-CoA reductase (Gibson & Parker, 1985).

The mechanism by which cholesterol mediates these effects is unclear. The exposure of UT-1 cells to LDL in culture diminishes the level of HMG-CoA reductase mRNA (Chin et al., 1982b; Luskey et al., 1982, 1983). A decrease in the level of HMG-CoA reductase mRNA (also accompanied by a decrease in immunoprecipitable protein) is observed in vivo in the liver of cholestyramine mevinolin-treated rats fed a

cholesterol-supplemented diet (Liscum et al., 1983b). These observations suggest that cholesterol affects the control of transcription of the HMG-CoA reductase gene and it may be relevant that Erickson et al. (1975) have previously shown that the concentration of non-esterified cholesterol associated with chromatin in the rat liver increases prior to the normal diurnal decrease in the activity of HMG-CoA reductase (see Section 4.2; Gould, 1977).

1.3.1 (ii) Oxysterols

Oxygenated cholesterol derivatives (oxysterols), in particular 25-hydroxycholesterol (Kandutsch & Chen, 1975) and 7-ketocholesterol (Brown & Goldstein, 1974), have been shown to be powerful inhibitors of HMG-CoA reductase activity (for review see Gibbons, 1983a). Culture of cells in the presence of 25-hydroxycholesterol results in the inhibition of the synthesis of HMG-CoA reductase (Faust et al., 1982; Tanaka et al., 1983; Sinensky et al., 1981; Luskey et al., 1983); this is associated with regulation both at the level of transcription (decrease in HMG-CoA reductase mRNA, Luskey et al., 1983) and translation (no change in mRNA; Tanaka et al., 1983). In addition, 25-hydroxycholesterol also enhances enzyme degradation (Faust et al., 1982; Tanaka et al., 1983) and may involve other 'mediating-proteins' since this effect is diminished in the presence of protein synthesis inhibitors (Chang et al., 1981; Chen et al., 1982). The possible physiological significance of these effects observed in vitro is indicated by the fact that 25-hydroxycholesterol can reproduce any of the effects of LDL-cholesterol on cellular activity i.e. suppression of cholesterol

biosynthetic enzymes ACAT and the LDL-receptor (see Section 1.1.2; Gibbons et al., 1983b). Therefore, the similarity of the effects of these related moieties suggests that oxysterols, possibly formed by the action of the P450-cytochrome oxidase on the microsomal membrane (Gupta et al., 1985), may be the mechanism through which changes in the availability of cholesterol are transmitted to the systems controlling the rates of synthesis and/or degradation of HMG-CoA reductase. Inhibition of the formation of polar sterols in cultured rat intestinal epithelial cells was shown to result in the de-repression of the activity of HMG-CoA reductase (Gupta et al., 1985; see also Sexton et al., 1983).

1.3.1 (iii) Bile Acids and the Enterohepatic Circulation (EHC)

The interruption of the enterohepatic circulation by use of the bile acid sequestrant, cholestyramine (see Section 1.1.2), results in the 5- to 8-fold increase in the activity of HMG-CoA reductase (Goldfarb & Pitot, 1972; Tanaka et al., 1982). This is the consequence both of the activation of existing enzyme (Tanaka et al., 1982) and of the increased amount of enzyme protein (Goldfarb & Pitot, 1972; Hardgrave et al., 1979). Cholestyramine-treatment of animals increases the amount of mRNA for HMG-CoA reductase (Clarke et al., 1983) and the rate of synthesis of the enzyme protein (Edwards et al., 1983). Since cholesterol and oxysterol derivatives control the amount of enzyme protein by operating at the level of transcription and translation, it seems likely that cholestyramine produces its effects by the depletion of the hepatic pool of free cholesterol. In addition, bile acids may have a direct effect

through inhibition of enzyme synthesis (Barth & Hillmar, 1980; see Myant, 1981).

1.3.1 (iv) Mevalonate and its derivatives

The addition of mevalonate (or its lactone, which is rapidly hydrolyzed to mevalonate in the cell; Mitchell & Avigan, 1981) to cell cultures has the same effect as the addition of LDL with respect to the decrease in the concentration of HMG-CoA reductase (due to increased enzyme protein synthesis and increased degradation) (Edwards et al., 1983; Haro et al., 1985). A marked reduction in the levels of HMG-CoA reductase mRNA was observed in UT-1 cells exposed to mevalonate (Luskey et al., 1983) and in the liver of cholestyramine-treated animals to which mevalonate had been administered by stomach-tube (Clarke et al., 1983).

The mechanism of the mevalonate effect may be in part identical to that of LDL-cholesterol since mevalonate can potentially give rise to cholesterol and its derivatives in the liver. However, in cells in which HMG-CoA reductase is not subject to feedback regulation by exogenously added cholesterol (e.g. Drosophila embryo cells) the effect of mevalonate persists (Brown et al., 1983). This suggests that mevalonate (or a non-sterol derivative thereof) is capable of acting on HMG-CoA reductase synthesis independently of its role as substrate for cholesterol synthesis. This effector is likely to act at the translational level since its effects occur in the absence of any decrease in the quantity of mRNA coding for HMG-CoA reductase protein (Peffley & Sinensky, 1985). It is noteworthy that in Swiss 3T3 cells labelled mevalonate is

incorporated covalently into a set of proteins (Schmidt et al., 1984). This has been suggested to represent protein terpenylation (Peffley & Sinensky, 1985) which would possibly explain the observed involvement of the monoterpenes, cineole and menthol in the feedback repression of HMG-CoA reductase activity (Clegg et al., 1982).

1.3.2 Regulation of HMG-CoA Reductase by Modulation of Expressed Activity

(i) Microenvironment of HMG-CoA reductase in the endoplasmic reticulum

HMG-CoA reductase is an integral protein component of the endoplasmic reticulum (see Section 1.2.2). Consequently, its activity is capable of being modulated by changes in the properties of the membrane in which it resides. Mitropoulos (1983 a,b) has recently reviewed evidence which suggests that HMG-CoA reductase could be modulated by interactions with the immediate lipid environment. Thus a decrease in activity of HMG-CoA reductase, measured in microsomes isolated from the liver of animals fed a diet supplemented with cholesterol, was accompanied by distinct changes in the Arrhenius plot for the activity of the enzyme in the microsomes isolated from the liver of these animals. The changes were attributed to an increase in the pool-size of non-esterified cholesterol in the membrane. Another microsomal enzyme, ACAT (see Section 1.1.2), shows a direct relationship between activity and the membrane content of free cholesterol (Mitropoulos et al., 1981; 1983a,b; Hashimoto et al., 1983).

Similar changes to the Arrhenius plot of HMG-CoA reductase activity are also observed in microsomes obtained from the liver of rats which have been starved (Mitropoulos 1983b), infused (intravenously) with mevalonic acid (Mitropoulos et al., 1978) or fed an unsaturated fat-supplemented diet (Mitropoulos et al., 1980).

Moreover, work from a number of laboratories (e.g. Sabine & James, 1976; Sipat & Sabine, 1981; Finkel & Volpe, 1979; Ramirez et al., 1984; Richert et al., 1984) supports the concept that the modulation of HMG-CoA reductase activity by cholesterol (detected by changes in the Arrhenius plot) is achieved by virtue of its ability to alter the fluidity of the endoplasmic reticulum membrane. Thus Davis & Poznansky (1985) demonstrated that when the fluidity of the microsomal membrane fraction was altered without changing the cholesterol content by enrichment with a saturated phosphatidylcholine preparation, the activity of HMG-CoA reductase was still depressed. However, this mechanism does not appear to effect the changes observed in other cholesterol metabolizing enzymes (e.g. ACAT; see above) or the effects of starvation on HMG-CoA reductase (see above; Sipat & Sabine, 1981). This suggests that both mechanisms exist to regulate microsomal enzymes (see Hashimoto et al., 1983; Van Huesden & Wirtz, 1984).

(ii) Effector proteins and metabolites

A number of liver cytosolic proteins are known to influence the activity of HMG-CoA reductase. Amongst these are a number of sterol and lipid carrier proteins that seem to function directly in the multi-enzyme process of lanosterol conversion to

cholesterol in the microsomal membranes (see Trzaskos & Gaylor, 1983). An iron-containing protein has also been found to inhibit HMG-CoA reductase activity (Menon et al., 1982). HMG-CoA reductase is also allosterically activated by thiol agents including glutathione (GSH) and dithiothreitol (DTT) (Roitelman & Schechter, 1984); these effects may have physiological significance since marked diurnal variations are observed in the liver ratios of reduced (GSH) and oxidized (GSSG) forms of glutathione (Roitelman & Schechter, 1984). This mechanism could be involved in the regulation of a proposed conversion of an inactive, dimer of the '97k' HMG-CoA reductase to an active, monomer form under reducing conditions (Ness et al., 1984; Edwards et al., 1985).

(iii) Covalent modification of HMG-CoA reductase

The reversible phosphorylation of serine and threonine amino acid residues of proteins is one of the major mechanisms by which metabolic processes are controlled by neural and hormonal stimuli. More than forty enzymes are now known to be regulated in this manner (Krebs, 1983) including important regulatory enzymes of carbohydrate and lipid metabolism in the liver (see Geelen et al., 1980). In addition phosphorylation of tyrosine residues appears to be involved in the function of certain cell surface hormone receptors (Hunter & Cooper, 1985). Sections 1.4 and 1.5 present the evidence to date which suggests that HMG-CoA reductase is also regulated by reversible phosphorylation.

1.3.3 Modulation of Hepatic HMG-CoA Reductase Activity in the Physiological Setting: The Diurnal Rhythm

The best documented example of changes in the concentration of HMG-CoA reductase in the microsomal fraction of rat liver under different physiological conditions is the diurnal variation observed when rats are kept under a 12h light:12h dark cycle and fed *ad libitum*. At the peak of this rhythm, which occurs at the middle of the dark period (and coincides with active feeding) the activity of HMG-CoA reductase is 5- to 10-fold higher than that at the nadir of the rhythm during the light period (see Rodwell *et al.*, 1976; Gibbons *et al.*, 1982) as the consequence of equivalent changes in the concentration of enzyme protein (Clarke *et al.*, 1984). The physiological role of this rhythm is discussed in detail in Chapter 4.

From experiments involving the determination of the incorporation of [^{3}H]-leucine into enzyme protein (Higgins *et al.*, 1974), the effects of cycloheximide administration (see Rodwell *et al.*, 1976) and the turnover rate of the enzyme (Dugan *et al.*, 1972; Edwards & Gould, 1974) it was concluded that changes in the rate of enzyme synthesis were responsible for diurnal changes in HMG-CoA reductase activity. The rate of enzyme degradation appeared to be uniform throughout the day (see Rodwell *et al.*, 1976). Further studies revealed that the diurnal increase in HMG-CoA reductase depends on an initial period of *de novo* RNA synthesis (Edwards & Gould, 1974) and that changes in the amount of HMG-CoA reductase enzyme protein are accompanied by parallel changes in the functional mRNA content of the liver throughout the diurnal cycle (Clarke *et al.*, 1984).

Since HMG-CoA reductase has a very short half-life in the cell (approximately 2.2h; see Rodwell et al., 1976) it is possible that the sharp decline in HMG-CoA reductase activity observed from the peak of the rhythm is fully accounted for by the cessation of enzyme synthesis (see Dugan, 1981). This does not, however, exclude the possibility that the rate of enzyme degradation may be raised during the diurnal cycle. Such modulation of the rate of degradation is evident under conditions of greatly increased rates of enzyme synthesis e.g. cholestyramine plus mevinolin treatment in vitro (Tanaka et al., 1982). Using cholestyramine alone, a dissociation between mRNA content and peak activity is evident (Tanaka et al., 1982). Therefore mevinolin may induce a specific inhibition of the degradation of HMG-CoA reductase, possibly as a result of a decreased cellular concentration of an endogenously produced metabolite of mevalonate (Edwards et al., 1983b). This observation, coupled with the demonstration that mevalonate will accelerate enzyme degradation (Edwards et al., 1983a&b; Section 1.3.1(iv)) suggests that this variation in the cellular concentration of a metabolite(s) of mevalonate may have an important regulatory control over the diurnal rhythm of HMG-CoA reductase in vivo.

A number of hormones may be involved in the control of the diurnal rhythm of HMG-CoA reductase protein and activity (for reviews see Rodwell et al., 1976; Dugan & Porter, 1977). Thus, because of the pronounced diurnal rhythm observed in the plasma concentration of insulin and glucocorticoids, these hormones would appear to be plausible candidates for the generation of signals in the liver that result in the rhythm in enzyme concentration discussed above. The

secretion of insulin would be expected to be more closely related to food intake and therefore to approximate to the rhythm in HMG-CoA reductase activity observed (which peaks in the dark, feeding period). Insulin deficiency induced either by treatment with streptozotocin (Nepokroeff et al., 1974) or alloxan (Huber et al., 1973) results in the abolition of the rhythm of a much reduced HMG-CoA reductase activity. The rhythm is restored by the daily administration of insulin (Nepokroeff et al., 1974; see Rodwell et al., 1976).

Moreover, insulin stimulates the increase in HMG-CoA reductase activity and induces proportional increases in cholesterogenesis both in animals in which the normal rhythm is approaching a minimum or in animals from which food had been withdrawn 6h earlier (Lakshmanan et al., 1973). That these effects are due to increased protein synthesis is suggested by studies in isolated hepatocytes which demonstrated that insulin stimulates the synthesis of both HMG-CoA reductase mRNA and enzyme protein (cf. work on fibroblasts, Avigan, 1977; Bhathena et al., 1974).

Glucagon also shows a diurnal variation in plasma concentrations (Murakami et al., 1981) but it is still not clear whether glucagon, through its intracellular messenger, cyclic AMP, is a physiological regulator of HMG-CoA reductase protein synthesis. Little effect of this hormone is observed on the activity of HMG-CoA reductase in the liver of animals during the light period (Lakshmanan et al., 1973) or in perfused liver preparations (Raskin et al., 1974). However, administrations of glucagon to conscious rats suppresses the normal diurnal rise in hepatic HMG-CoA reductase

substantially (around 50%, Nepokroeff et al., 1974; Dugan et al., 1974; Huber et al., 1973; Lakshmanan et al., 1973). Also, increased intracellular concentrations of cyclic AMP suppress the diurnal rise in HMG-CoA reductase activity in rat liver in vivo.

The diurnal peak in plasma concentrations of corticosterone, the major glucocorticoid in the rat, coincides with the commencement of the diurnal increase in HMG-CoA reductase activity towards the end of the light period (Knox et al., 1979; cf. Rodwell et al., 1976; see Section 4.2). Therefore it is possible that, by analogy with the effects of glucocorticoids on the synthesis of several hepatic enzymes e.g. tyrosine aminotransferase (TAT; Olson et al., 1980), they also stimulate the synthesis of HMG-CoA reductase.

Experiments designed to determine a possible role of glucocorticoids on the diurnal rhythm of HMG-CoA reductase activity have produced conflicting reports. For example, adrenalectomy has been reported to abolish (Hickman et al., 1972; Edwards, 1973), diminish (Mitropoulos & Balasubramanian, 1976) or have no effect on (Neprokroeff et al., 1974; Nervi & Dietschy, 1974) this rhythm. Equally contradictory results have been reported for the effects of added glucocorticoids either in whole rats or isolated hepatocytes. The injection of hydrocortisone into normal rats suppressed the diurnal-, insulin- and triiodothyronine (T_3)-induced increases in HMG-CoA reductase activity in normal (Nepokroeff et al., 1974), diabetic (Dugan et al., 1974) or hypophysectomized rats (Dugan et al., 1974; Ness et al., 1985) respectively. By contrast,

other groups of workers using either natural glucocorticoids (e.g. cortisol; Mitropoulos & Balasubramaniam, 1976) in whole animals, or synthetic glucocorticoids (e.g. dexamethasone; Lin & Snodgrass, 1982) in whole animals, HeLa cells (Cavanee et al., 1978) or hepatocytes (Lin & Snodgrass, 1977; 1982; Barth & Hillmar, 1980) have presented evidence to suggest that the hormones could positively effect a diurnal increase in HMG-CoA reductase activity in vivo. It is clear, however, that glucocorticoids cannot be exclusively responsible for the diurnal rythym in HMG-CoA reductase since both of the effects on whole animals in vivo are dependent on the administration of the hormone being made during a particular phase of the light-dark cycle.

Other hormones e.g. triiodothyronine (T_3), whilst not thought to have a role in the initiation of the diurnal rhythm in HMG-CoA reductase synthesis, may play a permissive or potentiating role for more direct-acting hormones. T_3 restores the diurnal rhythm of HMG-CoA reductase activity to hypophysectomized rats although this treatment produces a supranormal stimulation of this activity (Ness et al., 1973; Fig. 1.7) and has recently been shown to be the consequence of increased HMG-CoA reductase mRNA and protein (Ness et al., 1985). Similarly, the administration of T_3 to euthyroid rats abolishes the same rhythm at the level of the diurnal peak after a time-lag of 2 days (Ness et al., 1973; Fig. 1.7). Also, prior administration of T_3 is required to restore the ability of insulin to elevate HMG-CoA reductase activity in hypophysectomized (Huber et al., 1974) or in diabetic-hypophysectomized rats (Dugan et al.,

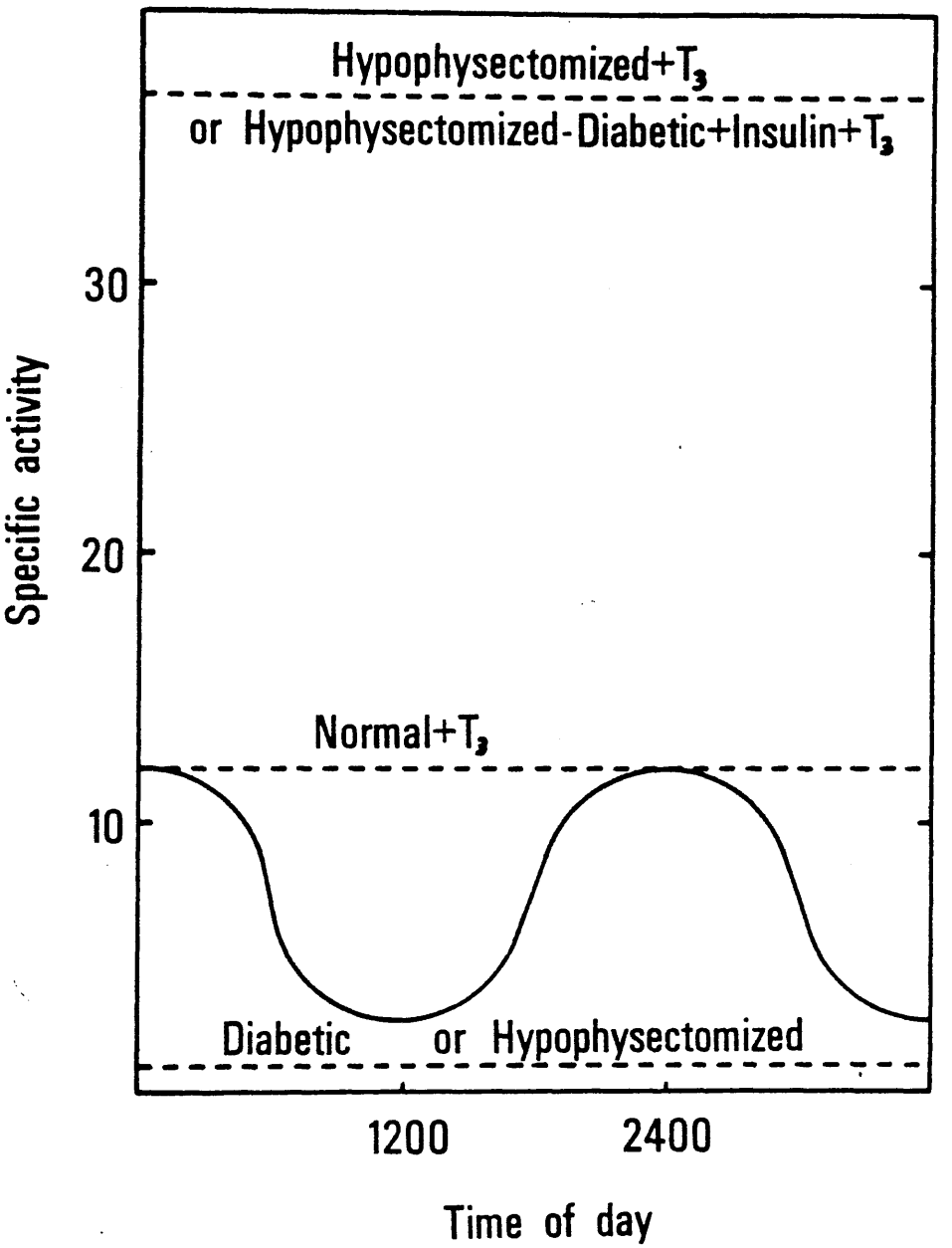


Fig. 1.7 Rat liver HMG-CoA reductase activity in various hormonal states

Solid line represents the diurnal rhythm of total HMG-CoA reductase activity (nmoles/h per mg of microsomal protein) in normal rats or diabetic rats treated with insulin. L-Triiodothyronine (T_3; lug/g body wt.) was administered 54th before the animals were killed.

(Taken from Dugan et al. 1974)

1974). However, it is not clear whether these are direct effects of the hormone since the effects of T_3 can be mimicked by other experimental treatments such as high carbohydrate diets, or the injection of growth hormone (see Rodwell et al., 1976).

Whereas most of the hormonal effects discussed above occur through the stimulation or repression of enzyme synthesis it is to be appreciated that some of these effects may occur indirectly through hormonal effects on cholesterol dynamics in the intact animal (e.g. see Lakshmanan et al., 1975; Ness et al., 1985). As mentioned above, this is possible because the cholesterol status can affect the concentration of HMG-CoA reductase and hence activity by feeding back on the rate of synthesis of HMG-CoA reductase (see Section 1.3.1(i)).

1.4 Reversible Phosphorylation of HMG-CoA Reductase In Vitro

1.4.1 Historical Survey

In 1973, Beg et al. reported that HMG-CoA reductase activity in freshly isolated, washed rat liver microsomes was inhibited by preincubation with Mg^{2+}:ATP and a protein fraction separated from the liver cytosol. The inhibition was time-dependent and was partially reversed by treatment of the microsomes with a second protein fraction of the cytosol. These investigators therefore proposed that HMG-CoA reductase might undergo interconversion between two forms of differing specific activities and suggested protein phosphorylation as a potential mechanism (Beg et al., 1973).

Independently, Higgins and Rudney (1973) suggested that the response of HMG-CoA reductase activity to dietary manipulation (i.e. cholesterol feeding) involved variations in the degree of activation

of the enzyme in addition to changes in its concentration. Goodwin & Margolis (1973) observed that the incubation of the post-mitochondrial supernatant of rat liver homogenate at 37°C (prior to assay) increased the rate of ^{14}C-acetate but not ^{14}C-mevalonate incorporation into cholesterol by 8- to 20-fold. Since these initial observations, work in numerous laboratories has yielded evidence that supports the concept of the existence of active and inactive forms of HMG-CoA reductase in rat liver (Bove and Hegardt 1978; Chow et al. 1975; Ingebritsen et al. 1978; Nordstrom et al. 1977; Berndt et al. 1976; Hunter & Rodwell 1980; Saucier & Kandutsch 1979).

In the initial stages of the characterization of the Mg:ATP-dependent inactivation system, Brown et al. (1975), working on human fibroblasts and rat liver, demonstrated that the factor involved was heat labile, non-dialyzable and precipitable with ammonium sulphate. Kinetic analysis showed that the inactivation produced an 8- to 20-fold decrease in the V_{max} of HMG-CoA reductase without any change in the K_m for HMG-CoA (Brown et al., 1975; Nordstrom et al., 1977).

The activation process of HMG-CoA reductase was shown to be blocked by NaF, a potent inhibitor of protein phosphatase (Nordstrom et al., 1977; Berndt et al., 1976; Erickson et al., 1980; Ingebritsen et al., 1978; Beg et al., 1978). Ingebritsen et al. (1978, 1981) confirmed the involvement of a protein phosphatase by achieving the reactivation of Mg^{2+}:ATP-inactivated HMG-CoA reductase by incubation with a partially purified cytosolic phosphorylase a phosphatase. These observations were consistent with the proposal that the activity of HMG-CoA reductase could be regulated by reversible phosphorylation.

This phenomenon has since been confirmed for the enzyme from a wide variety of tissues including liver (e.g. Ingebritsen et al., 1978; Berndt et al., 1976) brain (Shah, 1981), leucocytes (Harwood, et al., 1984) and intestine (Oku et al., 1984; Panini & Rudney, 1980) in a variety of animal species including pigs, dogs, rodents, chickens, frogs, fish (Hunter et al., 1980), insects (Brown et al., 1983) and humans (Harwood et al., 1984; Beg et al., 1984a).

The involvement of reversible phosphorylation in the regulation of HMG-CoA reductase was not universally accepted because of difficulties encountered in certain experiments. Thus, whereas the freeze-thaw solubilized HMG-CoA reductase, then thought to be the native form of the enzyme, could be inactivated by Mg^{2+}:ATP and a cytosolic preparation of reductase kinase (Nordstrom et al., 1977) thus had proved surprisingly difficult as compared to the microsomally-bound enzyme (Brown et al., 1975; Nordstrom et al., 1977). Also Ness et al. (1981, 1982, 1983) pointed out that the decrease in the rate of mevalonate formation observed for microsomes preincubated in the presence of Mg^{2+}:ATP could be due to the removal of mevalonate by the ATP-dependent activity of mevalonate kinase, the enzyme immediately distal to HMG-CoA reductase in the cholesterogenic pathway (Fig. 1.1). Harwood & Rodwell (1982) and Beg et al. (1982) subsequently showed that mevalonate kinase and reductase kinase activities were readily distinguishable; this has recently been confirmed by Ferrer & Hegardt (1984); (for a review see Kennelly & Rodwell, 1985).

Definitive proof of reversible phosphorylation was obtained from *in vivo* and *in vitro* experiments in which the incorporation of [^{32}P]-phosphate into HMG-CoA reductase was shown to be directly proportional to inactivation of the enzyme. This was shown to be true for microsomally bound (Bove *et al.*, 1978; Beg *et al.*, 1978; Gil *et al.*, 1980; Keith *et al.*, 1979, 1983) and for immunoprecipitated, electrophoretically pure '53K' enzyme (Beg *et al.*, 1980; Ferrer & Hegardt, 1984). Phosphorylation was shown to occur on serine residues. Furthermore, numerous laboratories have demonstrated that the activation of [^{32}P]-labelled HMG-CoA reductase is accompanied by the concomitant release of [^{32}P]-phosphate from the enzyme (Beg *et al.*, 1978; Gil *et al.*, 1980; 1981a; 1981b; Brown & Rodwell, 1983; Sitges *et al.*, 1984; Ferrer & Hegardt, 1984).

The precise location of the phosphorylation site(s) on HMG-CoA reductase has not been resolved although they are presumed to be situated in the cytosolic domain of the '53K' HMG-CoA reductase which has access to the modulating enzymes (Fig. 1.6). Tryptic digestion of [^{32}P]-labelled '53K' fragment yields two phosphorylated peptides indicating that the enzyme contains at least two structurally distinct phosphorylation sites. However, in two recent reports, evidence was presented that only one site is directly involved in the depression of catalytic activity of HMG-CoA reductase. First, Brown & Rodwell (1983) found that when partially inactivated microsomal HMG-CoA reductase was treated with either of two partially purified, high molecular weight cytosolic HMG-CoA reductase phosphatases (see

Section 1.4.3.2) full reactivation of the enzyme followed the release of only about 50% of the bound [^{32}P]-phosphate. Second, treatment of homogeneous '53K'-enzyme with a purified microsomal HMG-CoA reductase kinase yielded [^{32}P]-labelled enzyme phosphorylated at only one site, as determined by analysis of the tryptic phosphopeptides (Ferrer & Hegardt, 1984). This single-site phosphorylation was apparently sufficient to produce total inactivation of the enzyme (Ferrer & Hegardt, 1984). The function of the putative second phosphorylation site is unclear. However it is possible that it may have a role in controlling the rate of (de)phosphorylation of the site that affects catalytic competence of the enzyme such as occurs in pyruvate dehydrogenase (Cohen, 1982). Alternatively, it may produce some conformational change in HMG-CoA reductase enzyme which alters its ability to act as a substrate for endogenous proteases, and thus affect its rate of degradation.

1.4.2 Bicyclic Mechanism of Reversible Phosphorylation

Further studies conducted on the activity of rat liver reductase kinase have revealed that this enzyme is also regulated by reversible phosphorylation-dephosphorylation (Ingebritsen *et al.*, 1978; Gibson & Ingebritsen, 1978; Beg *et al.*, 1979): the phosphorylated enzyme is active whereas the dephosphorylated enzyme is inactive. It was directly demonstrated that the incorporation of [^{32}P]-phosphate from [γ-^{32}P]-ATP:Mg^{2+} into homogeneous reductase kinase using reductase kinase kinase was associated with a concomitant activation of the enzyme (Beg *et al.*, 1979). This process is reversed by protein phosphatase (Ingebritsen *et al.*,

1981). On the basis of these observations it was suggested that the regulation of HMG-CoA reductase activity by reversible phosphorylation is mediated through a bicyclic system consisting of two separate protein kinases (see below) and at least one phosphatase (but the dephosphorylation of two separate phosphoproteins) (Fig. 1.8). A similar system is thought to operate in human liver (Beg et al., 1984).

1.4.3 Properties of Protein Kinases and Phosphatases that act on HMG-CoA Reductase

1.4.3.1 Reductase Kinase and Reductase Kinase Kinase

Several lines of evidence indicate that reductase kinase and reductase kinase kinase are distinct enzymes. First the two activities can be resolved by chromatography on DEAE-cellulose (Ingebritsen et al., 1981). Second, reductase kinase kinase does not catalyse the inactivation of HMG-CoA reductase (Ingebritsen et al., 1981). Third, reductase kinase kinase activity is inhibited in high-ionic-strength buffers that are routinely used to assay both HMG-CoA reductase and reductase kinase (Ingebritsen et al., 1978). However, both reductase kinase and reductase kinase kinase appear to belong to a class of cyclic nucleotide-independent protein kinases (Beg & Brewer, 1981). Neither activity is influenced by cyclic AMP, cyclic CMP or cyclic IMP in a concentration range of 10^{-5}-10^{-3}M or the specific heat stable inhibitor of cyclic AMP-dependent protein kinase (Ingebritsen et al., 1981).

The initial studies demonstrating the inactivation of microsomal HMG-CoA reductase (Beg et al., 1973; Nordstrom et al.,

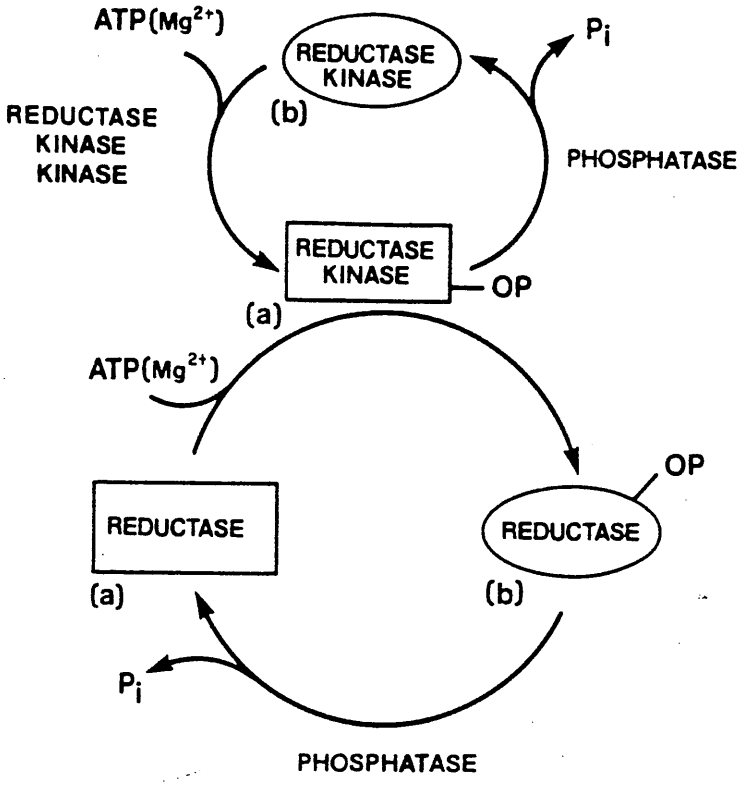


Fig. 1.8 Bicyclic phosphorylation system for the regulation of HMG-CoA reductase activity

(a) = active enzyme; (b) = inactive enzyme

(Taken from Ingebritsen et al. 1981)

1977) indicated that the reductase kinase was present both in the microsomal fraction and in the cytosol of liver. After detailed examination of the cellular distribution of this kinase and of reductase kinase kinase (Ingebritsen et al., 1981) more than 80% of each activity was found to be in the cytosolic fraction. The remaining activity which sedimented with the microsomal fraction was readily extracted under conditions which did not solubilize HMG-CoA reductase (Ingebritsen et al., 1981). It is considered that these enzymes are primarily cytosolic and that the activities observed in the microsomal fraction result from adventitious binding of these enzymes with the microsomal membrane.

Both reductase kinase and reductase kinase kinase utilize ATP as phosphoryl donor. Reductase kinase has some activity with GTP but is virtually inactive with CTP, ITP and UTP (Brown et al., 1975; Harwood et al., 1984); the nucleotide specificity of reductase kinase kinase is presently unknown. Interestingly, Brown et al. (1975) and Nordstrom et al. (1977) demonstrated that ADP was required for full reductase kinase activity. This property was later confirmed using purified (cytosolic) reductase kinase-mediated inactivation of either microsomal '53K' HMG-CoA reductase (Harwood et al., 1984) or detergent-solublized '97K' enzyme (Kennelly et al., 1983). Using '53K' HMG-CoA reductase as substrate, the K_a of reductase kinase for ADP has been determined to be 0.1-1.4mM (Harwood et al., 1984; Caelles et al., 1985) and thus suggesting that this could be a physiologically important effect. (Total intracellular concentration of ADP is approximately 0.8 mM; Harwood et al., 1984). This property appears to be unique for protein kinases and exclusive

to cytosolic reductase kinase (Gibson & Parker, 1985). This suggests that cytosolic and microsomal reductase kinase may have different properties but the significance of this is unclear.

Beg et al. (1979), in a preliminary publication containing a procedure for purifying reductase kinase to apparent homogeneity from microsomal extracts, reported that the enzyme possessed a molecular weight (Mr) of 380K as judged by gel filtration on Sepharose 6B and was hexameric with subunit Mr of 58K as determined by SDS-PAGE. Ferrer & Hegardt (1984) have recently purified rat liver reductase kinase to homogeneity from microsomal extracts but reported that the enzyme possessed a native molecular weight (Mr) of 205K and monomeric Mr of 105K on gel filtration and SDS-PAGE respectively. The relationship between these two preparations is unclear. As yet the equivalent properties of reductase kinase kinase have not been assessed.

Apart from reductase kinase, Beg and co-workers have shown that a purified Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase-C) (Beg et al., 1985a) and a Ca^{2+}-calmodulin-dependent protein kinase (Beg et al., 1985b) also catalyze the phosphorylation and consequent inactivation of both microsomal '97K' and purified, soluble '53K' HMG-CoA reductase. These effects are clearly independent of the involvement of reductase kinase because none of the properties exhibited by these protein kinases are possessed by reductase kinase. Therefore, this could represent a mechanism through which HMG-CoA reductase activity could be modulated in response to an increase in intracellular Ca^{2+} such as occurs following the stimulation of hepatocytes with α_1-adrenergic

agonists (See Exton, 1985), vasopressin or angiotension II (Charest et al., 1983) further to the regulation of reductase kinase (via reductase kinase kinase). This regulation of HMG-CoA reductase activity by multiple protein kinases is is illustrated in Fig. 1.9.

Reductase Phosphatase(s)

The ability of phosphorylated HMG-CoA reductase to act as substrate for a range of protein phosphatases that occur in mammalian tissues has recently been investigated (Ingebritsen & Cohen, 1983; Ingebritsen et al., 1983a, 1983b). The four major protein phosphatases have been termed phosphatases-1, -2A, -2B and -2C (Ingebritsen & Cohen, 1983). HMG-CoA reductase-phosphate and reductase kinase phosphate were shown to be substrates for protein phosphatases-1, -2A and -2C (Ingebritsen & Cohen, 1983).

These three phosphatases can be distinguished by virtue of their different properties, e.g. protein phosphatase-1 is inhibited by protein inhibitors (inhibitors-1 and -2) whereas protein-phosphatases-2 are insensitive to these inhibitors. The phosphatases also have different substrate specificities e.g. protein phosphatase-1 preferentially dephosphorylates the β-subunit of phosphorylase kinase whereas type-2 phosphatases dephosphorylate the α-subunit of phosphorylase kinase (Ingebritsen & Cohen, 1983).

Protein phosphatase-2A, which acts on HMG-CoA reductase phosphate, is resolved by chromatography on DEAE-cellulose into three species, termed protein phosphatase-$2A_0$, -$2A_1$ and -$2A_2$ with Mr = 210K, 210K and 150K respectively (as determined by gel filtration; see Ingebritsen, 1983). Each contain the same catalytic subunit (Mr

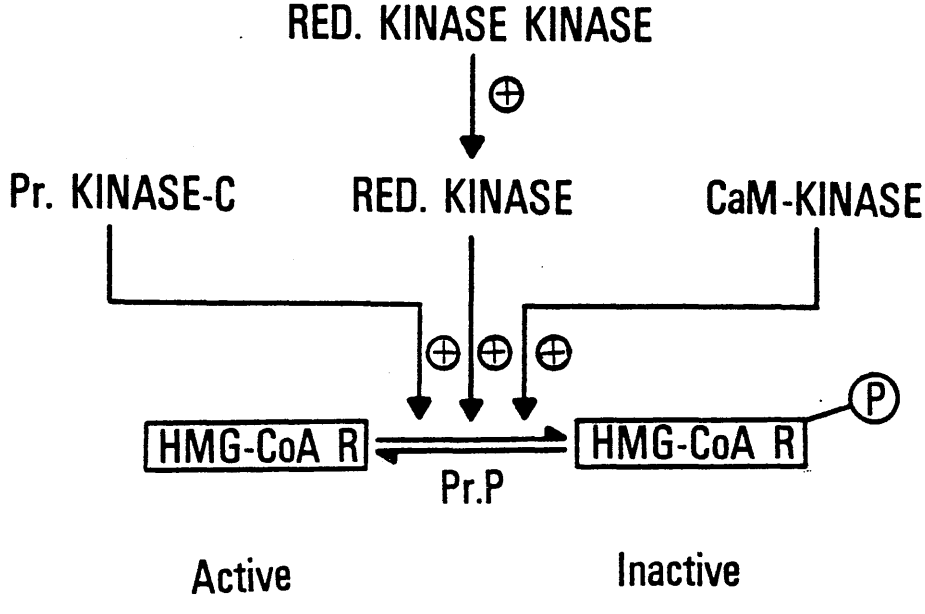


Fig. 1.9 Multivectorial regulation of phosphorylation of HMG-CoA reductase (HMG-CoA R) by kinases.

Pr. kinase-C, Ca^{2+}-activated, phospholipid-dependent kinase; CaM-kinase, Ca^{2+}-calmodulin-dependent protein kinase; Red. kinase, reductase kinase. Pr.P, reductase protein phosphatase.

= 35K). Protein phosphatase -2C is composed of a single subunit (Mr = 46K) and is dependent on Mg^{2+} (K_a = 1.0 mM). The mechanisms for regulating protein phosphatases-2A and -2C are presently unknown although the observed activation of the catalytic subunit of protein phosphatase-2A (Mr = 35K) following its dissociation from the other subunits subsequent to freeze-thaw treatment of the phosphatase preparation (Ingebritsen et al., 1983c), suggests that mechanisms may exist for the regulation of this phophatase in vivo.

Two important mechanisms apparently exist for the regulation of protein phosphatase-1:

(i) Protein phosphatase activity is inhibited by the binding of inhibitor-1 only subsequent to its phosphorylation and concomitant activation by the phosphorylation of a specific threonine residue by cAMP-dependent protein kinase (Huang & Glinsman, 1975; Cohen et al., 1977; Nimmo & Cohen, 1978). In skeletal muscle the extent of phosphorylation of inhibitor-1 is increased by epinephrine treatment (Foulkes & Cohen, 1979) and decreased by insulin, which antagonizes the effects of low concentrations of β-adrenergic agonists (Foulkes et al., 1982) on proteins which are substrates for protein phosphatase-1 and which are phosphorylated by cyclic AMP-dependent protein kinase e.g. glycogen synthase (site 2) (Cohen, 1982). This represents a mechanism by which inhibitor-1 may prevent dephosphorylation by protein phosphatase-1 and so amplify the effects of cyclic AMP on their phosphorylation. In addition, since protein phosphatase-1 also acts on proteins phosphorylated by other protein kinases e.g. glycogen synthase (sites 3a+b+c) (Parker et al., 1982) the inhibition of this enzyme by phospho-inhibitor-1 may allow cyclic

AMP to modulate the phosphorylation of these substrates. A protein with properties that are very similar to those of inhibitor-1 has been isolated from liver (Knight & Tal, 1980; Goris et al., 1978) and therefore it is possible that this mechanism may exist for the regulation of HMG-CoA reductase and reductase kinase by reversible phosphorylation.

(2) The second mechanism known for the regulation of protein phosphatase-1 involves the reversible association of the catalytic subunit with inhibitor-2 (Hemmings et al., 1982; Resink et al., 1983). Dissociation of this inhibitor activates protein phosphatase-1 and depends on the phosphorylation of the latter on a threonine residue (Hemmings et al., 1982). Consequently, when associated with inhibitor-2, phosphatase-1 activity is ATP-dependent. Although inhibitor-2 (Khandelwal & Zinman, 1978; Goris et al., 1979) and a suitable protein kinase, thought to be glycogen synthase kinase-3 (Hemmings et al., 1981; Yang et al., 1980), appear to be present in the liver, the physiological role of this interconversion system in liver or other tissue remains to be established.

The relative contributions of protein phosphatases-1, -2A and -2C to the dephosphorylation activity towards HMG-CoA reductase and reductase kinase in vivo remains unclear. Protein phosphatase-2C shows the greatest activity on HMG-CoA reductase and reductase kinase-phosphate accounting for 60-70% and 95-100% of the dephosphorylating activity at high and low dilutions of liver homogenates respectively (Ingebritsen et al., 1983b). The prominence of protein phosphatase-2C action may be due to the inhibition of protein phosphatases-1 and -2A in concentrated

extracts; however, such a degree of inhibition may not exist *in vivo*. It is noteworthy that a significant fraction of protein phosphatase-1 alone among four phosphatases in the liver (20%) is associated with the microsomal fraction (Ingebritsen *et al.*, 1983b) and consequently this phosphatase would be expected to be most likely to act as a microsomal membrane enzyme such as HMG-CoA reductase *in vivo*.

1.5 Intracellular Phosphorylation of HMG-CoA Reductase

Numerous previous investigations have been performed to determine whether the phosphorylation state of HMG-CoA reductase is capable of modulation within the intact cell. Ingebritsen *et al.* (1979) demonstrated that the phosphorylation state of the enzyme could be modulated by hormones in isolated rat hepatocytes. These workers deduced these changes in the phosphorylation state from changes in the rates of HMG-CoA reductase activity measured in microsomes isolated from cell homogenates prepared in the presence of F^- and EDTA, to block endogenous protein phosphatase and kinase activities respectively ('expressed' activity; 'E'; see Section 2.8.3) to activity determined after the incubation of microsomes prepared in the absence of F^- (but with EDTA) with sufficient added partially purified protein phosphatase to fully activate the enzyme ('Total' activity, 'T' see Section 2.8.3); this ratio is designated as the E/T ratio (see Section 2.8.3) In control cells i.e. incubated in the absence of hormones the E/T ratio of HMG-CoA reductase activity which was initially low (approximately 20%) showed a marked increase (to approximately 95%) between 90 and 150 min of incubation.

Whereas the addition of glucagon to this incubation completely antagonized the rise, an effect mimicked by dibutyryl-cyclic AMP, insulin markedly enhanced this increase. Glucagon and insulin also had opposing effects (enhancement and inhibition respectively) on a spontaneous decline in total activity observed in control cell incubations.

Recently, Henneberg and Rodwell (1981, 1985) have further showed that glucagon can specifically lower the E/T ratio of HMG-CoA reductase rather than merely antagonize a spontaneous increase in this parameter as indicated in the study of Inegbritsen et al. (Henneberg & Rodwell, 1985). These effects were also mimicked by cyclic AMP and cyclic GMP. The effects of glucagon represent a paradox since both reductase kinase and reductase kinase kinase belong to the cyclic nucleotide-independent class of protein kinases (see Section 1.4.3.1). Nevertheless, Ingebritsen et al. (1979) showed that glucagon (and insulin) stimulated (and insulin diminished) the expressed activity of reductase kinase and subsequently proposed the involvement of protein inhibitor-1 through its phosphorylation by cyclic AMP-dependent protein kinase (see Section 1.4.3.2(i) and Chapter 5).

Subsequently two groups of workers demonstrated that changes in the phosphorylation state of HMG-CoA reductase could be provoked in the intact rat in vivo by the acute administration of pharmacological doses of glucagon or large amounts of mevalonalactone or cholesterol. Beg and coworkers demonstrated that the incorporation of [^{32}P]-phosphate from [γ-^{32}P]-ATP into HMG-CoA reductase protein was increased following the acute intraperitoneal

injection of glucagon (Beg et al., 1980) or the oral administration of mevalonolactone by stomach intubation (Beg & Brewer, 1982). In the case of this latter treatment and similar treatments of cholesterol, the increased [^{32}P]-phosphate content of HMG-CoA reductase was accompanied by changes in the activity of the enzyme (Beg & Brewer, 1982). These changes which were confirmed by immunotitration studies (Arebalo et al., 1981, 1982). (The studies concerned with the effects of cholesterol and mevalonate are discussed in detail in Chapter 6).

Whilst these observations suggest that changes in the phosphorylation state occur in vivo, attempts at detecting such changes in the liver in vivo in response to normal physiological hormonal or nutritional changes e.g. the diurnal rhythm (Jenke et al., 1981) or short-term starvation (Brown et al., 1979) have been unsuccessful. Studies by Nordstrom et al. (1977), Brown et al. (1979) and Dugan et al. (1982) led to the conclusion that under normal physiological conditions HMG-CoA reductase is always largely (about 85%) inhibited in spite of the large range of the rates of hepatic cholesterol synthesis in vivo (see e.g. Rodwell et al., 1976). Consequently, the view that the modulation of the phosphorylation state of HMG-CoA reductase such as observed in hepatocytes or in response to acute stimuli is not relevant to the control of the enzyme in normal physiological conditions has persisted (see e.g. Berndt et al., 1976; Hardgrave et al., 1979; Kleinsek et al., 1980; Jenke et al., 1981; Dugan et al., 1982; Liscum et al., 1983b; Gregg & Wilce, 1983; Ness, 1983) in spite of some evidence to the contrary (Edwards et al., 1980). In many of

these studies, it has been suggested that the observed changes in the expressed activity of HMG-CoA reductase could be fully accounted for by changes in the amount of enzyme protein in the liver.

By analogy with the physiologically induced changes in the activity state *in vivo* of other enzymes regulated through a phosphorylation-dephosphorylation mechanism (e.g. see Cohen, 1982) it is highly unlikely that the bicyclic protein phosphorylation mechanism described for the regulation of HMG-CoA reductase activity goes largely unused *in vivo*. Therefore it is possible that the paradoxical situation evident in the literature could be explained if the expected changes in the phosphorylation state of HMG-CoA reductase induced by physiological stimuli such as occur during the diurnal cycle of feeding and short-term starvation have been overlooked. If this is the case then the modest change observed in the livers of animals challenged acutely with unphysiological concentrations of hormone or dietary manipulation could be only the residual effects of much larger responses. Therefore, the main aim of the work reported in this present thesis was to resolve definitively the controversy as to whether changes in the phosphorylation state of HMG-CoA reductase do occur *in vivo* and to investigate quantitatively any changes in the phosphorylation state that occur in response to physiological stimuli.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

The rats used in this study were of the Wistar strain obtained from A. Tuck & Son, Battlesbridge, Essex, U.K. The animal house was maintained at temperatures between 20 and 25°C and on a 12h light:12h dark cycle. The timing of the cycle was adjusted to satisfy the requirement of each experiment but a period of at least 10 days was allowed for the animals to adapt to any new lighting regimen. Male rats used in experiments described in Chapters 3, 4 and 5 were housed from birth in this lighting regimen. The animals were fed Oxoid breeding diet (Oxoid, Basingstoke, Hants, U.K.) ad libitum and were used within a weight range of 160-200g (i.e. 5-6 wks old). The male rats used in Chapter 6 were delivered to the animal house at a weight of 70-90g and kept for 2 weeks on the same lighting regimen before use at the same weight as above (i.e. 160-200g). These animals were maintained on a Labsure diet (K. & K.-Greeff Ltd., Croydon, U.K.).

Female rats used for this study into the effects of pregnancy and lactation were transferred to the reversed lighting regimen during the first week of pregnancy except for animals used on day 12 of pregnancy which were time-mated under the required lighting regimen. The rats weighed 280-300g at day 12 of pregnancy, 230-270g and 340-370g on day-1 and day-12 of lactation respectively. The day of conception, determined from the appearance of vaginal plugs, was

counted as day-0 and animals littered on day-21. Animals used to study the effects of weaning had their pups removed on day-11 of lactation 24h before being killed.

Animals were trained individually to handling for several minutes each day for 4 days prior to killing in order to minimize the effects of stress on the day of the experiment. Anaesthesia for normal rats was achieved through the intraperitoneal injection of pentobarbital (60mg/kg body wt.) solution prepared in physiological (0.9% NaCl) strength saline immediately before use. Animals were allowed to settle for 20 min before the abdominal cavity was opened. A reduced (67% of normal) and an increased (125-150% of normal) dose of pentobarbital was used to anaesthetize diabetic and late-pregnant animals respectively. These doses were required to achieve the same degree of anaesthesia achieved for animals in other physiological states.

2.2 Induction of Experimental Diabetes

Diabetes was induced in male animals (140-160g) by intraperitoneal injection of the β-cell cytotoxic drug, streptozotocin (150mg/kg body wt.). Solutions of the drug (40mg/ml) were prepared immediately before use in citrate-saline (pH 4.5). Diabetes was allowed to develop in the animals and they were used 7-8 days later. For the first 2 days after treatment, the animals were given a 1% glucose solution to drink. This treatment produced an elevation in blood glucose concentrations between approximately 10-40mM.

2.3 Stomach Intubation

The rats were anaesthetized with 80% of the normal dose of pentobarbital and closely observed. When the animals became relaxed but still able to swallow a tube inserted into their mouths, they were intubated. This procedure facilitated the guidance of the plastic tube into the stomach and deleterious effects in the normal respiratory pattern of the animals were minimized.

Mevalonolactone (750-780mg/kg body wt.) and four fractions of spray-dried milk (whole milk, buttermilk, whey proteins and skim milk; 1.8g/kg body wt.; see Section 2.14) were administered in distilled water; control animals were given distilled water only. Further anaesthesia was given as required in order to induce full anaesthesia after the intubation was completed. The abdominal cavities were opened and the livers of these animals sampled 20-30 min later.

2.4 Hormonal Treatment

Insulin (30U/kg body wt.), glucagon (1.25mg/kg body wt.) and epinephrine (0.6-1.5μg/kg body wt.), were administered to anaesthetized rats intravenously via a Monoject fine-guaged needle (27G x ½"; Sherwood Medical Industries, Crawley, West Sussex, U.K.) inserted into the femoral vein previously exposed by a small incision of the skin. When administered intraperitoneally, the dose of insulin was higher (120U/kg). Insulin and glucagon were dissolved in acidified saline (0.9% NaCl, pH 3-4; 1mg/ml). Epinephrine was prepared at 1000-times the required concentration (0.8mg/ml) in 10mM ascorbic acid and diluted immediately prior to use in saline. Saline

or diluted ascorbic acid minus hormone was administered to control rats as appropriate.

Somatostatin (cyclic) was infused intravenously to suppress the endogenous secretion of insulin and glucagon from the pancreas on subsequent administration of either of these hormones. Using a syringe pump (Sage, Model 341, A.R. Horwell Ltd, Kilburn High Rd., London, SW6 2BP) a continuous infusion (100-200 μg/kg body wt. per h) was maintained for 20 min through a catheter dwelling in the femoral vein.

2.5 Administration of Adrenergic Antagonists

Prazosin (50 μg/kg body wt. in 0.8mM-lactic acid) and propranolol (125 μg/kg body wt. in saline), specific α_1 and non-selective β-adrenergic receptor blockers respectively were administered by intravenous infusion to rats anaesthetized with ether 1h prior to killing (as above). Immediately after the infusion a local anaesthetic, Lignovet (C-Vet Limited, Bury St. Edmunds, U.K.) was administered to the skin in the area surrounding the incision and the wound stitched using a Mersilk silk suture (4% guage, round bodied 16mm needle; Ethicon, Edinburgh, Scotland, U.K.). The animals, which showed no signs of discomfort, were anaesthetized as described above after 40 min and the liver sampled 20 min later.

2.6 Biochemicals

Inorganic chemicals used were of the highest grade commercially available and were generally obtained from Fisons Scientific Apparatus (Loughborough, Leics., LE11 ORG, U.K.), (for

details see Easom & Zammit, 1984). In addition, dicyclohexylcarbodiimide was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, SP8 4JL, U.K.). Insulin (crystalline, pig), epinephrine (free base), and propranolol-HCl (crystalline) were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Crystalline pig glucagon and streptozotocin were generously given by Lilly Research Laboratory (Indianapolis, I.N., U.S.A.) and the Upjohn Co. (Kalamazoo, N.I., U.S.A.) respectively. Cyclic somatostatin was obtained from Bachem U.K. (Saffron Walden, Essex, CB10 1AA, U.K.). Prazosin was a generous gift from Pfizer Central Research (Sandwich, Kent, CT13 9NJ, U.K.).

The following radioactive biochemicals were obtained from Amersham International plc (Amersham, Bucks., HP7 9LL, U.K.): 3-hydroxy-3-methyl[3-^{14}C] glutaric acid, DL-[2-^{3}H] mevalonic acid lactone and 3′,5′-cyclic [8-^{3}H] AMP.

2.7 Synthesis of 3-hydroxy-3-methyl[3-^{14}C] glutaryl-coenzyme A

3-Hydroxy-3-methyl[3-^{14}C] glutaryl-coenzyme A (HMG[3-^{14}C]-CoA) was synthesized by either of two methods: (i) The first involved the formation of a high energy anhydride intermediate after the nucleophilic reaction of HMG acid on dicyclohexylcarbodiimide (DCCD) as described by Goldfarb & Pitot (1971). HMG[3-^{14}C]-CoA was purified by paper chromatography. Elution from the chromatogram with ice-cold distilled water was accelerated using centrifugal force (1500g, 5 min) as described by Edstrom (1968). This method resulted in yields of 62-68% with final specific activity ranging from 0.30-0.70 Ci/mol.

(ii) The second method was essentially the same as that described above but with the omission of the recrystallization of HMG-anhydride. This method (described by Williamson & Rodwell, 1981) involved the separation of HMG[3-^{14}C]-CoA by ion-exchange column chromatography using DEAE-cellulose (DE-52, 20cm x 2.5cm Whatman, Maidstone, Kent, U.K.). Elution was performed with a gradient (0.1-0.4M) of ammonium formate, pH 4.4. The recovery of radioactivity from the column (routinely at 0.20-0.22M formate) approached 100% as ensured by the inclusion of a 4-fold excess of lithium-CoA in the reaction with HMG-anhydride. The yield of HMG[3-^{14}C]-CoA was 70-75%. The specific activity of HMG-[3-^{14}C]-CoA synthesized by this method was much higher (15-18 Ci/mol) and after suitable dilution with cold HMG-CoA was used at a final specific activity of 1.25 Ci/mol for studies in animals characterized by low activities of HMG-CoA reductase e.g. in diabetic animals.

The concentration of HMG [3-^{14}C]-CoA was determined by the complete conversion to [3-^{14}C]-acetoacetate after incubation with either sonicated rat liver mitochondria or freeze-clamped liver homogenate (50μl). The assay medium contained 55mM-Tris/HCl (pH 8.5) and 5.5mM-$MgCl_2$; the total volume was 450μl. The reaction was stopped after 120 min with 3% PCA (500μl). The denatured protein was sedimented by centrifugation (8000g, 1 min) in an Eppendorf 4312 centrifuge, and the supernatant neutralized by the addition of solid $KHCO_3$. After repeating the centrifugation step, a portion of the supernatant was assayed for acetoacetate by the spectrophotometric method described by Mellanby & Williams (1974).

The assay medium contained 50mM-KH_2PO_4 pH 7.0; 0.2mM-NADH in a total volume of 2ml. The reaction was started by the addition of 0.1U -hydroxybutyrate dehydrogenase and the difference in absorbance at 340nm determined after the reaction was complete.

2.8 Determination of Hepatic HMG-CoA Reductase Activity

2.8.1 Preparation of Liver Homogenate

Unless otherwise indicated, liver was sampled and homogenized by the cold-clamping technique described in Chapter 3 (see Easom & Zammit, 1984). The clamped liver sample was homogenized by three passes in a Teflon/glass homogenizer (approximately 25ml) (radial clearance 0.20-0.25mm) driven by a constant-torque motor (Tri-R Stir-R, Model K43, Camlab Ltd., Cambridge, CB4 1TH, U.K.) operated at 1000 rev/min. The ice-cold homogenization medium contained 50mM-sucrose, 10mM-Tris/HCl (pH 7.4), 100mM-KF, 10mM-EDTA, 1mM-DTT and 0.5mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was centrifuged at 5000g for 10 min; the procedure was then repeated. These were optimal conditions for the maximal removal of intact mitochondria concomitant with minimal loss from the supernatant of microsomal cytochrome-c oxido-reductase activity (Fig. 2.1).

The final supernatant was divided into two parts, and both were centrifuged at 100,000g for 45 min. One of the resulting microsomal pellets was resuspended in a buffer containing 50mM-KH_2PO_4, pH 7.25, 50mM-KCl, 100mM-KF and 1mM-EDTA and the other in a similar buffer in which the KF had been replaced by an equivalent concentration of KCl. After a further centrifugation at 100,000g for 45 min, the pellets were resuspended in the respective

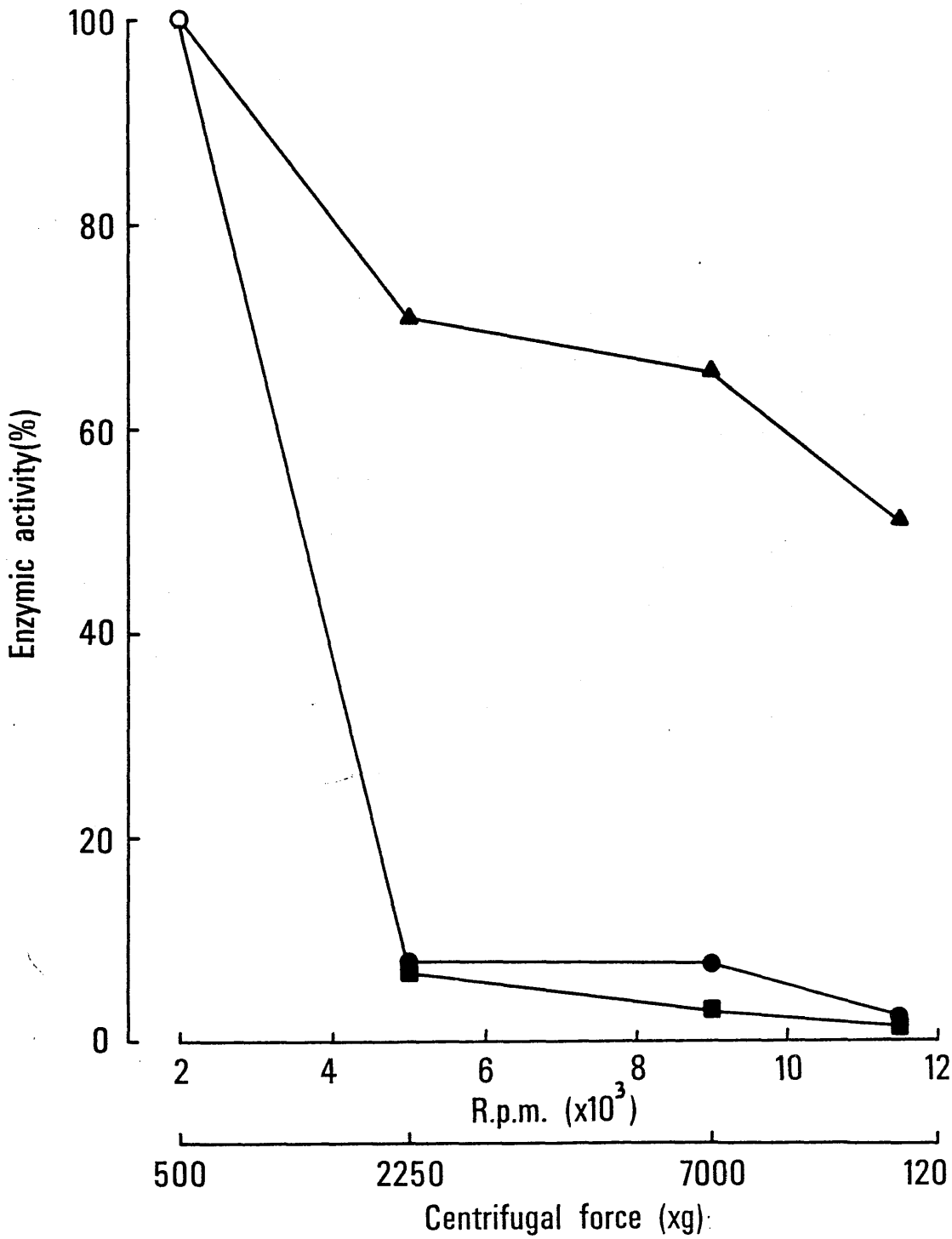


Fig. 2.1 Determination of optimal conditions for selective separation of mitochondria from microsomal membranes

Mitochondria marker enzymes, citrate synthase (inner membrane, ●), glutamate dehydrogenase (matrix, ■), and microsomal marker enzyme, cytochr c-oxido-reductase (▲) were determined in the supernatant of cold-clamped liver samples (see Chapter 3) subsequent to centrifugation at the indicate speed (10 min, 4°C). Values are represented as a percentage of enzyme activities recorded in the same volume of homogenate subsequent to the sedimentation of cell debris by centrifugation at 2000 rpm (~500xg).

buffers to a concentration of approximately 8-10mg of microsomal protein/ml and used for the assay of HMG-CoA reductase.

Freeze-clamped liver samples were processed by pulverization in an aluminium mortar and pestle and then homogenized (2x10s) with a Polytron disintegrator (Kinematic, Basle, Switzerland) in ice-cold buffer (50mM-KH_2PO_4, pH 7.4), 15mM-EDTA, 15mM-EGTA, 10mM-DTT, 0.5mM-PMSF and 0.1mg/ml of each of trypsin inhibitor, antipain, pepstatin and leupeptin (Sigma Chemical Co., Poole, Dorset, U.K.)). The homogenate was centrifuged (8000g; 30s) and the supernatant used for the assay of HMG-CoA reductase.

2.8.2 Assay of HMG-CoA Reductase

HMG-CoA reductase activity was assayed at 37°C essentially as described by Shapiro et al., 1974, except that the assay volume was 75µl. Microsomes (20µl) were preincubated for 2 min in a total volume of 50µl and the reaction was started by the addition of 25µl of cofactor solution containing 2.5 moles glucose-6-phosphate, 0.15U glucose-6-phosphate dehydrogenase, 225 nmoles NADP , 25 nmoles DL-HMG[3-^{14}C]-CoA and 11,000-19,000 dpm DL-[2-^{3}H] mevalonic acid lactone. The reaction was stopped after 15 min by the addition of 25µl of 5M-HCl. The tubes were centrifuged for 1 min to sediment denatured protein. After incubation at 37°C to effect complete lactonization, samples (75µl) were applied to activated t.l.c. plates (silica gel G; 0.5mm thickness) and chromatographed vertically in toluene/acetone (1:1, v/v). In this system, the non-polar product mevalonate (R_f = 0.55-0.75) is clearly resolved from the more polar HMG-CoA (R_f = 0.1-0.3). The product (located by visual detection of a concentrated mevalonolactone standard marker run in parallel on the

same plates) was recovered from the gel scraped off the plates by extraction with 2 vol. (7.5ml) of diethyl ether.

The combined extracts were dried down at 40°C and the residue was dissolved in water (400 l) to which 10ml of scintillant (Scintillator 299, Packard) was added. The amount of radioactivity due to ^{3}H and ^{14}C was measured in a Packard Tri-Carb liquid-scintillation counter and the results were corrected for the efficiency of extraction using the added [^{3}H] mevalonolactone as an internal standard. This was found routinely to be 70-80%.

The formation of mevalonolactone product was linear with respect to both time (Fig. 2.2) and microsomal protein concentration (Fig. 2.2) over the ranges used in the experiments. Controls from which $NADP^{+}$ was omitted were run concurrently. These allowed the subtraction from the total ^{14}C-labelled products formed of the activity of HMG-CoA lyase (see Chapter 3).

2.8.3 Activation of HMG-CoA Reductase by Exogenous Purified Protein Phosphatase to Obtain Total Activity

Total dephosphorylation and activation of the enzyme was achieved by incubating microsomes (160-200 g of protein) with 1-4U of exogenous protein phosphatase partially purified from rat liver (see Section 2.12) in the presence of 2mM-$MnCl_2$ (concentration of free Mn ions was approximately 1mM). Under these conditions the activation was rapid and complete after approximately 5 min; a typical progress curve of the phosphatase-induced activation of HMG-CoA is shown in Fig. 2.3. Routinely, microsomes were incubated for 10-15 min to ensure the attainment of maximal activity (total

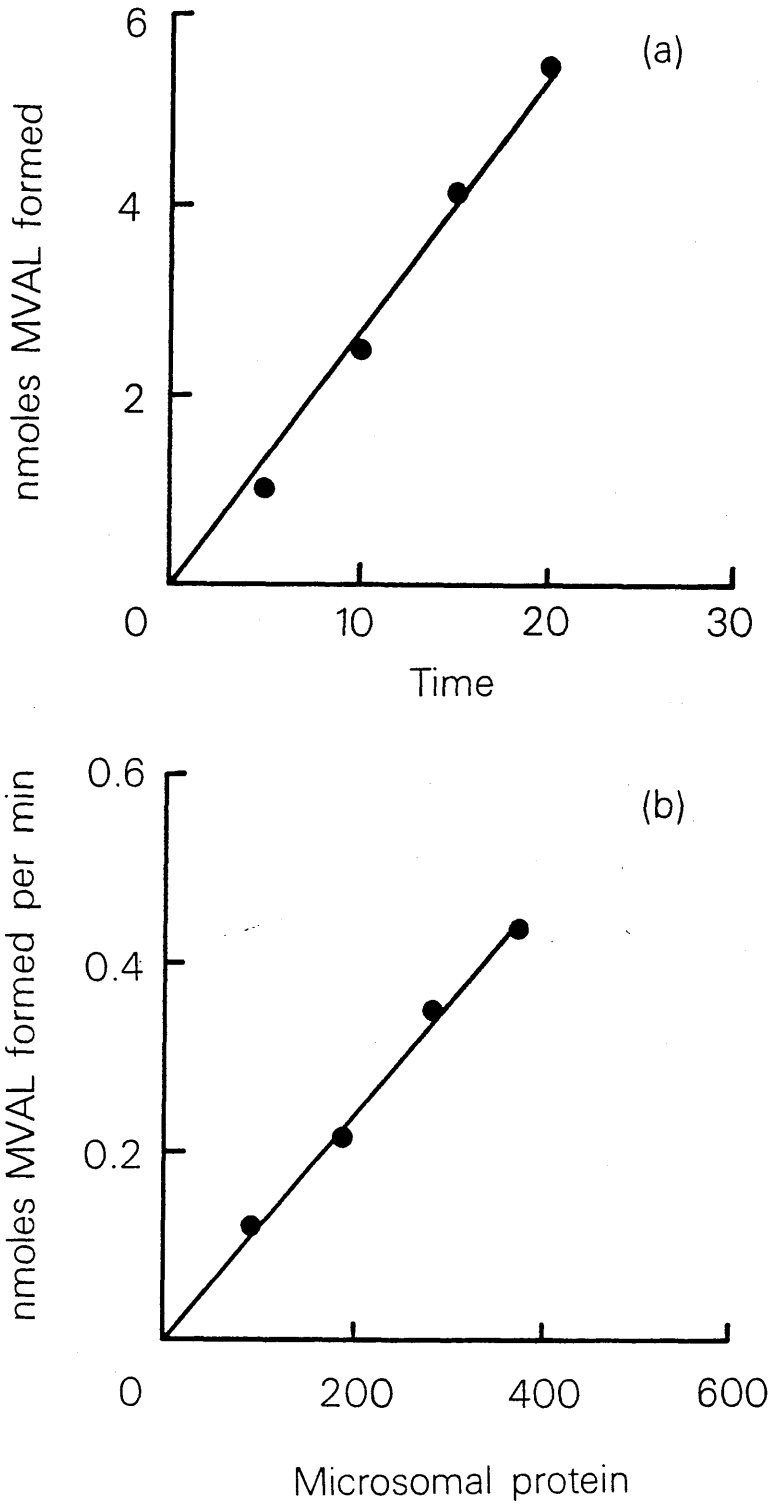


Fig. 2.2 Effect of (a) time and (b) protein concentration on HMG-CoA reductase activity in isolated rat liver microsomes

Microsomes were prepared from homogenate of cold-clamped liver sample and assayed for HMG-CoA reductase activity subsequent to activation by partially purified protein phosphatase (Section 2.8). (a) Assay mix (75µl) contained 112µg microsomal protein and (b) assay time was 15 min. MVAL; mevalonolactone.

activity) of HMG-CoA reductase. Controls from which protein phosphatase was omitted, but in which 100mM-KF was included were run concurrently. No activation of HMG-CoA reductase was evident in these samples (Fig. 2.3). (The incubation media for phosphatase-treated and non-phosphatase-treated microsomes were equalized with respect to KF concentration before assay of HMG-CoA reductase activity).

2.8.4 Expression of Activity of HMG-CoA Reductase

By analogy with terminology used for other enzymes that are regulated by covalent modification, the activity of HMG-CoA reductase obtained before full activation with phosphatase treatment is referred to as the 'expressed' activity, whereas that of the fully activated enzyme is designated 'total' activity. The fraction of the enzyme in the active form is presented as the expressed/total-activity ratio (E/T ratio). Absolute values of HMG-CoA reductase for both expressed and total activities are presented as nmoles/min per mg of microsomal protein at 37°C.

2.9 Assay of Other Enzymes

2.9.1 Radiochemical Assay of HMG-CoA Lyase

The activity of HMG-CoA lyase in homogenates of freeze-clamped liver samples was determined as described by Clinkenbeard et al., (1975a). The assay medium contained 50mM-Tris/HCl (pH 8.5), 5mM $MgCl_2$, 1.5mM $CaCl_2$, 0.2mM-HMG[3-^{14}C]-CoA in a final volume of 100µl. The incubations at 30°C were started with homogenate (10µl) and after

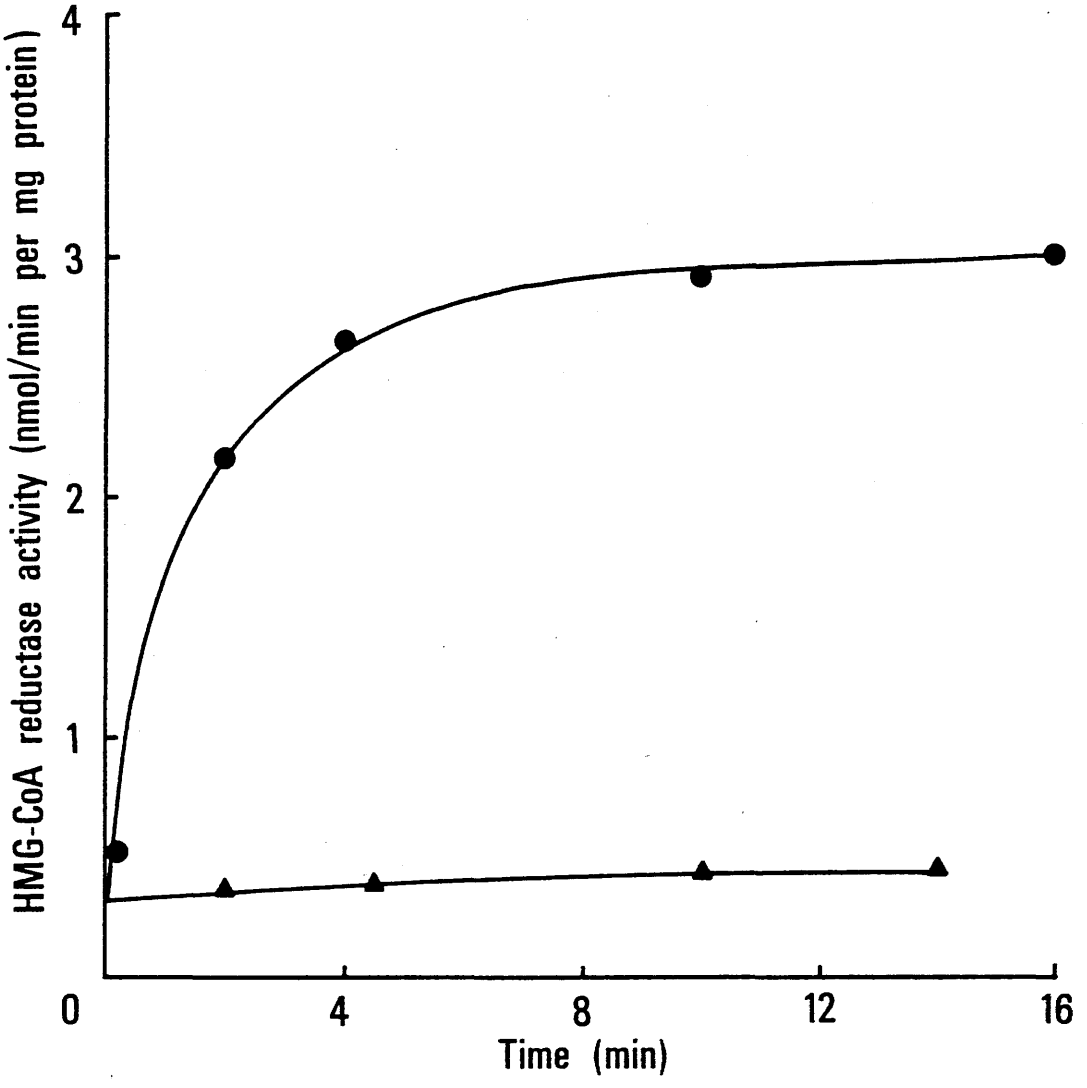


Fig. 2.3 Time course of activation of HMG-CoA reductase in rat liver microsomes by exogenous, partially purified liver protein phosphatase

Microsomes prepared from the liver of a rat killed by cervical dislocation were incubated with protein phosphatase (approx. 4U) in the presence of 100mM-KCl (●) or 100mM-KF (▲). At the intervals shown, samples of the incubation mixtures were assayed for activity of HMG-CoA reductase.

various lengths of time (0-10 min, to ensure linearity of reaction) 0.08ml aliquots were transferred to glass vials containing 0.2ml 6N HCl. The volatile product [3-^{14}C]-acetoacetate was removed by taking this aliquot to dryness at 95^{o}C under a constant flow of air and the non-volatile HMG [3-^{14}C]-CoA remaining was dissolved in water (0.4ml) and the radioactivity counted as described above (Section 2.8.2).

2.9.2 Pyruvate Kinase

Pyruvate kinase activity was measured as described by Zammit et al. (1978). The assay coupled pyruvate formation via lactate dehydrogenase to NADH oxidation. The change in absorbance at 314nm was followed at 25^{o}C using a recording spectrophotometer. The assay medium contained 160mM-TRA, 15mM-MgCl , 80mM-KCl, 5mM-ADP, 0.2mM-NADH, 3-6U lactate dehydrogenase and various concentrations of phosphoenolpyruvate (PEP; Tricyclohexylammonium salt) (see section 3.3.2). The reaction was started by the addition of PEP. Controls omitting ADP were run concurrently.

Phosphorylation of pyruvate kinase in fluoride-containing homogenates of freeze-clamped liver (100µl) was performed by addition of a mixture (100µl) containing 10mM-ATP, 5mM-$MgCl_2$, 100µm-cyclic AMP and 2 units of catalytic subunit of cyclic AMP-dependent protein kinase. In preliminary experiments the mixture was incubated at 37^{o}C and samples were taken at intervals and assayed for pyruvate kinase activity (with 0.6mM PEP) until a constant minimum activity was obtained. Routinely, phosphorylation was performed for 20-30 min. The homogenates (termed 'phosphorylated') were then used to determine the activity of pyruvate kinase at various concentrations of PEP.

2.9.3 Citrate Synthase

Citrate synthase was assayed spectrophotometrically by the method of Srere et al. (1963). The rate of release of CoASH from acetyl-CoA was followed at 412nm and 25°C by use of DTNB. The assay medium contained 50mM-Tris/HCl (pH 7.3), 0.2mM-DTNB, 0.1mM-acetyl-CoA, 0.2mM-oxaloacetate and 0.5% (v/v) Triton X-100. The total volume was 1.05ml. The reaction was started with homogenate (10 l). Controls, omitting oxaloacetate, were run concurrently.

2.9.4 Glutamate Dehydrogenase

The assay for glutamate dehydrogenase was based on that described by Schmidt (1963). The assay medium contained 70mM-Tris HCl (pH 7.5), 100mM-NH acetate, 2.5mM EDTA, 0.2mM-NADH, 1mM-ADP, 0.1% (v/v)-Triton X-100, and 2.5% (w/v) antimycin A to which was added 10 l sample. The total volume was 2.0 ml. The reaction was started by the addition of 2-oxoglutarate (20 l; final concentration 8mM) and the decrease in absorbance at 340nm due to oxidation of NADH was followed continuously at 25°C. Controls omitting 2-oxoglutarate were run concurrently.

2.9.5 Cytochrome-c (NADPH) Oxido-Reductase

The assay for cytochrome-c oxido-reductase was based on that described by Brown et al. (1976). The assay medium contained 900mM-K_2HPO_4 (pH 7.6), 4.05mM-EDTA, 0.09%-Triton X-100, 50mM-NaN_3, 0.4mg/ml-cytochrome-c to which was added 10 l of sample. The total

volume was 1.0ml. The reaction was started with NADPH (20μl; final concentration 0.2mM) and the NADPH-dependent reduction of cytochrome-c was followed continuously at 550nm and at 25^{o}C using a recording spectrophotometer. Controls omitting NADPH were run concurrently.

2.10 Measurement of the Rate of Mitochondrial Oxygen Consumption

Oxygen upake was measured using a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) as described by Zammit (1980b). The oxygen electrode was standardized by determinations of the change in the output from the electrodes on addition of portions of spectrophotometrically standardized NADH to 2.2ml of 'basal' medium (see below) containing lysed mitochondria (2mg of mitochondrial protein) and 1mg of cytochrome-c. Mitochondrial preparations (0.2ml) were added to a basal medium (2.0ml) containing 8mM-Tris/HCl (pH 7.4), 176mM-sucrose, 32mM-KCl, 0.8mM-EGTA, 2.5mM KH_2PO_4 and 10mg/ml-BSA. Oxygen consumption was measured after the addition of ADP (state-3) and its exhaustion (state-4) using malate and glutarate (10μl of 1M solution of each as substrates).

2.11 Determination of Metabolites

2.11.1 Determination of Blood Glucose

Whole blood samples obtained either from the tail (incision of distal tip of tail) or the aorta (immediately after sampling the liver) were mixed with an equal volume of ice-cold 3% (v/v) $HClO_4$. The concentration of glucose in the neutralized supernatant obtained after sedimentation of the protein was measured as described by

Bergmeyer et al. (1974). The assay coupled the hexokinase-catalyzed formation of glucose-6-phosphate (G6P), via glucose-6-phosphate dehydrogenase (G6PDH), to the reduction of $NADP^+$. The assay medium contained 200mM-Tris/HCl (pH 7.4), 100mM-KCl, 20mM-$MgSO_4$, 0.25mM-$NADP^+$, 2.0mM-ATP, 3.5U-glucose-6-phosphate dehydrogenase and 4U-hexokinase. The total volume was 2.0ml. The reaction was started with hexokinase (1µl) and the reaction followed continuously using a recording spectrophotometer at 25°C.

2.11.2 Cyclic AMP in Freeze-Clamped Liver

Freeze-clamped liver samples were processed as described in section 2.8.1 but homogenization was performed in 5% PCA and for 40s at setting 4. The resulting samples were diluted to 40mg fresh wt./ml with 5% PCA and then centrifuged for 6 min at 8,000g and 0-4°C in a micro-centrifuge. The supernatant (pellet discarded) was neutralized with solid $KHCO_3$ and centrifuged for a further 3 mins. Aliquots (50µl) of this resultant supernatant were then assayed for cyclic AMP content using the method initially described by Gilman (1970) and modified by Tovey et al. (1974). The assay is based on the competition between the unlabelled cyclic AMP in the sample and a fixed amount of [^{3}H]-labelled nucleotide for binding to a protein which has a high affinity for cyclic AMP. The amount of labelled protein-cyclic AMP complex formed is inversely related to the amount of unlabelled cyclic AMP present in the assay sample. Each assay (final volume 0.2ml) contained 0.025µCi of cyclic [^{3}H] AMP. A

preparation of cyclic AMP-dependent protein kinase was used, at a final concentration of 25μg of protein/ml, as the source of cAMP binding protein. Equilibrium binding of cyclic [^{3}H] AMP in samples containing up to 20pmol of cyclic AMP was achieved in 3h at 0°C.

2.12 Partial Purification of Protein Phosphatase from Rat Liver

Protein phosphatase was prepared from rat livers by the method of Brandt et al. (1975) up to and including the first DEAE-cellulose step. The functions obtained from the column containing phosphatase activity were pooled and dialyzed overnight against 60% glycerol in imidazole buffer (50mM-immidazole/HCl (pH 7.45), 5mM-EDTA, 0.5mM-DTT). The dialysate was concentrated further to a final specific activity of 109-469U/ml by an ultrafiltration process initially through a Diaflo PM10>10,000Mr membrane (Amicon Ltd., Stonehouse, Glos., U.K.) and then to a smaller volume through an Ulvac, GIOT>10,000 Mr membrane (Chemlab Instruments Ltd., Hornchurch, Essex, U.K.). The final preparation was stored at -20°C in small batches which were dialysed for 3h against a buffer containing 50mM-immidazole/HCl (pH 7.4), 250mM-KCl and 1mM-EDTA immediately before being used. This preparation involves a 95%-ethanol precipitation step which is known to cause the dissociation of the catalytic subunits of protein phosphatase-1 and -2A from their native complexes (Ingebritsen et al., 1983a; see Section 1.4.3.2). Therefore, it is thought that this preparation is a mixture of the catalytic subunits Mr = 37kDa and 35kDa from phosphatases-1 and -2A.

Protein phosphatase activity was determined by the measurement of inorganic phosphate released from phosphorylase concomitant with

its inactivation as described by Brandt et al. (1975). The unit of activity is the amount of enzyme which releases 1 mol of P_i min at 30°C.

2.13 Preparation of Guinea-Pig Anti-Insulin Serum

Anti-insulin serum was raised in guinea pigs (300-400g) by the serial injection of porcine insulin (Leoretard, Leo Laboratories Ltd., Princess Risborough, Bucks., U.K.), in a paraffin oil mono-oleate emulsion by the method described by Robinson & Wright (1961). For the initial injection, insulin (20U/ml) was emulsified in approximately 3 vols of complete Freunds adjuvant (Miles Laboratories Ltd., Slough, U.K.) containing Mycobacterium butyricum to induce an immune response. This emulsion was administered subcutaneously to 6 distinct areas of the abdomen of the guinea pigs because of the susceptibility of the skin to ulceration. Subsequent injections of insulin (20U/ml) in similar proportions of incomplete adjuvant (i.e. without bacteria) were made at 21-day intervals. Blood (approximately 10ml) was obtained by cardiac puncture of conscious animals 14-21 days after the 3rd injection and each subsequent injection of insulin. Serum was separated from clotted blood by centrifugation (5000g, 10min, Coolspin, Fisons Scientific Apparatus, Loughborough, Leics., U.K.) and stored at -20°C. Control serum was obtained from untreated guinea-pigs by the same method.

2.14 Preparation and Analysis of Milk Fractions

2.14.1 Preparation

Whole cow's milk was supplied by the farm at HRI. Skim milk

(prepared by centrifugal separation of cream from whole milk), buttermilk (a by-product of butter-making) and a concentrated preparation of soluble whey proteins (40% total solids) were purchased from Mauchline Creamery, Ayrshire, Scotland, U.K.). Whole milk and bought-in products were pasteurized at 74°C and held for 15s. Whole milk, skim milk and buttermilk fractions were concentrated to approximately 40%, 45% and 45% total solids respectively by falling-film evaporation. The concentrated fractions were finally spray-dried using a gas-fired tall-form spray drier with nozzle atomization. Each fraction was fed into the drier at a temperature of 45°C and at a flow rate of 10gal/hr; the inlet temperature was approximately 190°C and the powder was collected at a temperature of 94°C. These preparations were kindly performed by Mr I.G. West, HRI.

Crude protein was assayed by an improved Kjeldahl nitrogen analysis devised by Dr J.C.D. White at HRI. On digestion of the milk (5ml) with concentrated sulphuric acid (25ml) in the presence of copper sulphate (50mg) and potassium sulphate (5g), nitrogen was converted to ammonium sulphate. Sodium hydroxide (57ml, 48% w/w) was added and the liberated ammonia was distilled into boric acid and subsequently titrated with 0.1N hydrochloric acid using 3 drops of a mixture (1:1, v/v) of methyl red (0.2% in 95% ethanol, v/v) and methylene blue (0.1% in 95% ethanol, v/v) as indicator. Crude protein was estimated as nitrogen (g/100g x 6.38). These analyses were kindly performed by Miss E. Noble, at HRI.

2.14.2 Compositional Analyses of the Milk Fractions

'Total solids' is a term applied to the dry residue obtained after the complete evaporation of water. The total solids content of each of the four milk fractions was estimated by the technique detailed in 'Methods for Chemical Analysis of Liquid Milk and Cream' (British Standard, 1741:1963). The only modification made to this method was that, following the initial drying step (in a boiling-water bath) samples were incubated in an oven at 102 $\pm$ 1°C for 3h.

Fat content was measured by the Gerber butyrometric method (British Standard, 1741:1963).

Lactose was assayed polarimetrically (Grimbleby, 1950) and calculation of results was by the procedure of Biggs & Szijarto (1963). An automatic polarimeter (Thorn Automation Ltd., Nottingham, Model 143D) was employed for this work and was calibrated using standard solutions of sucrose ('Analar' grade).

2.15 Determination of Protein Concentration

Protein concentrations were assayed by the method of Lowry et al. (1951).

CHAPTER 3

THE DEVELOPMENT OF COLD-CLAMPING: A TECHNIQUE SUITABLE FOR THE STUDY OF THE INTERCONVERSION OF HMG-CoA REDUCTASE BETWEEN ACTIVE AND INACTIVE STATES IN VIVO

3.1 Introduction

A paradoxical situation occurs in the literature in that whilst there is evidence to suggest that changes in the phosphorylation state of hepatic HMG-CoA reductase can be induced in in vitro systems e.g. isolated hepatocytes (see Ingebritsen, 1983) or in vivo by acute and non-physiological stimuli e.g. pharmacological doses of glucagon (see Beg & Brewer, 1982), no such changes can be demonstrated in the enzyme in vitro by varying the physiological situation of the animal despite large changes in the cholesterogenic capacity of the liver (see e.g. Brown et al., 1979); the enzyme was always observed to be highly phosphorylated and therefore mostly (> 80%) inactive (see Chapter 1; Section 1.5). However, as recognized by Ingebritsen et al. (1979) the major difference between these physiological studies in vivo and the studies on isolated cells in vitro was the ease and rapidity with which cells could be harvested and the enzyme assayed. The cells were homogenized in an appropriate medium containing inhibitors of protein kinase (i.e. EDTA) and phosphatase (i.e. fluoride ions; F^-) activities in order to arrest or slow down the metabolic changes which may otherwise alter the phosphorylation status of an enzyme such as HMG-CoA reductase (see Section 1.4 &1.5). Consideration of

the studies of the isolated cell system raised the possibility that the observation that HMG-CoA reductase consistently existed in a highly phosphorylated state *in vivo* (Brown *et al.*, 1979) was artifactual and was the possible consequence of the lengthy method of sampling used in the *in vivo* studies i.e. the conventional method of mincing, rinsing and homogenization of the tissue prior to preparation of microsomes by differential centrifugation.

These types of experimental problems have been encountered previously in the study of metabolic systems capable of very rapid changes *in vivo* both for the determination of the activity of enzymes which are regulated through reversible phosphorylation-dephosphorylation and for the measurement of intracellular metabolite concentrations. These studies require that the metabolic processes that affect the parameters studied namely enzyme activity (in the case of metabolites) and in the activity of protein kinases and phosphatases (in the case of phosphorylated-dephosphorylated enzymes) be instantaneously arrested. Artefacts arise from any delay between sampling of the liver and the cessation of metabolic activity which is likely to alter the state of the metabolic system from that existing *in vivo*. To minimize these artefacts extensive use has been made of the freeze-clamping technique (Wollenberger *et al.*, 1960) in which whole animals (e.g. insects) or tissues (e.g. liver, mammary gland) of anaesthetized larger animals are pressed between two metal blocks cooled in liquid N_2; this results in the very rapid cooling to $-197^{o}C$ and cessation of metabolic activity within the tissue and thus preserves the instantaneous state of metabolic concentrations and activity status

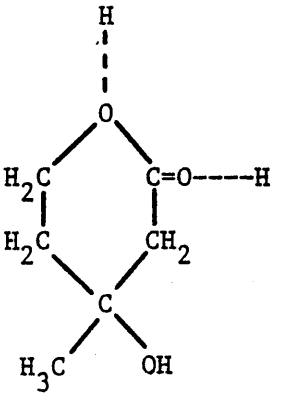
of the enzymes. Subsequent homogenization of the frozen tissue samples is performed in a medium which either destroys enzyme activity (e.g. in perchloric acid for the measurement of metabolites) or preserves the activity status of rapidly interconvertible enzymes (see above).

This technique has been successfully employed to demonstrate the modulation of the activity of a number of regulatory enzymes by phosphorylation-dephosphorylation in the liver and other tissues *in vivo*. For example, it has been used to demonstrate that the fraction of pyruvate dehydrogenase, an enzyme which regulates the formation of acetyl-CoA from pyruvate, that exists in the active (dephosphorylated) form varies in different physiological conditions e.g. starvation and refeeding in rat liver and adipose tissue (Stansbie *et al.*, 1976a). Also, in similar metabolic conditions, the involvement of equivalent alterations in the phosphorylation state in the regulation of acetyl-CoA carboxylase in the mammary gland (Zammit & Corstorphine, 1982) and in the liver (Stansbie *et al.*, 1976b) and of pyruvate kinase in the liver (see Engstrom, 1978) have also been successfully demonstrated.

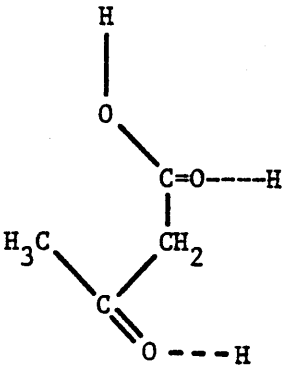
The inevitable consequence, however, of the freeze-clamping of e.g. liver samples is the disruption, on subsequent thawing and homogenization of the membranes of intracellular structures such as mitochondria. Therefore, the success of this technique in the determination of the activities of HMG-CoA reductase and other enzymes not only requires that the enzyme is stable to the actual process of freezing but also that the subsequent measurement of enzyme activity in the resulting crude homogenate is not impaired by

mutually interfering enzymes which, *in vivo*, occur in different cell compartments.

Such interference was in fact, encountered in the preliminary attempts to adopt the freeze-clamping technique to study the changes in the phosphorylation state of HMG-CoA reductase *in vivo*. This was the consequence of the formation from HMG-CoA of acetoacetate by HMG-CoA lyase, an enzyme of the ketogenic pathway of the liver after its leakage from the lysed mitochondria of homogenates of freeze-clamped tissue. After its visualization using the 2,4-dinitrophenylhydrazine reagent for the detection of ketoacids, aldehydes and ketones, it was shown that acetoacetate migrates with the same R_f value (0.55-0.75) as that of mevalonolactone in the TLC developing system used (see Methods). This is presumably due to the similarity of the polar parts of the two molecules (see Fig. 3.1). Because the activity of HMG-CoA lyase (per g wet wt. of liver) is approximately 2 orders of magnitude greater than that of HMG-CoA reductase (Table 3.1) in crude homogenates of freeze-clamped liver samples the formation of ^{14}C-acetoacetate (which appears as NADP-independent ^{14}C-radioactivity at R_f 0.55-0.75) always produced a high radioactivity background. Thus, using an estimate of the total microsomal protein content of liver (23.41 $\pm$ 1.75 mg/g wet wt.) determined by careful isolation of microsomes from tissue homogenized in the conventional way (see above), the incorporation of ^{14}C-material into R_f 0.55-0.75 was approximately 10-fold greater when freeze-clamped homogenate rather than an isolated microsomal membrane preparation was used in the HMG-CoA reductase assay conditions (i.e. 14.5 v 1.5 nmoles/min per mg of microsomal protein).



MEVALONOLACTONE



ACETOACETATE

Fig. 3.1 Similarity between the products of HMG-CoA reductase (mevalonolactone) and HMG-CoA lyase(acetoacetate) activities as shown in acid conditions.

TABLE 3.1

Comparison of the Absolute Activities of HMG-CoA Reductase and HMG-CoA Lyase in Rat Liver

Portions of a single liver were either freeze-clamped and used for the assay of HMG-CoA lyase activity, or excised for the preparation of microsomes in the conventional manner and assayed for HMG-CoA reductase activity. The expression of HMG-CoA reductase in the same units as HMG-CoA lyase (μmoles/g wet wt.) was enabled by the quantitative determination of the microsomal protein content of liver (see text).

	Range of Activities (μmoles/g wet wt)
HMG-CoA lyase	6.120-9.480
HMG-CoA reductase	0.035-0.058

In the intact cell *in vivo*, the chronic interference between the two enzymes is prevented by the physical separation of the two respective pathways in the cytosol (cholesterogenesis) and the mitochondria (ketogenesis) (Clinkenbeard *et al.*, 1975a see Fig. 3.2). Each pathway has been shown to be supplied by different pools of acetyl-CoA (Dietschy & McGarry, 1974) and to contain molecularly and catalytically distinct species of HMG-CoA synthase (Reed *et al.*, 1975; Clinkenbeard *et al.*, 1975b) and acetoacetyl-thiolase (Clinkenbeard *et al.*, 1973). In the conventional method of liver sampling (see above) the interference of HMG-CoA lyase in the assay of HMG-CoA reductase can be effectively eliminated by the subcellular fractionation of the HMG-CoA lyase-containing mitochondria from the reductase-containing microsomal fraction. In the case of the freeze-clamping technique however, unless there is some way in which HMG-CoA lyase can be eliminated from the crude homogenate formed, this technique must be considered unsuitable for the study of this enzyme.

These problems led to the development of a technique that was designed to satisfy the necessity to rapidly sample tissue and to arrest metabolic activity, but that allowed the subcellular fractionation of the liver. Mitochondria (and therefore HMG-CoA lyase) could thus be separated from microsomal membranes for the accurate determination of HMG-CoA reductase activity.

3.2 Elimination of HMG-CoA Lyase from Homogenates of Freeze-clamped Liver

Two approaches were made in an attempt to eliminate the

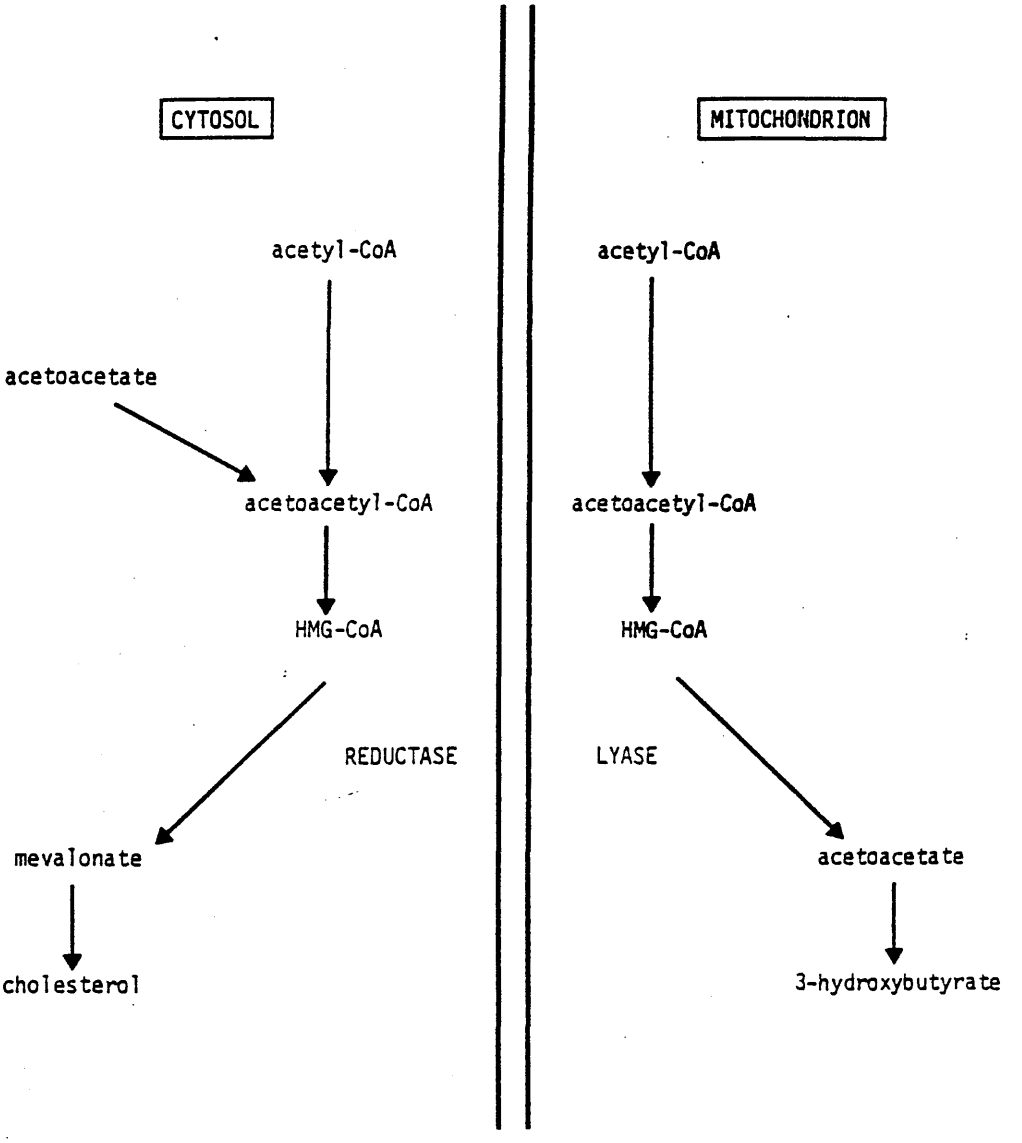


Fig. 3.2 Subcellular location of HMG-CoA reductase and HMG-CoA lyase enzymes and their involvement in the pathways of cholesterogenesis and ketogenesis respectively

interfering activity of HMG-CoA lyase from homogenation of freeze-clamped liver.

3.2.1 Ca^{2+}-sequestration

It has previously been demonstrated that HMG-CoA lyase of chicken liver is a Ca^{2+}-containing metalloprotein and that its activity is rapidly lost at $4^{o}C$ in the absence of this ion (Clinkenbeard et al., 1975a). Therefore the effects of the sequestration of Ca^{2+} (and Mg^{2+}) using specific chelators for these ions (EGTA and EDTA) on the activity of HMG-CoA lyase in homogenates of freeze-clamped liver were investigated in an attempt to specifically inhibit this enzyme and facilitate the assay of HMG-CoA reductase.

HMG-CoA lyase activity was measured spectrophotometrically (see Methods) in homogenates prepared in media containing either 50 mM-Tris/HCl pH 7.5, 250 mM-Sucrose, 2 mM-DTT (Buffer A) or 50 mM-K_2HPO_4 pH 7.4, 15 mM-EDTA, 15 mM-EGTA, 250 mM-NaCl, 2 mM-DTT (Buffer B). HMG-CoA lyase activity was markedly depressed when prepared in buffer containing the divalent cation chelators (Buffer B) to 33% of the activity observed in the control homogenate incubated in the presence of endogenous Mg^{2+} and Ca^{2+} (Fig. 3.3). When similar measurements were made after an incubation period of 3h at $0-4^{o}C$, HMG-CoA lyase activity in the presence of EDTA and EGTA was only 15% of the activity observed in the control medium; both activities had declined gradually over the 3h period (Fig. 3.3).

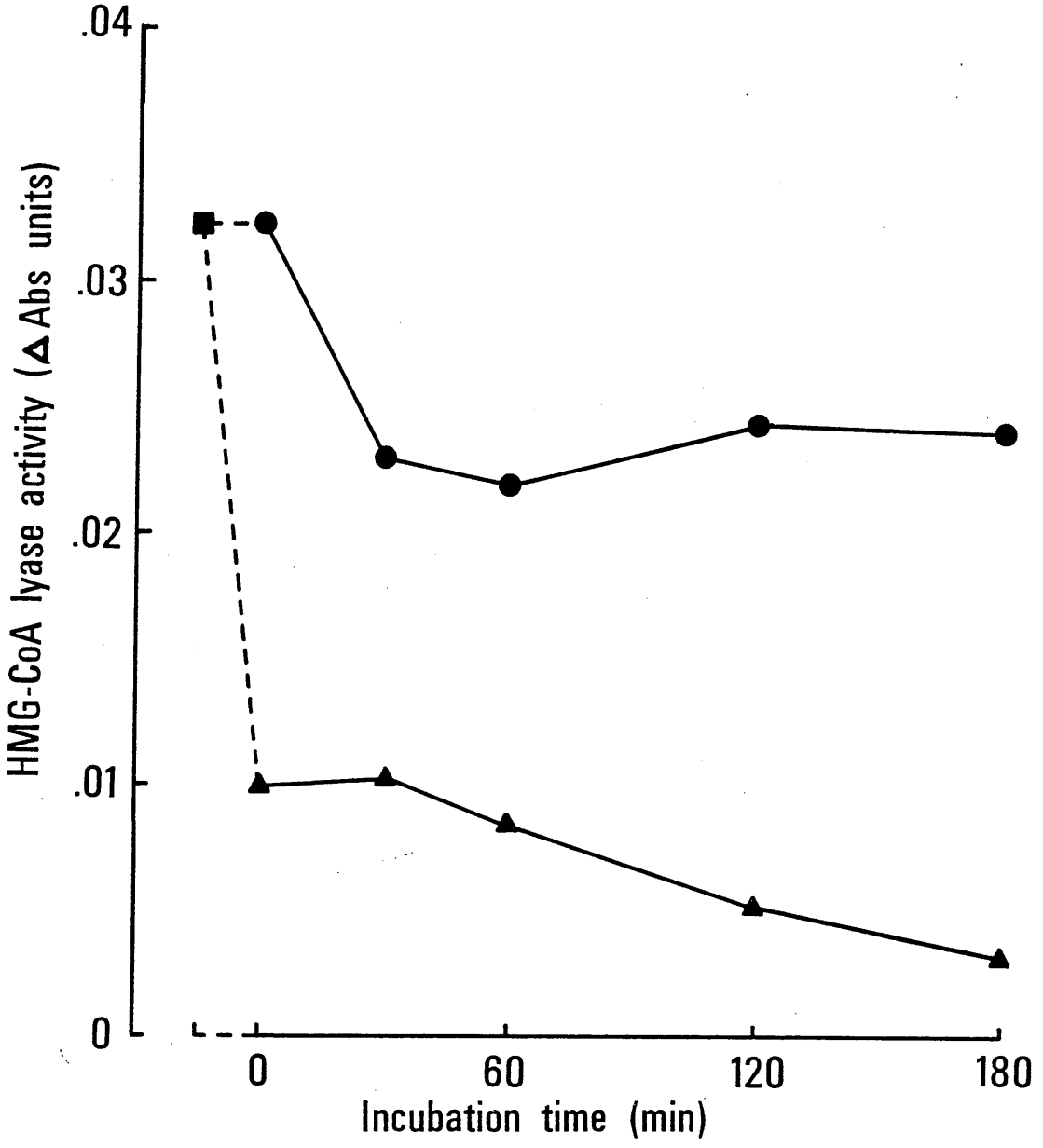


Fig. 3.3 Effect of chelation of Mg^{2+} and Ca^{2+} ions on HMG-CoA lyase activity in freeze-clamped homogenate of rat liver

A concentrated homogenate was prepared in buffer containing 50mM-Tris HCl (pH 7.5), 250mM-Sucrose, 2mM-DTT and diluted (10-fold) into the same buffer (●) or buffer containing 50mM-K_2HPO_4 (pH 7.4), 15mM-EDTA, 15mM-EGTA, 250mM-NaCl, 2mM-DTT (▲). At the intervals shown, samples of the incubation mixtures were assayed spectrophotometrically for HMG-CoA lyase (see Section 2.7).

Although it is evident that a considerable proportion of HMG-CoA lyase activity in freeze-clamped liver homogenates was suppressed following the chelation of Ca^{2+} (and Mg^{2+}), the residual activity was still in approximately 20-fold excess of the activity of HMG-CoA reductase in the liver. This level of HMG-CoA lyase activity would still interfere severely with any subsequent determinations of HMG-CoA reductase activity in these homogenates. Furthermore, it is probable that the loss of activity of HMG-CoA lyase following the lengthy incubations (at 0-4^{o}C) (Fig. 3.3) was the result of proteolytic degradation. Since it is known that HMG-CoA reductase is also susceptible to proteolysis (Ness et al., 1981) these types of incubations may lead to the dissociation of the catalytic domain of HMG-CoA reductase ('53K' reductase; see Section 1.2.2) from the endoplasmic reticulum and possibly to a change in catalytic activity, rate of degradation or ability of the enzyme to be regulated (see Section 1.3). Although proteolysis inhibitors can be added to homogenates it is not always possible to inhibit all types of proteases during such lengthy incubations.

3.2.2 Isolation of Microsomal Membranes

HMG-CoA lyase is a soluble enzyme of the mitochondrial matrix (Clinkenbeard et al., 1975a) and therefore it should be possible to separate microsomes and hence HMG-CoA reductase from the lyase-containing supernatant (due to lysis of mitochondria). To test this, a microsomal fraction was obtained from the homogenate of freeze-clamped liver by standard centrifugation steps (see Methods) and the effect of washing the pellet by resuspension and centrifugation was investigated.

Table 3.2 demonstrates that when unwashed microsomes were assayed for HMG-CoA reductase using ^{14}C-HMG-CoA as substrate, 80% of the ^{14}C-labelled material that migrated with the same R_f as mevalonolactone was the result of NADP-independent reactions and concluded to be the product of HMG-CoA lyase. This level of NADP-independent radioactivity was reduced to 25% by a further washing of the membranes (Table 3.2) but this was still approximately 5-fold greater than the level (i.e. 5-10%) routinely encountered in hand-homogenized tissue and therefore unsuitable for the accurate determination of HMG-CoA reductase activity required.

A further disadvantage of using the freeze-clamped technique in this way is that the disruption of intracellular membranes as the result of freezing (see above) would lead to a more diverse membrane population. This in turn could lead to a greater variation in the quantitation of HMG-CoA reductase activity with respect to membrane protein.

3.2.3 Conclusions

From these results it is evident that the freeze-clamping technique is unsuitable for the determination of the activity of HMG-CoA reductase *in vivo*, because the vast excess of HMG-CoA lyase released into the homogenate during the homogenization process can never be sufficiently removed. Consequently it was concluded that the only way to eliminate the interference of HMG-CoA lyase was to remove the intact mitochondria by differential centrifugation.

TABLE 3.2

Partition of NADP-Dependent and NADP-Independent Formation of ^{14}C Material with R_f 0.55-0.75 from ^{14}C-HMG-CoA between the Supernatant and Membrane Pellet of Unwashed and Washed Microsomal Fractions Isolated from a Sample of Freeze-Clamped Liver

Various fractions of freeze-clamped liver homogenates were assayed for HMG-CoA reductase activity (see Section 2.8). Controls from which NADP was omitted were run concurrently.

Fraction obtained by centrifugation		^{14}C-material recovered with R_f 0.55-0.75 (d.p.m.)		
		+NADP	-NADP	NADP-DEPENDENT
Total homogenate		46140	29940	16200
Unwashed microsomal	pellet	13380	10771	2609
	supernatant	32820	33960	-
Washed microsomal	pellet	4805	1206	3599
	supernatant	4770	3960	810

3.3 The Cold-clamping Technique

The emergence of the requirement for the retention of mitochondrial integrity coupled with the fundamental requirement of a rapid sampling technique (see above) led to the development of the cold-clamping technique. In this technique, mitochondrial lysis is avoided by rapidly cooling (rather than freezing) the liver to $0^{o}C$ followed by the rapid homogenization in a medium designed to preserve the phosphorylation status of regulatory enzyme *in vivo* (see above). Using this method, intact mitochondria and hence HMG-CoA lyase could be removed from the homogenate by differential centrifugation and HMG-CoA reductase assayed relatively free from interference.

3.3.1 Development of the Cold-clamping Technique

(a) Description of clamps

The clamps designed for this technique consisted of two circular stainless-steel blocks (diam. 5 cm) carried at the ends of the two arms of tongs which could be freely shut and opened. Each block had a raised cutting edge (height 1.5 mm) down one side and extending approximately 4/9 of the circumference (Fig. 3.4) and a groove of the same, but reciprocal shape (1.5 mm deep) symmetrically opposite. The raised edge and the groove on each block were confluent at one point, but were separated by a distance equivalent to approximately 1/9 of the circumference at the diametrically opposite side of the blocks. The blocks were mounted on the arms of the tongs in such a manner that the raised edge of the one fitted into the groove of the other, with the confluent cutting edges and diametrically opposite gaps being positioned perpendicularly to the

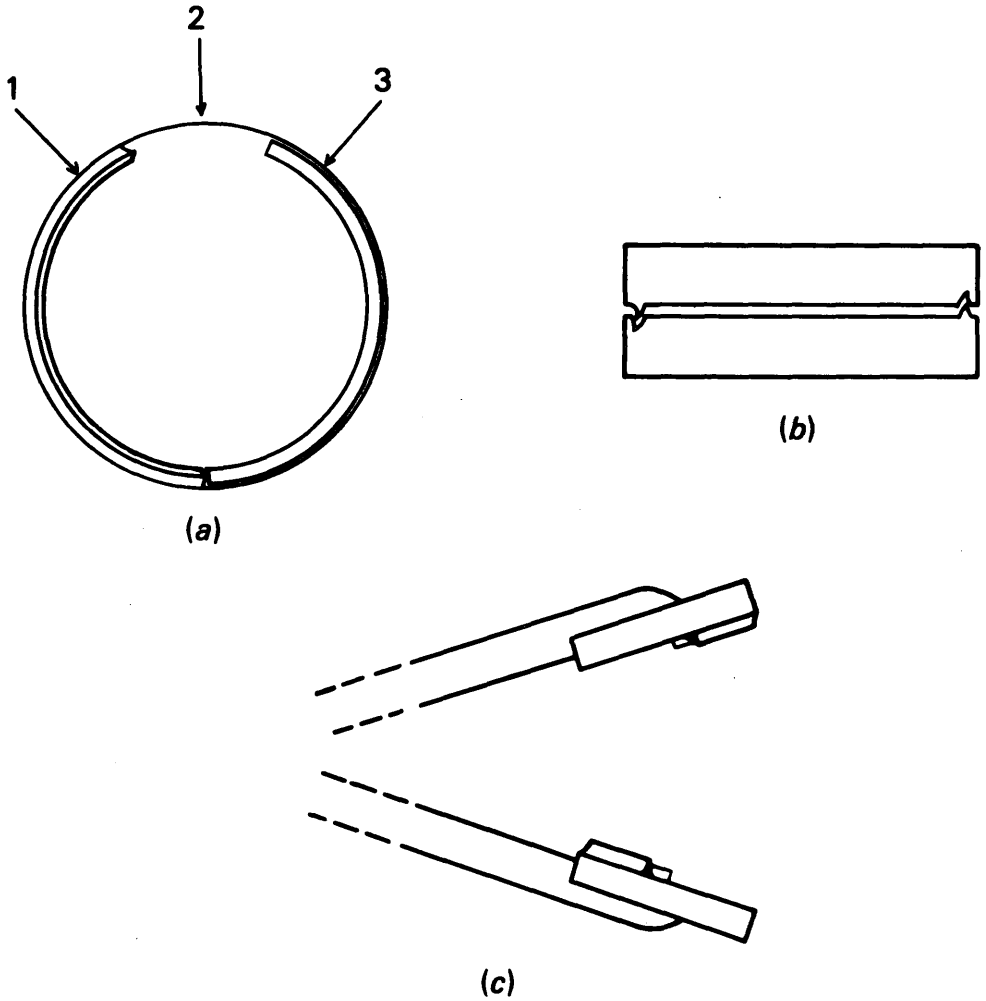


Fig. 3.4 *Details of construction of stainless-steel blocks used for obtaining samples of rat liver by cold-clamping* (*a*) Face of one block: 1, cutting edge; 2, gap; 3, groove. (*b*) Transverse (equatorial) section through the two blocks with clamps shut. (*c*) Top view of clamps to show mounting of blocks on handles.

plane of closure of the tongs. In use the tongs were held with the gap pointing vertically upwards so that excess liver sample squashed between the two blocks could escape through the gap. The effect of shutting the tongs was to produce a circular cutting edge throughout the lower and lateral parts of the two apposed blocks; the clamping action simultaneously cut a sample (approximately 1 g) of the liver lobe and smeared it in a thin layer over the cooled surfaces of the blocks.

(b) Cold-clamping of liver

A schematic representation of the procedure is given in Fig. 3.5 and also demonstrated photographically in Fig. 3.6. Animals were anaesthetized with pentobarbital (60 mg/kg body wt.) and allowed to stabilize for 15-20 min (see Section 2.1). The abdominal cavity was opened and the liver was dissected free of surrounding tissue by cutting the mesenteries. The left lateral lobe of the liver was threaded 2-3 mm from the edge with a Mersilk silk suture (4/0 gauge, round bodied 16 mm needle, Ethicon, Edinburgh, Scotland, U.K.). This allowed the lobe to be lifted out vertically from the abdominal cavity. (The use of a suture rather than a pair of forceps to lift the liver lobe was necessitated by the interference of forceps with the action of the cutting edges of the clamps; routinely no bleeding of the liver or any other adverse effects of the technique were apparent.) The lobe was clamped between ice-cooled stainless-steel blocks (see above). The shut clamps containing the liver sample were immediately held over the mouth of a plastic funnel inserted into the top of the body of a Teflon/glass homogenizer. The

Fig. 3.5 Diagrammatic representation of cold-clamping of rat liver samples

(a) Lifting of left lateral lobe of liver and positioning of cold-clamps (vertical section); (b) sampling of liver; (c) flushing of liver sample into body of homogenizer with extraction medium; (d) homogenization of tissue with a Teflon/glass homogenizer. The liver is drawn as the cross-hatched area. The scale at the bottom of the diagram gives the approximate times taken routinely to perform the different parts of the procedure.

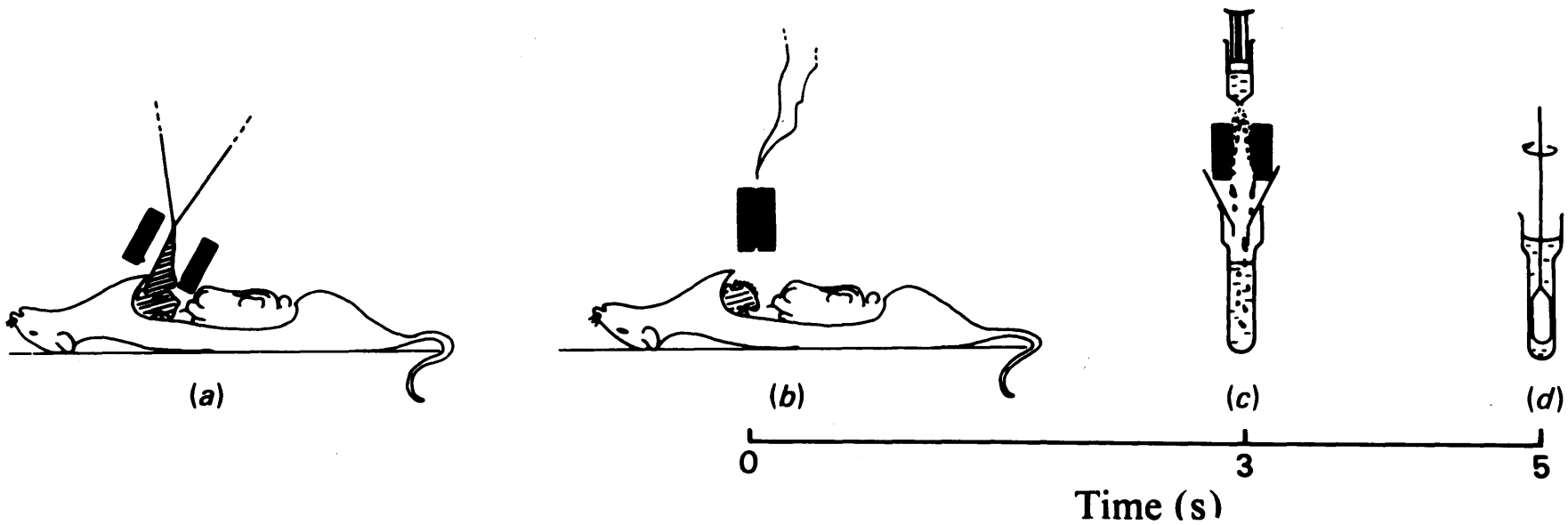
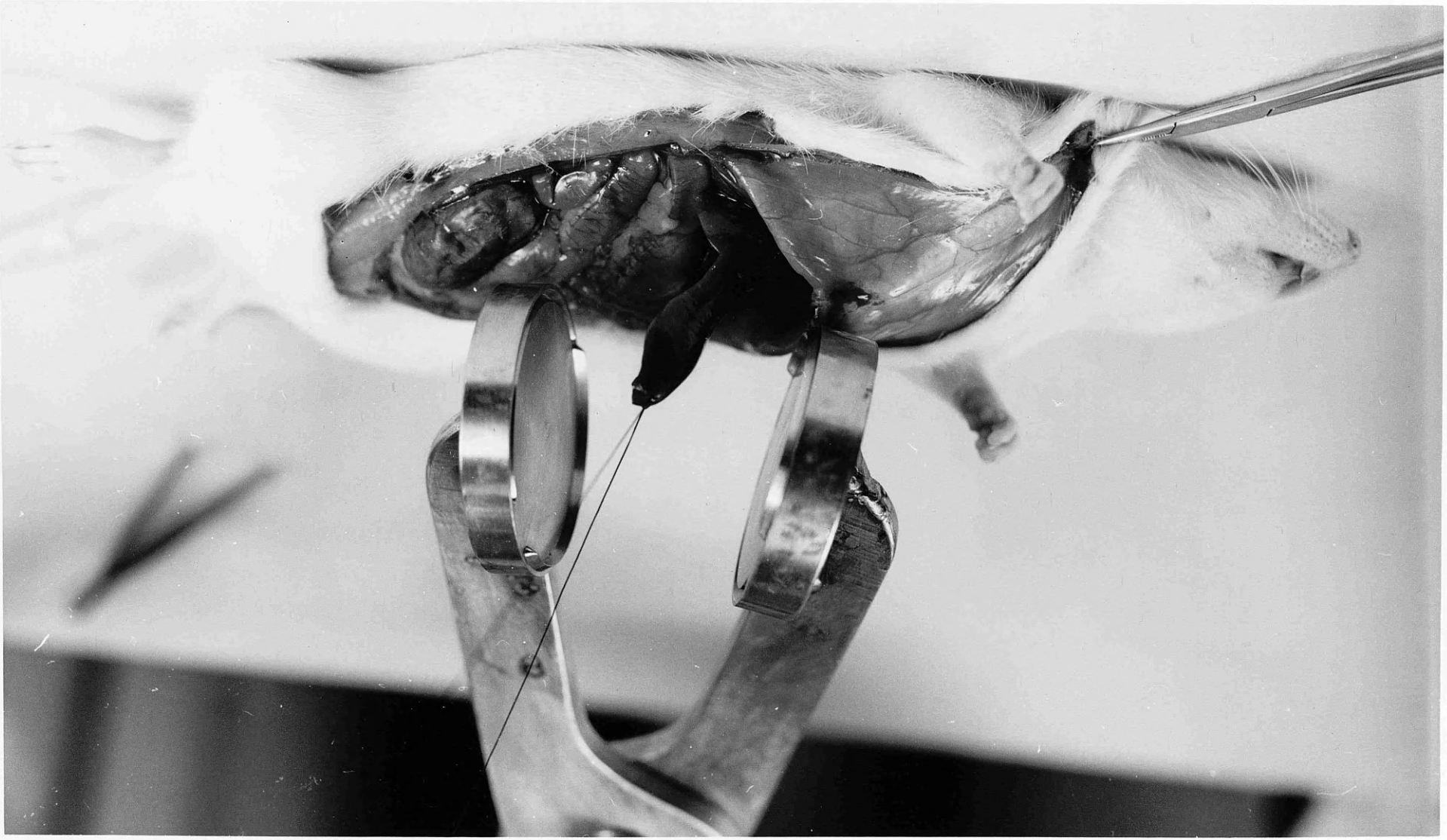

(a)
(b)
(c)
(d)
0
3
5
Time (s)

Fig. 3.6 Photographic representation of the cold-clamping technique

Left lateral lobe of liver is lifted between the cooled faces of the blocks. Arrow denotes cutting edge of blocks.



clamps were opened and the liver sample was washed directly into the homogenizer by squirting 25 ml of ice-cold extraction medium over the inner surfaces of the metal blocks. The tissue was then homogenized for 5s (three passes) with a motor-driven pestle (1000 rev./min). The entire procedure was completed in less than 10s. The resulting homogenate was used as a source of mitochondria, for the studies in mitochondrial respiration (see Section 2.10), or microsomes, for the assay of HMG-CoA reductase as described in Methods (section 2.8.2).

3.3.2 Validation of the Cold-clamping Technique

Two criteria were used in order to ascertain whether the cold-clamping technique was suitable for the study of changes in the phosphorylation state of HMG-CoA reductase *in vivo*. (i) The efficiency of the technique to preserve the phosphorylation state of a cytosolic enzyme which is known to undergo reversible phosphorylation-dephosphorylation, pyruvate kinase, was tested by direct comparison with the freeze clamping technique (pyruvate kinase is free from interferences from other enzymes in crude homogenates). (ii) The cold-clamping technique was used to investigate whether artefactual changes in the phosphorylation state of HMG-CoA reductase occurred, as suspected, when liver samples were obtained in the conventional manner (see section 3.1).

(a) Retention of pyruvate kinase kinetics

In these experiments the liver of a rat was sampled in three ways: (i) one lobe was freeze-clamped, (ii) a second lobe was cold-clamped, and (iii) a third was excised and minced (for practical

reasons cold-clamping was always performed on the left lobe, but the use of the other lobes was randomized so as to eliminate the possibility of variations between lobes, although no such variations were ever observed). All subsequent treatment of the tissue was identical, except that freeze-clamped tissue was homogenized with a Polytron tissue disintegrator (see Section 2.8.1). The activity of pyruvate kinase of the three samples was measured at several suboptimal concentrations of phosphoenolpyruvate (see Table 3.3) and at 3 mM-phosphoenolpyruvate, which was ascertained to measure maximal activity of the enzyme. In addition, the activity of pyruvate kinase in a portion of the freeze-clamped sample homogenate which had been incubated with a protein-phosphorylating mixture (see section 2.9.2) was also measured at the same phosphoenolpyruvate concentrations to obtain information about the kinetics of the fully phosphorylated enzyme with respect to phosphoenolpyruvate concentration. The results of three such experiments are given in Table 3.3; activities of pyruvate kinase at suboptimal phosphoenolpyruvate concentrations are expressed as a percentage of the maximal activity. It is evident that, whereas the kinetics of pyruvate kinase at suboptimal phosphoenolpyruvate concentrations were very similar in homogenates of freeze-clamped and cold-clamped samples, those for the minced liver samples were very different, and were much more similar to those of the fully phosphorylated enzyme. These results demonstrated that cold-clamping preserved almost intact (compared with freeze-clamping) the activity state of pyruvate kinase *in vivo*, and therefore appeared to be an equally effective sampling technique in minimizing the artefactual apparent increase in phosphorylation of

TABLE 3.3

Comparison of Activity of Pyruvate Kinase Activity at Sub-Optimal Phosphoenolpyruvate Concentrations in Homogenates Prepared from Freeze-Clamped, Cold-Clamped and Minced Liver Samples

Activities at three suboptimal phosphenolpyruvate concentrations are expressed as a percentage of the maximal activity measured at 3mM-phosphoenolpyruvate (average activity 46.6 μmol/min per g of liver at 25°C). The activities of the enzyme in homogenate of freeze-clamped samples which were incubated with a protein-phosphorylating mixture are also given.

Expt. no.	Phosphoenolpyruvate conc. (mM)	Pyruvate kinase activity (% of maximal)			
		Freeze-clamped	Cold-clamped	Minced	Phosphorylated
1	0.6	8.8	10.0	7.8	4.3
	0.8	17.3	19.2	12.3	11.6
	1.0	23.8	23.0	16.8	-
2	0.6	11.6	11.6	9.4	6.8
	0.8	19.1	18.1	13.8	-
	1.0	33.3	32.1	24.3	-
3	0.6	17.2	14.0	5.6	4.4
	0.8	28.6	23.1	13.3	5.7
	1.0	33.2	27.4	22.2	-

pyruvate kinase that occurred in the time taken to mince the tissue. If the rapidity of change in phosphorylation status of pyruvate kinase during sampling is representative of that of other enzymes that are regulated by phosphorylation-dephosphorylation, the above results would suggest that cold-clamping is as valid a technique as freeze-clamping for the sampling of tissue *in situ* in studies on the effects of different physiological conditions on the activity state of these enzymes *in vivo*. Both techniques are superior in this respect to excision and mincing of the liver followed by homogenization.

(b) Preservation of expressed/total activity (E/T) ratio for HMG-CoA reductase by cold-clamping

These experiments were performed when the activity of HMG-CoA reductase was maximal i.e. at 6h into the dark phase of the diurnal rhythm (D-6) (see section 4.2). Rats were anaesthetized 20 min before sampling of the liver. The left lateral lobe of the liver was sampled by cold-clamping followed by immediate homogenization in medium containing EDTA and F^-. The right lateral lobe was excised and minced on an ice-cold Petri dish and homogenized in the same medium. All subsequent treatments of the two homogenates and microsomal pellets were identical (see Methods; Section 2.8.1). The results of four such experiments are given in Table 3.4. It is evident that the E/T ratios were always appreciably higher in homogenates of cold-clamped liver samples than in minced samples. Moreover, this difference was solely due to a lower expressed

TABLE 3.4

Expressed (E) and Total (T) Activities of HMG-CoA Reductase

in Microsomes Isolated from Homogenates of Cold-Clamped and Minced Rat Liver Samples

Liver microsomes were prepared at D-6 from cold-clamped homogenates or homogenates which had been prepared by conventional mincing and homogenization (see Section 3.1). HMG-CoA reductase activity is expressed as nmole/min per mg of microsomal protein at 37°C.

	Cold-clamped samples			Minced samples		
	HMG-CoA reductase activity			HMG-CoA reductase activity		
Expt. no.	Expressed (E)	Total (T)	E/T ratio (%)	Expressed (E)	Total (T)	E/T ratio (%)
1	0.87	1.39	62.6	0.58	1.34	43.0
2	1.85	2.38	77.8	1.36	1.97	68.7
3	1.22	1.63	68.7	0.73	1.79	40.5
4	1.52	1.95	77.6	1.17	2.25	52.0

activity in minced samples than in cold-clamped samples (0.96 ± 0.18 and 1.34 ± 0.22 nmol/min per mg of microsomal protein at 37°C respectively; $P < 0.005$, paired 't' test). The total HMG-CoA reductase activities for microsomes prepared from both types of samples were identical (1.84 ± 0.20 nmol/min per mg of microsomal protein). These experiments illustrated that the longer delay between sampling of the liver and homogenization in the F^- and EDTA-containing medium introduced by mincing of the tissue resulted in a markedly reduced expressed activity of HMG-CoA reductase, i.e. an increased proportion of the enzyme was phosphorylated. Thus it was concluded that when liver was excised and minced the relative amounts of the enzyme in phosphorylated and dephosphorylated states were modified from their respective values *in vivo* during the time that elapsed between liver sampling and homogenization in a F^-- and EDTA-containing medium. These marked changes did not occur when the liver was sampled by cold-clamping.

It is noteworthy that if animals were stunned by a blow to the head and then killed by cervical dislocation prior to the excision, mincing and homogenization of the liver (the conventional manner of sampling) the E/T ratio of HMG-CoA reductase was very much lower than the equivalent ratio recorded in the homogenates of minced liver obtained from anaethetized animals (Table 3.5.). This very much depressed ratio too was the consequence solely of a marked depression in the expressed activity ; the total activity remained identicial to the total activity observed in the homogenates of both minced and cold-clamped samples. The value obtained for the E/T ratio of HMG-CoA reductase in experiments in which rats were killed

TABLE 3.5

Comparison of 'Expressed'-(E) and 'Total'-(T) Activities of HMG-CoA Reductase in Homogenates of Liver Obtained from Anaesthetized or Cervically-Dislocated Rats

Values are means (+ S.E.M., where applicable). All homogenate were treated in an identical manner after liver sampling. HMG-CoA reductase activity is expressed as nmole/min per mg of microsomal protein at 37°C. Those marked (*) are significantly different ($P < 0.005$, paired 't' test) from values for cold-clamped liver samples.

	HMG-CoA Reductase Activity		
	(E)	(T)	%
(a) Cold-clamped	1.340 ± 0.217	1.838 ± 0.215	72.4 ± 2.4
(b) Minced anaesthetized	0.958 ± 0.183*	1.839 ± 0.192	52.5 ± 4.2*
(c) Minced (cervical dislocation)	0.181	1.850	9.8

by cervical dislocation was very similar to that obtained by Brown et al. (1979). Nordstrom et al. (1977) and Dugan et al. (1982).

3.3.3 Assessment of Mitochondrial Integrity in Cold-clamped Liver

Since the primary reason for using cold-clamping instead of freeze-clamping was to retain mitochondrial integrity an assessment was made of the intactness of mitochondria in homogenates of cold-clamped liver. This was performed by measuring the latency of mitochondrial marker enzymes and by measuring their O_2-consumption in the presence of ADP (state 3) and after its depletion (state 4) in the presence of malate and glutamate as substrates (see Methods, section 2.10).

(a) Latency of mitochondrial enzymes

The activities of glutamate dehydrogenase, a mitochondrialmatrix enzyme (Lehninger, 1982) and citrate synthase, an inner membrane enzyme (Weitzman & Danson, 1976) were determined in the supernatant fractions after intact mitochondria had been separated out by centrifugation (see Table 3.6). These two enzymes were chosen to provide information about: (i) the lysis of mitochondria i.e. release of glutamate dehydrogenase and (ii) the degree of disruption of the inner mitochondrial membrane i.e. exposure of citrate synthase.

The activities of both enzymes were almost completely latent in homogenates of cold-clamped rat liver samples. Routinely, approximately 7% of the total activity of citrate synthase and 3% of the total activity of glutamate dehydrogenase were overt in the

TABLE 3.6

Activities of Citrate Synthase and Glutamate Dehydrogenase in the Post-Mitochondrial Supernatant of Homogenates of Cold-Clamped Liver

Homogenates of cold-clamped liver samples were centrifuged at 500g for 5 mins (a) and then twice at 7000g (b) and (c). Citrate synthase and glutamate dehydrogenase activities were determined in the supernatant of the two high speed supernatants and expressed as a percentage of the activities obtained in the low speed supernatant. Enzymes were assayed as described in Methods (sections 2.9.3 and 2.9.4).

	Enzyme Activity (%)	
Centrifugation step	Citrate Synthase	Glutamate dehydrogenase
(a) 500g (5 mins)	100	100
(b) 7000g (10 mins)	6.9	2.9
(c) 7000g (10 mins)	6.4	2.7

mitochondrial supernatant (Table 3.6). These values are similar to latency obtained with conventional homogenization (Zammit, 1980a). Therefore the integrity of the mitochondrial inner membrane was largely preserved with respect to accessibility of the respective substrates to the two enzymes and of the leakage of glutamate dehydrogenase from mitochondrial matrix. It is unclear why citrate synthase should have an apparently higher activity within the mitochondrial supernatant.

(b) Respiratory control of mitochondria

In homogenates of cold-clamped liver samples, the rate of O_2-consumption by the mitochondria was 56.8 $\pm$ 8.0 nmol O_2/min per mg mitochondrial protein at 37°C (n=6) and the respiratory control ratio (state-3/state-4, see above) averaged 7.5, (12 determinations; range 4.0-12.5). These values were very similar to values obtained from mitochondria preparations obtained from liver sampled in the conventional manner (Zammit & Robinson, 1982; Zammit, 1980b).

3.4 Discussion

The cold-clamping technique provides rapid sampling and homogenization of liver with an apparent retention of structural and functional integrity of the mitochondria. This produces a system in which the activity of HMG-CoA reductase can be accurately determined without the interference of HMG-CoA lyase.

The use of this technique has enabled, for the first time the demonstration that HMG-CoA reductase exists largely in the active, dephosphorylated form *in vivo* at 6h into the dark period (D-6).

This is in contrast to in the observations of Brown et al. (1979) who reported that the majority of the enzyme was invariably phosphorylated. It is concluded that this observation is the result of the employment of this the rapid sampling technique and its efficiency in rapidly stopping the reversible phosphorylation-dephosphorylation of HMG-CoA reductase.

The observations made in this study confirm the suggestion made in Section 3(i) that the persistently low E/T ratio obtained by Brown et al. (1979) and others (see previously) in liver was artefactual. Furthermore, the direct comparison of the cold-clamping technique with the conventional method of preparing tissue homogenates has identified two possible factors which could account for the observations made by these workers:

(i) It is evident from Table 3.4 that the phosphorylation of HMG-CoA reductase is increased (i.e. E/T ratio decreased) in tissue that had been minced for a period of time before homogenization. Thus, the relative activities of the kinases and/or the phosphatases were modified in some way during this delay possibly through the effect of anoxia and emphasize the need of a rapid sampling method of tissue which is being actively perfused by blood.

The fact that the amount of HMG-CoA reductase in the phosphorylated form increased in the minced samples from anaesthetized rats was unexpected since this modulation requires ATP, the cellular concentration of which would fall rapidly following anoxia. This effect is also opposite to that observed in the case of other enzymes modulated by reversible phosphorylation e.g. pyruvate dehydrogenase (E. Kilgour, unpublished observations) which becomes

more dephosphorylated in similar conditions. It is possible that this effect is the result of an allosteric activation of reductase kinase by the inevitable increase in the intracellular concentrations of ADP during anoxia as recently proposed by Harwood et al. (1984).

(ii) It is evident from Table 3.5 that the added factor of killing the animal by cervical dislocation before the preparation of the tissue homogenate as in (i) above resulted in an even greater decrease in the proportion of HMG-CoA reductase in the dephosphorylated form. It is probable, therefore, that the phosphorylation system of HMG-CoA reductase is very sensitive to adrenergic stimulation such as would occur in rats acutely stressed by a stunning blow to the head (see Callingham & Barrand, 1979). The comparison of the E/T ratio of HMG-CoA reductase from these animals with the equivalent ratio obtained from animals treated as in (i) above reveals that this effect is greatly reduced by excising the liver from animals that had been anaesthetized 20 minutes earlier. Both of these factors are eliminated by the use of the cold-clamping technique. Moreover, in the light of the prominent effects of stress, further precautions were also taken in subsequent experiments and the animals were trained to being handled at least 4 days before they were used.

The retention of good respiratory control and full latency of inner-membrane and matrix enzymes by mitochondria isolated from homogenates of cold-clamped samples extends the possible fields of hepatic metabolic studies to which the cold-clamping technique could be applied. Thus it should be possible to study metabolic processes

in isolated mitochondria obtained after rapid cold-clamping and differential or gradient centrifugation. For example, this new technique could potentially be a method of investigating the regulation of the mitochondrial 2-oxoacid dehydrogenase complexes e.g. pyruvate, 2-oxoglutarate and branched-chain 2-oxoacid dehydrogenases (Randle et al., 1984). These complexes are known to be regulated by phosphorylation-dephosphorylation and to be controlled to some extent by the levels of free Ca^{2+} within the mitochondria. This technique would enable the study of these systems in intact mitochondria providing the kinases and phosphatases can be rapidly controlled for example by the combined use of Ca^{2+}-ionophores (e.g. A23187) and Ca^{2+}-chelators (e.g. EGTA) to remove Ca^{2+} from the mitochondria.

Similarly, the ability to rapidly prepare different cell fractions from cold-clamped rat liver samples could enable the sampling of liver in situ and the measurement of the relative amounts of soluble and membrane bound forms of enzymes, the regulation of which may be mediated partly by reversible interconversion between soluble and membrane bound forms (Wilson, 1978), e.g. CTP:choline phosphate cytidylyl transferase (Vance & Pelech, 1984).

CHAPTER 4

CHANGES IN THE PHOSPHORYLATION STATE OF HMG-CoA REDUCTASE IN DIFFERENT PHYSIOLOGICAL CONDITIONS

4.1 Introduction

The cold-clamping technique (see Chapter 3) overcomes the problems met by previous workers in assessing the involvement of reversible phosphorylation in the regulation of hepatic HMG-CoA reductase activity *in vivo* (Chapter 1). It makes possible the quantitation of the fraction (E/T ratio) of the hepatic enzyme in the active form *in vivo*, under different physiological conditions. In order to demonstrate that this parameter is responsive to normal, physiological stimuli, the expressed and total activities of HMG-CoA reductase were studied in several different physiological conditions which involve acute hormonal and nutritional variations. The three physiological conditions studied were: (i) the diurnal rhythm of feeding; (ii) the withdrawal of food for various, short and medium-term periods and (iii) the pregnant and lactating states. These conditions were chosen because they represent entirely physiological conditions characterized by different levels of food intake and hormonal status.

The diurnal pattern of feeding shown in normal rats kept in conditions of alternate 12h light:12h dark is thought to provide the main stimulus for the diurnal rhythm in the total activity of hepatic HMG-CoA reductase (i.e. amount of enzyme protein; see Section 1.3.3). In normal-fed rats, the total activity of HMG-CoA reductase

and the rate of cholesterogenesis rise subsequent to the availability of food even during the light period (Dugan et al., 1982; Edwards et al., 1972). In addition, the diurnal rises of both HMG-CoA reductase activity (Hamprecht et al., 1969; Dietschy & Brown, 1974; see Rodwell et al., 1976) and cholesterogenesis (Brown et al., 1979; Dietschy & Brown, 1974) have been reported to be markedly suppressed by prolonged fasting for 24h although there is also evidence that the rhythm of HMG-CoA reductase activity persists in these conditions although the diurnal peak of activity is much depressed (Hamprecht et al., 1969).

This diurnal increase in the rate of cholesterol formation in the liver during the period of active feeding in the rat is important in enabling the maintenance of the relatively stable compositional ratios of cholesterol (free and esterified) and triglycerides in the plasma lipoproteins. The cholesterol synthesized allows the secretion of endogenously formed triglyceride as VLDL. (Cholesterol formed in the intestinal mucosa is incorporated into chylomicrons that are secreted into the lymphatic system). Consistent with this function of cholesterol formation, the increased dietary intake of fat by the animal (Goldfarb & Pitot, 1972) or the increased delivery of fatty acids to perfused rat liver (Goh & Heimberg, 1979) generates an increased total activity of hepatic HMG-CoA reductase.

The diet-related signals which stimulate changes in the rate of HMG-CoA reductase synthesis have not been defined. In Chapter 1 (Section 1.3.3) the possible roles of insulin, glucagon and glucocorticoids were discussed because of the diurnal variations in the plasma concentrations shown by these hormones. They may be

involved not only in the generation of the diurnal rhythm in total activity but may also affect a variation in the fraction of the enzyme in the active form. Although Edwards et al. 1980 presented evidence that the specific activity of hepatic HMG-CoA reductase varied during the diurnal cycle, this view was not generally accepted (see e.g. Dugan et al., 1982; Section 3.1). However, in view of the observed ability of the phosphorylation state of HMG-CoA reductase to be modulated by hormones e.g. insulin and glucagon (see Ingebritsen, 1983; Section 1.5) and cholesterol (mevalonolactone) in isolated hepatocytes in vitro (see Parker et al., 1983; Section 6.1) and by pharmacological doses of glucagon and cholesterol in the whole animal in vivo (see Beg & Brewer, 1982), it was anticipated that changes in the E/T ratio of this enzyme would be observed in response to diurnal variations in the condition of the animals.

The diurnal feeding activity of the rat under normal conditions incorporates durations (4-8h) of reduced food intake (see Fig. 4.4). During these periods the most significant hormonal changes that occur are in the plasma concentrations of insulin and glucagon which fall and rise respectively in response to the modest fall in blood glucose (see White et al., 1984). Therefore the use of different variations of starvation may provide information about the likely period of diurnal fast that are central to the maintenance of the diurnal rhythms of total activity and the fraction of the enzyme in an active form.

Pregnancy and lactation represent interesting physiological conditions not only with respect to important hormonal changes that occur in the transition from the normal, unmated animal to the gravid

and lactating animal (see below) but also because of the hyperphagia that accompanies the two conditions with relative hypophagia around parturition (Cripps & Williams, 1975; Walker & Hahn, 1981). The increased food intake provides the additional nutritional requirements imposed on the mother by the growing foetus in pregnancy and by milk production in lactation.

In the rat, pregnancy is characterized by the progressive establishment of hyperinsulinaemia, lipidemia and an increased vulnerability to ketosis after food deprivation (referred to as 'accelerated starvation'; see Freinkel, 1980). In women, the hyperlipidemia, characterized by marked hypertriglyceridemia (Potter & Nestel, 1977; Darmady & Potter, 1982) and hypercholesterolaemia (Curiel et al., 1982; McMurry et al., 1981; Tolino et al., 1980; Skryten et al., 1980) has been shown to be due to the increased circulating levels of lipoproteins particularly VLDL and LDL (Hillman et al., 1975; Wrath & Knopp, 1977) with minimal increases in HDL (Curiel et al., 1982). Similar changes are evident in the rat (Bosch & Canejo, 1967; Ramirez et al., 1983; Lorenzo et al., 1979). However, it is noteworthy that rat and human are two of a small number of mammalian species in which hypercholesterolaemia accompanies the later phases of pregnancy (see Connor & Lin, 1967; Tietz et al., 1976); most other species are hypocholesterolaemic in the gravid state.

Triglyceridemia in the pregnant rat has been shown to be the result of increased de novo synthesis in the liver and, in late pregnancy, of the decreased utilization of lipoproteins in the periphery (Lorenzo et al., 1981; Hamosh et al., 1970). The increased synthesis is thought to be a response to the

hyperinsulinaemia characteristic of this stage of pregnancy (Lorenzo et al., 1981; Flint et al., 1979) as well as the increased circulating levels of progesterone and oestrogen (Kalkoff & Kim, 1979). Similarly, hypercholesterolaemia in the rat during late pregnancy may result from the increased cholesterol intake that accompanies hyperphagia (Cripps & Williams, 1975; Walker & Hahn, 1981) in addition to a decreased catabolism of plasma lipoproteins in the periphery (see above). However, whilst Feingold et al. (1983) have suggested that the rates of hepatic cholesterogenesis (determined by the incorporation of [^{3}H]-H_2O into cholesterol) increases towards the end of the gestation period, Leoni et al. (1984) have suggested that this rate (determined by [^{14}C]-acetate incorporation into digitonin precipitable sterols) remains constant throughout late pregnancy at the same value as observed in virgin animals and only decreases on the day of parturition.

The lipidaemia of pregnant rats is rapidly relieved at the onset of lactation (Scow et al., 1964; Hamosh et al., 1970) as the result both of a decrease in the rate of entry of triglycerides into the circulation (Agius et al., 1981) and a greatly increased clearance of triglyceride-rich lipoproteins by the mammary gland (Otway & Robinson, 1968). In further contrast to the pregnant animal, the lactating animal is mildly hypoinsulinaemic (Flint et al., 1979; Robinson et al., 1978) and is characterized by a high circulating concentration of prolactin (Flint et al., 1979; Amenomori et al., 1970;). In addition, the plasma concentrations of progesterone fall during late pregnancy and recover during early lactation (Flint et al., 1979).

Consequently, this transition period between pregnancy and lactation is characterized by marked hormonal (and nutritional) changes. It represents an interesting condition in which to study the physiological variations in expressed and total activities and in particular in the phosphorylation state of the enzyme. Changes in these parameters of HMG-CoA reductase activity would be expected because of involvement of hepatic cholesterogenesis in the provision of VLDL by the liver for utilization by the adipose tissue and mammary gland in pregnancy and lactation respectively.

4.2 Diurnal Rhythm

4.2.1 Experimental

Microsomes were isolated from cold-clamped liver homogenates obtained at 2h intervals throughout the diurnal light/dark cycle and the total and expressed activities of HMG-CoA reductase were determined as described in Methods (Section 2.8).

4.2.2 Results

(i) Diurnal variation in total activity

The total activity of HMG-CoA reductase showed a well-defined diurnal rhythm in agreement with work from other laboratories (see Sections 1.3.3 and 4.1). In the present study, this activity in microsomes obtained from rats sampled at the mid-dark time point (D-6) was approximately 5.3-fold higher than in microsomes obtained from rats sampled in the middle of the light phase (L-6), see Fig. 4.1. This high activity at D-6 was part of a plateau of activity which lasted for 8h in the dark phase, i.e. from D-2 to D-10. The

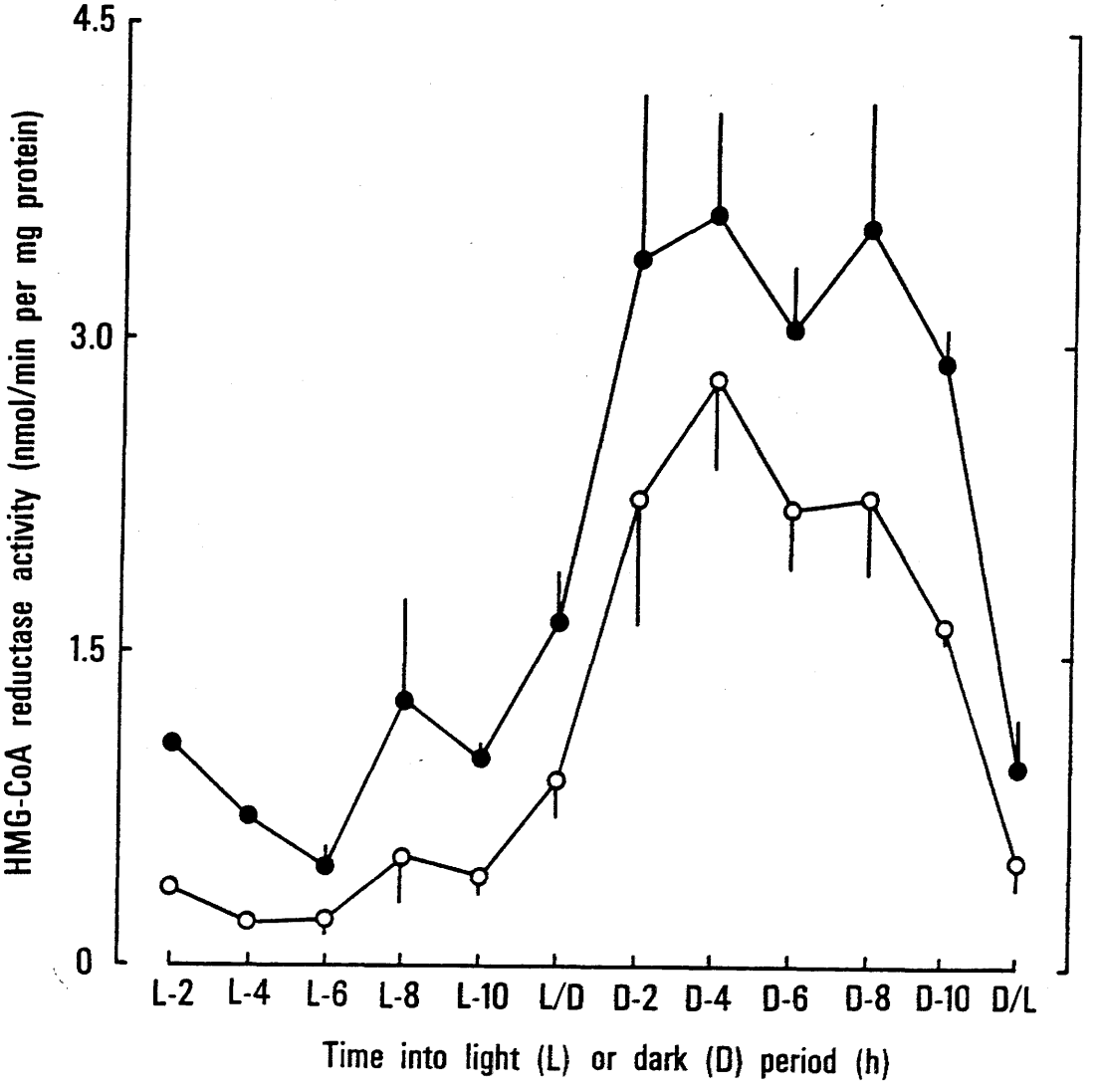


Fig. 4.1 Diurnal variation in the expressed (○) and total (●) activities of HMG-CoA reductase in rat liver microsomes

Values are means ± S.E.M. for three to seven determinations performed on microsomal preparations obtained from separate rats, except for L-2 and L-4 values, which are averages of two separate determinations which gave very similar results. L/D and D/L refer to the light/dark and dark/light transitions respectively.

plateau of high total activity consisted of two maxima, i.e. at D-4 and D-8, separated by a moderate decrease in activity at D-6. This observation was consistent with a previous report by Shapiro & Rodwell (1972) (see also Rodwell et al., 1973; 1976).

The rise of total HMG-CoA reductase activity to the value obtained during the plateau was very rapid and occurred primarily during the first 2h of the dark period, i.e. between the light/dark transition (L/D) and D-2. A similarly rapid and marked decrease in total HMG-CoA reductase activity occurred between D-10 and D/L. As a result of these rapid increases and decreases in total HMG-CoA reductase activity at the beginning and at the end of the dark period respectively, the diurnal peak of total HMG-CoA reductase activity is virtually symmetrical around the high activity that occurred between D-2 and D-10 (Fig. 4.1).

(ii) Diurnal variation in the fraction of HMG-CoA reductase in the phosphorylated form (E/T ratio)

The degree of activation of HMG-CoA reductase by protein phosphatase varied markedly depending on the phase of the light:dark cycle at which the microsomes were obtained from rats. Thus the enzyme in microsomes from animals the livers of which were sampled 4h into the light period (L-4) was activated 3.5-fold after phosphatase treatment whereas HMG-CoA reductase in microsomes prepared from livers of animals at D-4 was activated only 1.2-fold. Consequently the E/T ratio varied markedly in a diurnal pattern between 28% and 80% at the nadir and peak respectively of the cycle (Fig. 4.2) and demonstrated that the phosphorylation state of HMG-CoA reductase varies in response to normal physiological stimuli in vivo.

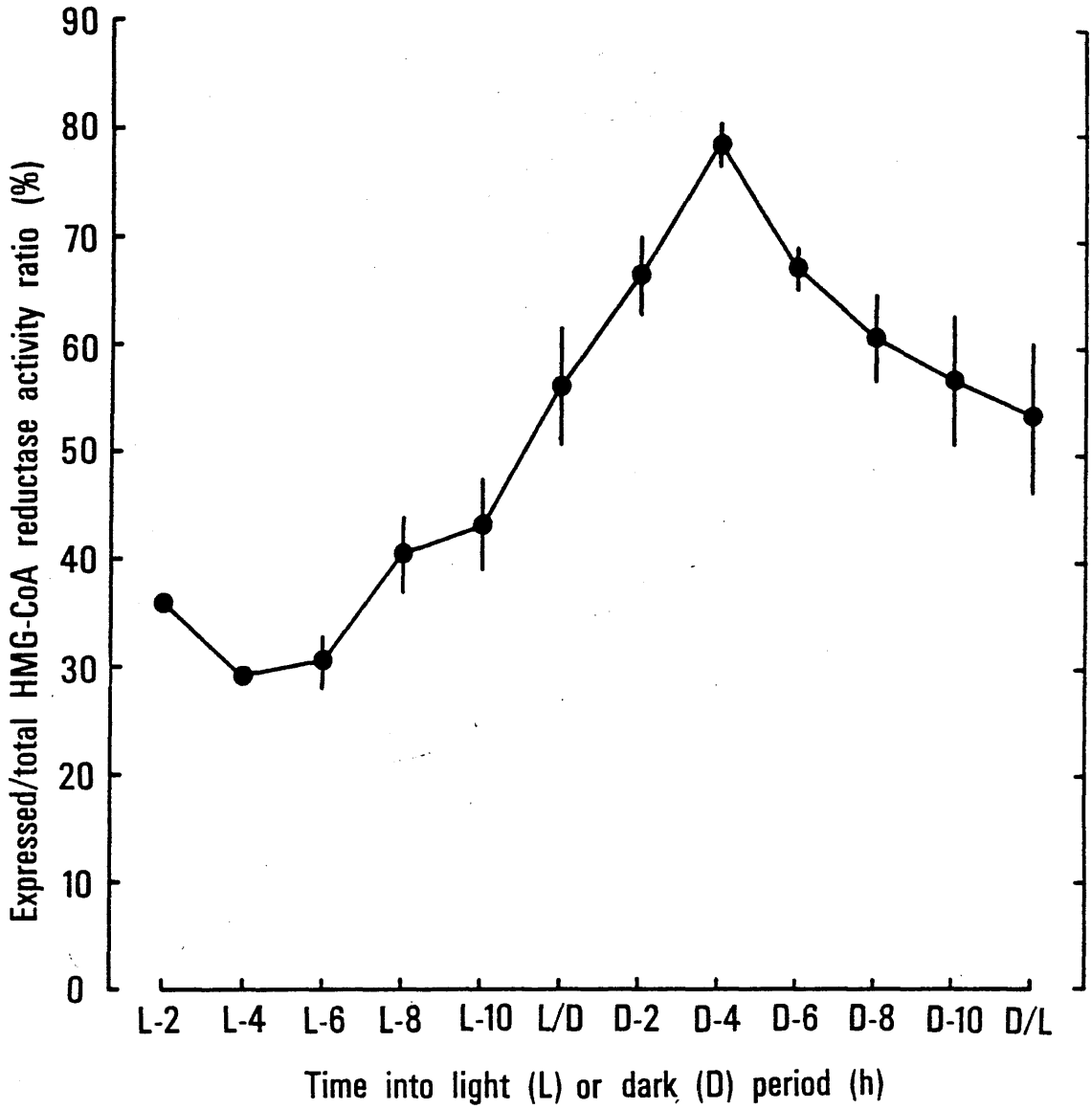


Fig. 4.2 Diurnal variation in the fraction of HMG-CoA reductase in the active form in rat liver microsomes

Microsomes prepared from cold-clamped livers were used to obtain 'expressed' and 'total' activities of HMG-CoA reductase at 2h intervals of the light/dark cycle in which the rats were maintained. Values are means ± S.E.M. for three to seven determinations performed on microsomal preparations obtained from separate rats, except for L-2 and L-4 values, which are averages of two separate determinations which gave very similar results.

This diurnal variation in the E/T ratio of HMG-CoA reductase had several important differences from the diurnal pattern of variation observed for total activity: (a) the E/T ratio had a pronounced well-defined peak at D-4 in contrast to the much broader (8h-long) plateau of the high total acitivity between D-2 and D-10. (b) The increase to and decline from this peak in E/T ratio were much more gradual than the corresponding phases of the cycle in the total activity above. Thus, the E/T ratio increased initially linearly with time over the 10h period between L-6 and D-4. The decrease in the E/T ratio after the D-4 peak was even more gradual such that the fraction of the enzyme in the active form was still over 50% at the D-L transition point. (c) The peak and nadir of the diurnal variation in E/T ratio did not coincide with those for the diurnal cycle for total activity (D-6 and L-6). The peak for the ratio occurred at D-4 and the nadir occurred at the corresponding time point in the light cycle 12h later i.e. at L-4. Therefore there was an apparent 2-3h phase difference between the two diurnal cycles in addition to the other differences described above.

The variation in the fraction of HMG-CoA reductase in the active form through the light/dark cycle had several effects on the resultant diurnal cycle in expressed activity of the enzyme (Fig. 4.1). Because of the increase in the E/T ratio, concomitantly with the increase in total activity of HMG-CoA reductase during the dark period the expressed activity of the enzyme increased 15-fold compared with only a 5.3-fold increase in total activity. In addition the effect of the relatively slow, steady increase in E/T ratio between L-6 and D-4 resulted in a more gradual increase and

decrease in the expressed activity of HMG-CoA reductase than in its total activity.

4.2.3 Discussion

This study demonstrated that the fraction of HMG-CoA reductase in the phosphorylated form, as determined by the E/T ratio, varies in the liver of rats *in vivo* in response to normal physiological stimuli that occur during the diurnal cycle. These observations contrasted with those made by Brown *et al.* (1979) and by Dugan *et al.* (1982) who reported that the protein phosphatase treatment of rat liver microsomes always resulted in an approximate 7-fold activation of HMG-CoA reductase activity irrespective of the phase in the light:dark cycle at which the animals were sampled.

The variations in the E/T ratio *in vivo* were, in general, similar to the diurnal variations shown in the total activity in that maxima and minima occurred in the mid-region of the dark and light periods respectively. Since the diurnal rhythm in total activity is thought to be regulated by factors related to the feeding activity of the animals (see Section 4.1) these observations suggest that the E/T ratio may be similarly responding to a diurnally varying stimulus (or group of stimuli) related to feeding.

Two major differences were evident, however, between the two rhythms: this may reflect either the different modes of action of a common stimulus (or stimuli) or the involvement of different stimuli in the maintenance of the two rhythms. Thus there was a phase difference of 2-3h between the T and E/T rhythms, the latter reaching a peak (and nadir) earlier than the former (see Fig. 4.3). But

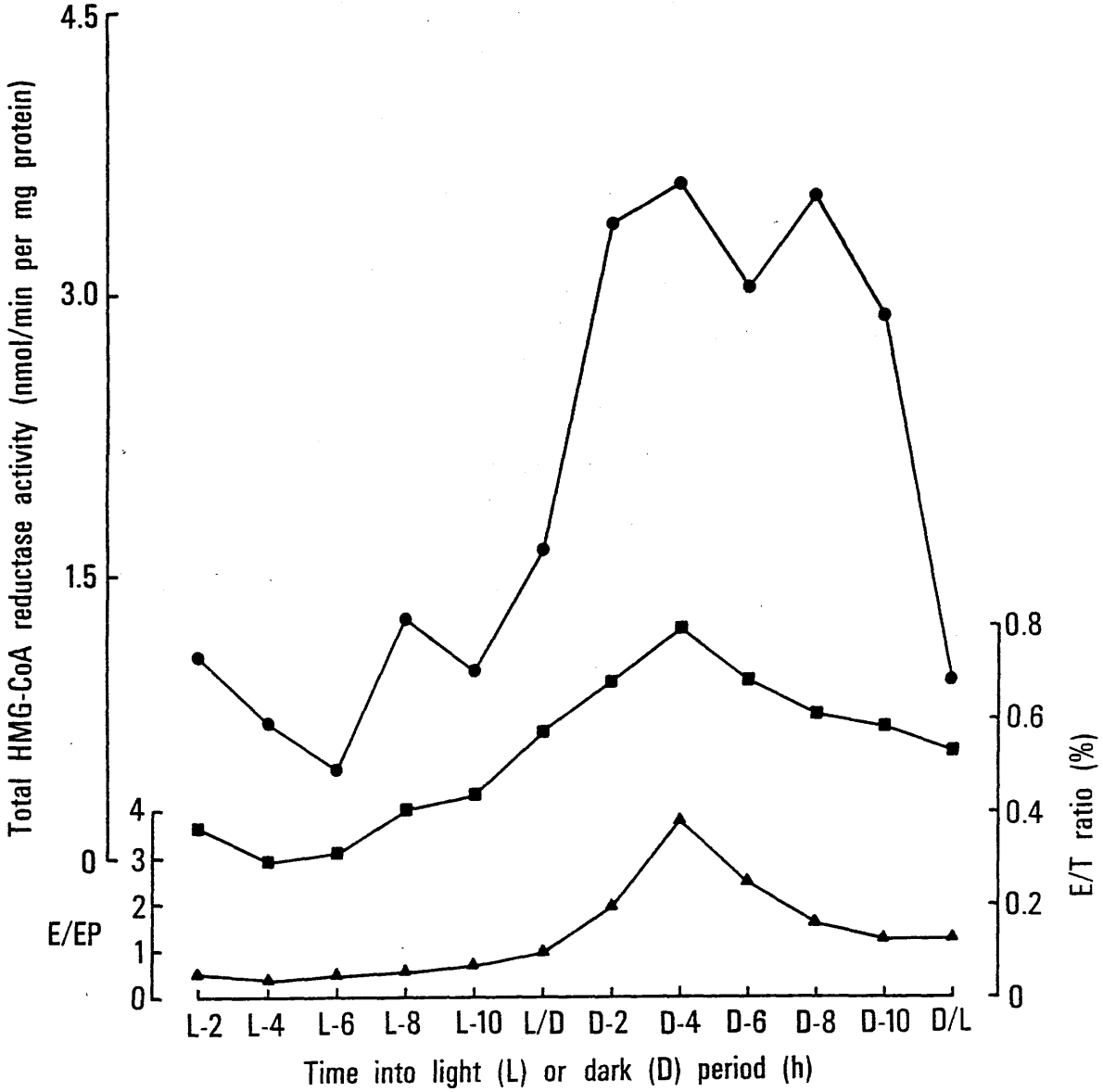


Fig. 4.3 Phase difference between the diurnal rhythms in total activity (●) and the E/T ratio (■)

Also illustrated (bottom curve) is the ratio of expressed activity to the calculated value of the inactive, phosphorylated enzyme (E/EP) (▲).

perhaps the most significant difference was the more gradual approach to and decline from a definite peak at D-4 shown by the E/T ratio compared to the very sharp increase and decrease to a broad, bimodal peak in total activity. In this respect, it is noteworthy that the gradual increase in the E/T ratio observed between L-6 and D-4 more closely parallels the gradual increase in food intake of the rats as the animals commence feeding in anticipation of the dark period (see Fig. 4.4; Peret et al., 1973; Bruckdorfer et al., 1974; Munday & Williamson, 1983). Hence when these distinct rhythms are combined (i.e. when expressed activity is considered), it is apparent that changes in the E/T ratio have an important role in producing a diurnal rise in effective HMG-CoA reductase activity in vivo which may more closely approximate to the feeding behaviour of the animals. In addition, it increases the range over which the expressed activity varies by nearly 3-fold (see Fig. 4.1).

A similar dampening effect on the diurnal cycle of the expressed activity was evident for the latter part of the dark phase when HMG-CoA reductase activity was decreasing due to a slower decrease in the E/T ratio than in total activity. This different behaviour of total activity and the E/T ratio from i.e. D-4 onwards, is further suggestive that the two diurnal rhythms are responding to different stimuli or responding differently to the same stimulus.

The observations that a progressive decrease in the E/T ratio occurs for approximately 4h prior to any decrease in total activity suggests that the increased phosphorylation of the enzyme presages the degradation of the enzyme protein (see Fig. 4.3). Thus, if phosphorylation of the enzyme identifies it as a better substrate

for intracellular processing (proteolysis), the increase in the proportion of the enzyme in the phosphorylated state that occurs after D-4 could facilitate the onset of the sharp decline in total activity that occurs between D-8 and D-L. Support for such a suggestion has emerged from reports that phosphorylated HMG-CoA reductase in microsomal membranes isolated from whole liver (Parker et al., 1984) or hepatocytes (Parker et al., 1985) is more susceptible to proteolytic attack in vitro by Ca^{2+}-dependent, leupeptin and calpastatin-sensitive protease calpain-II present in rat liver cytosol. Therefore changes in the phosphorylation state in vivo present a possible signal for the increased degradation of the enzyme during the latter part of the dark period (Fig. 4.3). Such a mechanism has previously been cited for liver pyruvate kinase (Engstrom et al., 1982) and yeast fructose-1-6-bisphosphatase (Müller & Hozer, 1981). A steady-state model has been proposed which recognizes fractional degradative rate constants for the phosphorylated and dephosphorylated species (Gibson et al., 1984). Similarly during the phase of the diurnal cycle in which HMG-CoA reductase activities increase (i.e. L-10 to D-4) the increase in the E/T ratio for HMG-CoA reductase could diminish the rate of enzyme degradation and therefore contribute to the diurnal rise in the concentration of enzyme protein in the microsomes.

The precise nature of the stimuli involved in generating the diurnal rhythm in the E/T ratio of HMG-CoA reductase is undetermined. However, insulin, because of its known effects on the E/T ratio of HMG-CoA reductase in isolated rat hepatocytes (see Ingebritsen, 1983) and the association of the secretion of this hormone with the diurnal

pattern of feeding activity (see Section 4.1) may have a major role. Indeed, the defined diurnal peak in the E/T ratio parallels closely the diurnal rhythm of the concentrations of insulin in the circulation (i.e. both peak at D-2 to D-4; Knox et al., 1979; Peret et al., 1973; Bruckdorfer et al., 1974; Fig. 4.2). (In preliminary experiments it was found that plasma insulin concentrations in the animals used in the present study were in fact, higher at D-4 than at D-2).

Insulin is also known to influence the synthesis of HMG-CoA reductase (i.e. the total activity; see Section 1.3.3). Hulcher et al. (1985) have recently demonstrated that the cyclic variations in insulin coincide with variations in the total activity of HMG-CoA reductase in primates in vivo. It is possible therefore the differences observed between the rhythms of the E/T ratio and the total activity are the result of the different mechanisms of action of insulin involved in the generation of each parameter: (i) covalent protein phosphorylation determining the E/T ratio which would be expected to reflect acutely the changing concentrations in the appropriate stimuli (e.g. insulin) in the blood, and (ii) a slower response required for the induction of enzyme protein synthesis. Support for this proposal, at least for the diurnal rise, has been obtained from studies in diabetic animals, (discussed in the next chapter), in which the administration of insulin produced a rapid increase in the E/T ratio followed after a lag period of approximately 2-3h by a marked increase in the concentration of enzyme protein in the liver (see Section 5.3.1 (iii)).

Further analysis of the data suggests, however, that other factors besides plasma insulin concentrations are probably also involved in establishing the rhythms in total activity and the E/T ratio. The bimodal peak in total activity is remarkably similar to the pattern shown in the feeding activity of the normal rat and reflected to a lesser extent by the plasma concentration of insulin (see Peret et al., 1973). The E/T ratio, however, appeared to be unaffected by this secondary period of increased feeding activity suggesting that this parameter may be responding to a further antagonistic factor to insulin. This could be the effect of mevalonate as the intracellular concentration of this metabolite increases i.e. as the consequence of the increased activity of HMG-CoA reductase (see Chapter 6). Alternatively such an effect could be mediated by the diurnal rhythm observed in the plasma concentrations of glucagon (Murakami et al., 1981).

A further complication inherent in the interpretation of the possible role of insulin in the diurnal rhythm of the E/T ratio and total activity is that it assumes a direct relationship between the concentration of insulin in the peripheral and portal blood. Thus Balks & Jungermann (1984) have indicated that whilst portal insulin concentrations do increase during the dark period of the diurnal cycle, the variations from basal level in the light period are only modest; greater changes occur in the portal concentrations of glucagon.

4.3 Food Deprivation

4.3.1 Experimental

Animals were sampled at D-4 and food was withdrawn from them for 3 different periods: 4h, 12h and 24h prior to sampling. The protocol is illustrated in Fig. 4.4.

4.3.2 Results

The withdrawal of food from animals 24h prior to the sampling of the liver by cold-clamping at D-4 resulted in a very marked reduction (85%) in the total activity of microsomal HMG-CoA reductase compared to animals fed ad lib. (Table 4.1;). This activity of HMG-CoA reductase after 24h starvation was very similar to that observed in the microsomes obtained from livers samples at the nadir of the normal cycle (i.e. 0.308 ± 0.056 v 0.360 ± 0.085 nmoles/min per mg of microsomal protein respectively;). Concomitant with the fall in total activity was a marked decrease in the E/T ratio from 79.9 ± 4.1% in control animals to 29.9 ± 5.0%. The value of this ratio in animals starved for 24h was also similar to that seen at the nadir of the normal cycle (i.e. 30.6 ± 3.0%).

The withdrawal of food from animals 12h before sampling (i.e. at L-4) also resulted in the depression of both the total activity and the E/T ratio from the values for control animals (Table 4.1;). However, the effects on these parameters were quantitatively less than those obtained after 24h starvation (see above). The total activity was depressed by about two-thirds (from 2.065 ± 0.357 to 0.691 ± 0.215 nmoles/min per mg of microsomal protein) and the E/T ratio was depressed to 49.5 ± 5.1%.

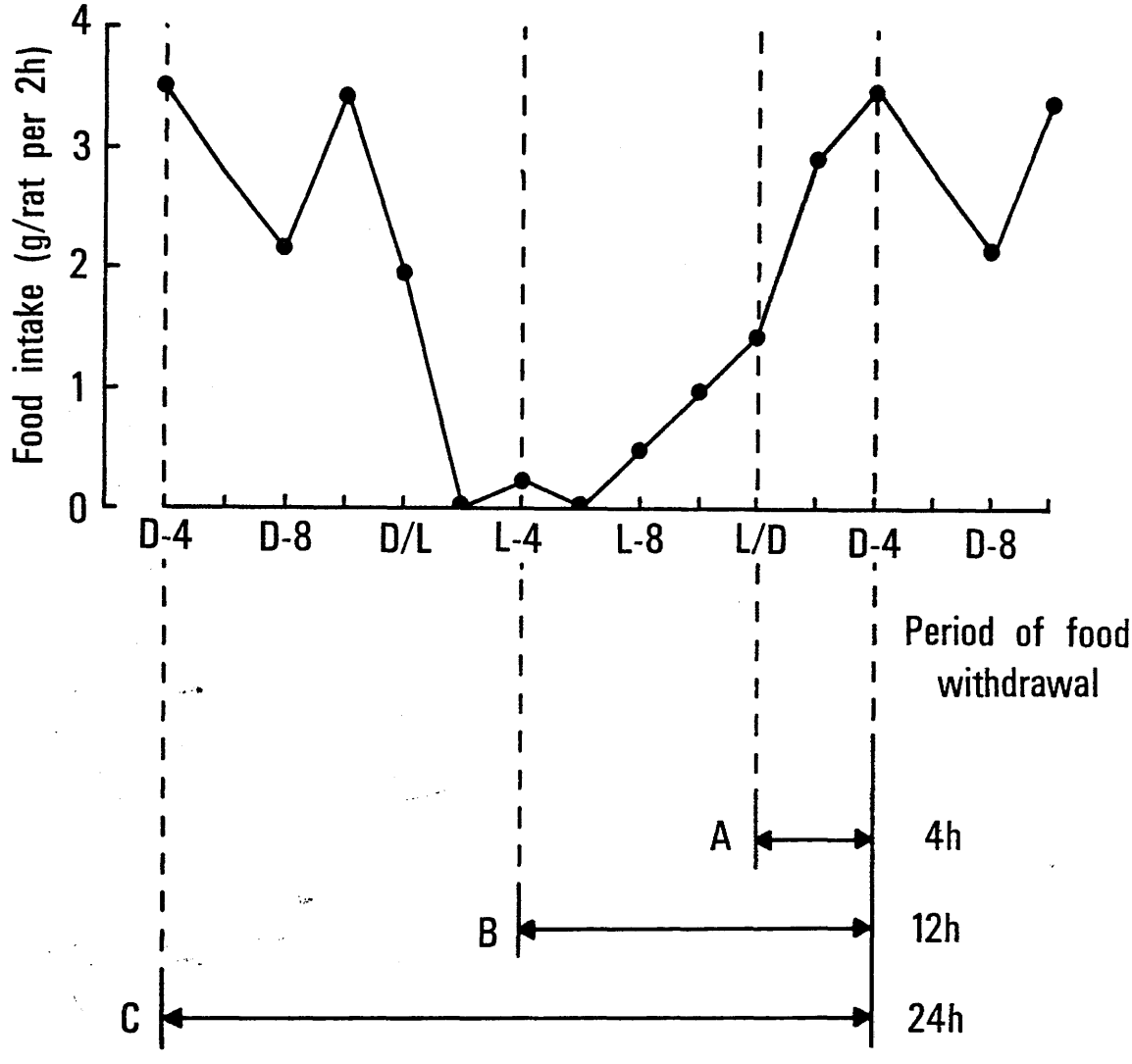


Fig. 4.4 Experimental protocol used for the assessment of the effect of various durations of food withdrawal on the activity of HMG-CoA reductase and the fraction of enzyme in the active form.

Food was withdrawn from normal rats at three different time points in the diurnal rhythm of food intake, namely L/D (A), L4 (B), and D4 (C), preceding a standard sampling time, i.e. D-4 to generate different durations of food deprivation, i.e. 4h (A), 12h (B), and 24h (C). HMG-CoA activity was determined in microsomes prepared from the liver at D-4. (Data for the food intake of rats was redrawn from Peret et al., 1973).

TABLE 4.1

Effects of Different Periods of Starvation on the Expressed and Total Activities of Hepatic HMG-CoA Reductase and the Fraction of Enzyme in the Active Form

Food was withdrawn from animals for various lengths of time (0, 4, 12 and 24h; see Fig. 4.4) before a sample of liver was obtained by cold-clamping at D-4 for the determination of the expressed and total activities of microsomal HMG-CoA reductase. Activities are expressed as nmole/min per mg of microsomal protein at 37°C. Values are means (± S.E.M.) for the numbers of separate determinations shown in parentheses. The levels of significance between values obtained for different conditions and those of control animals are indicated by: * $P > 0.100$; ** $P > 0.010$; *** $P > 0.002$ (two sample 't' test).

Condition		Activity of HMG-CoA Reductase		
		Expressed (E)	Total (T)	E/T (%)
Fed	(4)	1.661 ± 0.334	2.065 ± 0.357	79.9 ± 4.1
Starved 4h	(5)	0.785 ± 0.133**	1.553 ± 0.162*	50.0 ± 5.2***
Starved 12h	(9)	0.317 ± 0.092***	0.691 ± 0.215***	49.9 ± 5.1***
Starved 24h	(5)	0.092 ± 0.021	0.308 ± 0.056	29.9 ± 5.0

When food was withdrawn 4h prior to sampling i.e. at the beginning of the dark period (which normally coincides with the onset of the most active period of feeding, see Fig. 4.4), the only effect observed was on the E/T ratio which achieved values similar to those observed after a 12h period of starvation. There was only a minimal effect on total activity (Table 4.1). Thus, whereas the E/T ratio was depressed to 50.0 $\pm$ 5.2% (i.e the same value as after the starvation), the total activity was maintained at a level not significantly different from that of control animals.

It was evident therefore that the E/T ratio, and not the total activity, was capable of responding acutely to short-term (4h) starvation. Thus, it can be calculated that of the marked suppression (halving) in the resulting expressed activity (from 1.661 $\pm$ 0.334, in control animals, to 0.785 $\pm$ 0.133 nmoles/min per mg microsomal protein in starved animals) observed after 4h of starvation the increase in phosphorylation of the enzyme contributed the major part (73%). As the duration of starvation was prolonged, however, the contribution of the decline in total activity became more important such that the relative contribution of changes in the E/T ratio and total activity became approximately equal (47% and 53% respectively) after the 12h starvation. After 24h starvation the fall in total activity accounted for approximately 61% of the observed decrease in expressed activity. It is suggested that the latter observation resulted not from a decreased importance of the phosphorylation status of the enzyme but rather from the attainment of the maximal degree of phosphorylation obtainable *in vivo* (i.e. E/T ratio approximately 25% at the nadir of the diurnal rhythm, Section 4.2) such that its contribution could not be further increased.

4.3.3 Discussion

The effect of withdrawing food from an animal for 24h on the diurnal rise in total activity has been reported previously (see Rodwell et al., 1976; see Section 4.1). In this study, a similar, apparently complete, suppression of the rhythm in total activity was observed such that the level of total activity attained in the microsomes of liver sampled at D-4 was virtually identical to that normally seen at the nadir of the light:dark cycle. It was evident that the effect was progressive (Fig. 4.6) and did not merely reflect the non-resumption of active food intake during the L/D transition. Thus the withdrawal of food for 12h prior to D-4 did not suppress the total activity as much as when the food was withdrawn 24h prior to sampling. These observations suggest: (i) that the feeding activity between D-4 and D-12 of one diurnal cycle results in the generation of additional stimuli for the synthesis of HMG-CoA reductase (rise in total activity) of the subsequent cycle, and (ii) the diurnal rise in total activity in the dark period requires a continuous signal produced as the result of the gradually increasing intake of food from L-4 to D-4 (see below).

By contrast, the diurnal rise in the E/T ratio was disproportionately affected by the different periods of food withdrawal. The withdrawal of food from the animal at L/D resulted in an abrupt cessation of the diurnal rise in E/T and, indeed, partially reversed the rise observed during the normal diurnal rhythm such that the E/T ratio at D-4 was lower than the value of the ratio of control animals at L/D No additional effect on the

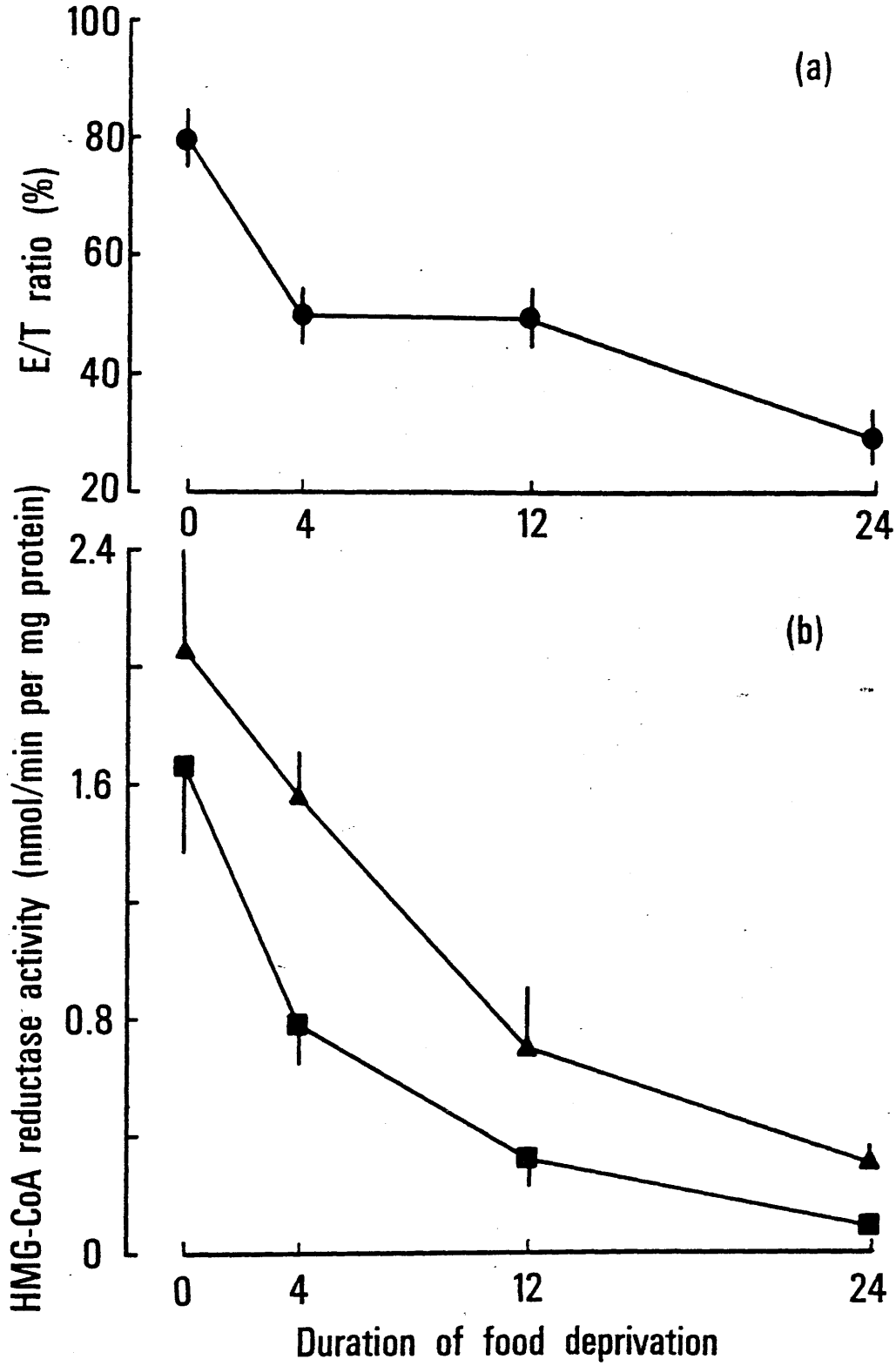


Fig. 4.6 Comparison of the effects of increasing durations of food withdraw on the total activity, (▲; panel b) expressed activity (■; panel and E/T ratio (●; panel a) of HMG-CoA reductase at D-4

See legends to Fig. 4.4

E/T ratio was produced by the withdrawal of food for a further period of 8h prior to sampling at D-4 (Fig. 4.6). Because of this distinct behaviour of the E/T ratio and the total activity in response to 4 and 12h starvation, their relative contributions towards the combined effects on the effective (expressed) HMG-CoA reductase in the liver in vivo was very dependent on the duration in starvation; changes in the E/T ratio were quantitatively more important in the adaptation of the expressed activity over short-term starvation (4h) whereas the converse was evident in conditions of long term starvation (24h) (see Fig. 4.6; cf. Beg & Brewer, 1981). These data are interpreted as suggesting that for livers sampled at D-4 induction of the synthesis of HMG-CoA reductase protein (i.e. total activity) is reponsive to stimuli that occur prior to L/D, the value of the E/T ratio at D-4 is primarily determined by stimuli that occur between L/D and D-4.

The factors responsible for these effects can only be surmized. However, it is noteworthy that the maxima observed in the diurnal rhythms of the circulating concentrations of glucocorticoids and insulin occur at L-10 and D-2 respectively (Knox et al., 1979; Bruckdorfer et al., 1974). Therefore if the above suggestion is valid, the generation of the separate rhythms in these hormones could provide the appropriate stimuli before and after the L/D transition point. The primary effects of glucocorticoids on cellular metabolism involve the control of gene expression through the modulation of the concentration of mRNA either through transcriptional or post-transcriptional control (see Rousseau, 1984). These observations are consistent with a primary involvement of circulating

glucocorticoid concentrations in the generation of HMG-CoA reductase mRNA (see Clarke et al., 1984) and ultimately enzyme protein, i.e. total activity. By contrast, insulin is known to influence the synthesis and covalent phosphorylation of enzyme proteins (for a review, see Goldfine, 1981) and in particular HMG-CoA reductase (Krone & Greten, 1984; Spence et al., 1985; see Section 1.3.3 & 1.5) Thus, as proposed in the previous study in this chapter, insulin may be the primary stimulus regulating the increased dephosphorylation of HMG-CoA reductase (increased E/T ratio) between L/D and D-4 but it may also provide the continuous signal apparently required for the maintenance of protein synthesis (see above). By analogy with its effects on another hepatic enzyme, tyrosine aminotransferase (TAT) (Krahl, 1974; Pilkis & Park, 1974; Fain, 1974), insulin could regulate the translation of HMG-CoA reductase mRNA (Krone & Greten, 1984) thus complementing the proposed transcriptional control of glucocorticoids (see Goldfine, 1981). It is suggested, therefore, that both insulin and glucocorticoids are jointly required for the establishment of the diurnal rhythms in total activity, whereas, of the two, insulin is the major determinant of the E/T ratio. Such a synergism between insulin and glucocorticoids has been previously reported for the maintenance of normal hepatic lipogenesis (Amatruda et al., 1983).

4.4 Pregnancy and Lactation

4.4.1 Experimental

Expressed and total activities of HMG-CoA reductase were measured in microsomes obtained from cold-clamped liver homogenates prepared at D-4 from female animals that were either unmated or at different stages of pregnancy and lactation. Animals were matched for age.

4.4.2 Results

The total activity and E/T ratio of HMG-CoA reductase observed in samples of liver taken from animals on day-12 of pregnancy were very similar to those observed in samples taken from the virgin animal, i.e. 1.4-1.5 nmoles/min per mg of microsomal protein and approximately 65% respectively (Fig. 4.7 & Fig. 4.8). This value for the E/T ratio in virgin animals at D-4 was slightly lower than the equivalent value in male animals (i.e. 65% vs 82%, approximate values, see Section 4.2.2).

Throughout the second half of pregnancy there was a gradual decrease in total HMG-CoA reductase activity (expressed on a unit microsomal protein basis; Fig. 4.7). However, this never reached less than 80% of the value observed for unmated animals. By contrast, there was a marked and rapid decrease in the E/T ratio during the last 2 days of pregnancy (i.e. from 60% to 25%, approximate values, Fig. 4.8).

After parturition both parameters increased in value (Fig. 4.7 & 4.8). However, the E/T ratio started to increase on the first day post-partum reaching the same level (approximately 55%) as

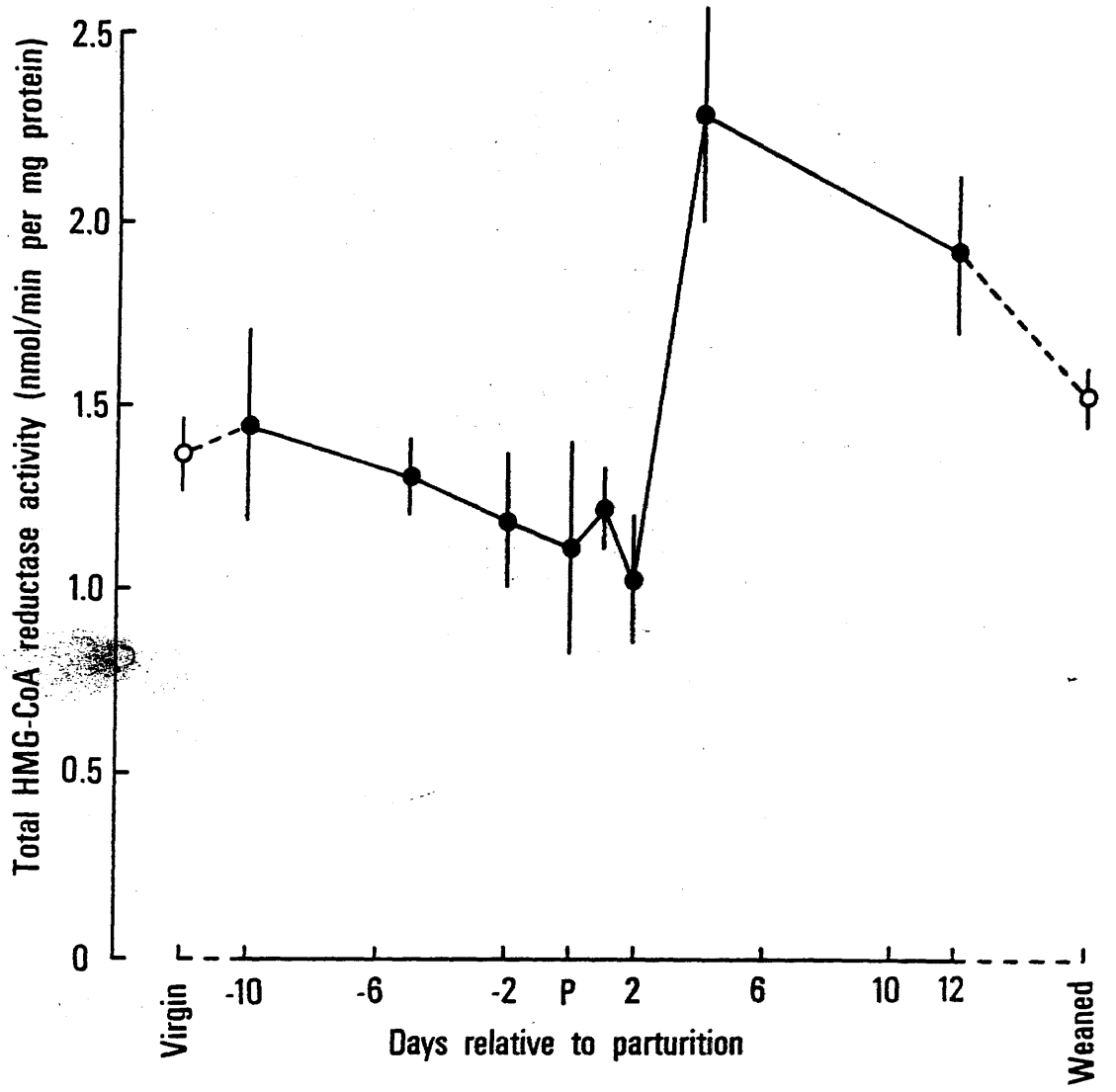


Fig. 4.7 Total activity of HMG-CoA reductase in isolated liver microsomes in virgin rats, rats at various stages of pregnancy and lactation and 24h-weaned rats

Total HMG-CoA reductase activity was determined (see Section 2.8) in microsomal membranes isolated from cold-clamped liver samples obtained 4h into the dark period. Values are means ± S.E.M. for three to six determinations obtained from separate rats.

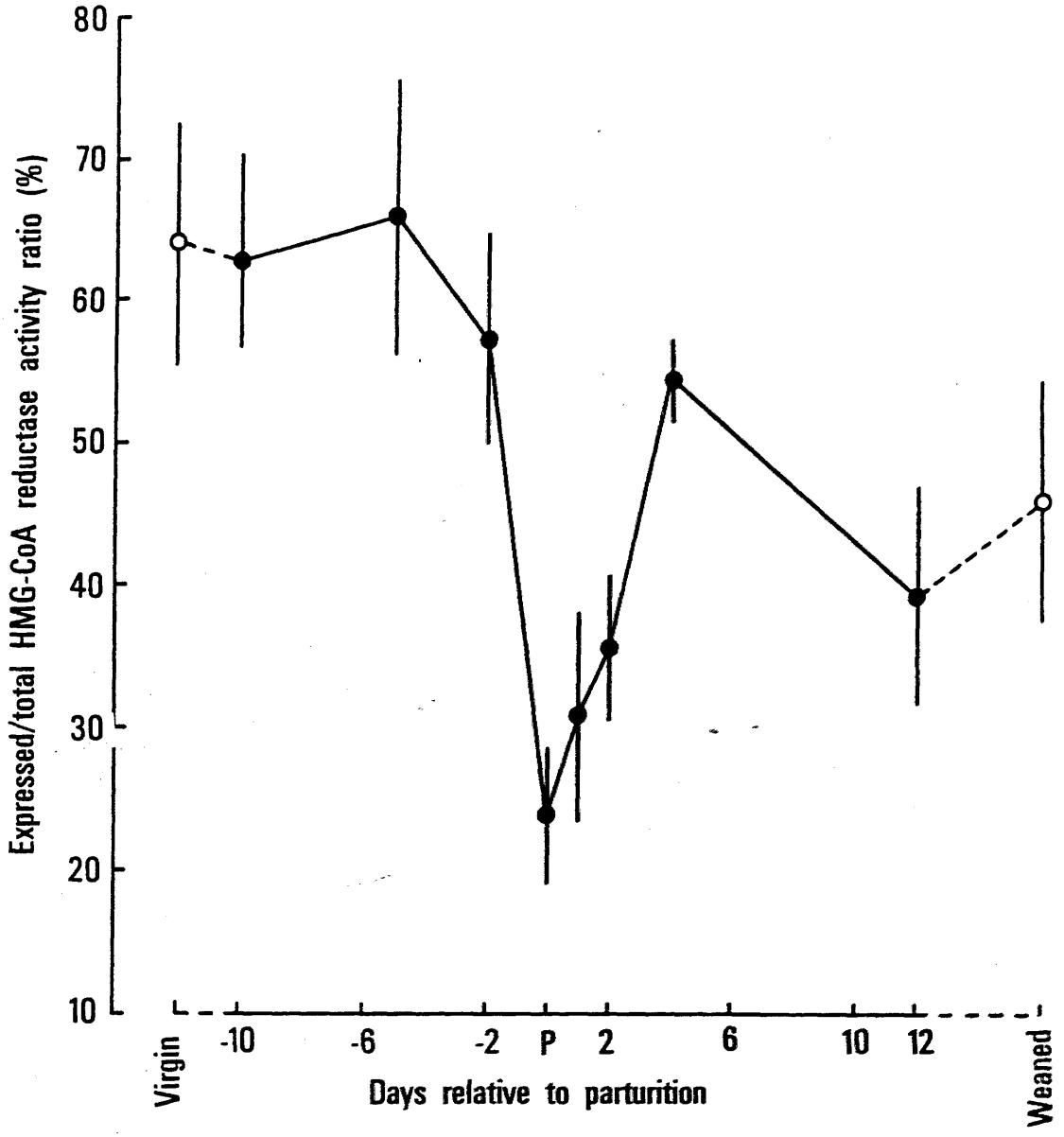


Fig. 4.8 Expressed/total activity ratio (E/T) of hepatic HMG-CoA reductase in virgin rats, rats at various stages of pregnancy and lactation and 24h weaned rats

Expressed (E) and total (T) activities were determined (see Section 2.8 in microsomes isolated from cold-clamped liver samples obtained 4h into the dark period. Values for the E/T ratio (percent) are means ± S.E.M. for thre to six determinations obtained from separate rats.

on day-14 of pregnancy after only 4 days (Fig. 4.8). The total activity, which was constant over the period of parturition, increased (doubled) only after the second day post-partum (Fig. 4.7).

By day-12 of lactation, the E/T ratio tended to decrease to a value lower than those achieved after 4 days post-partum and, indeed, of that found in virgin animals (i.e. 40% vs 65% approximate values, Fig. 4.8). Similarly, there was a gradual decline in total activity between days-4 and -12 of lactation although its value at day-12 was still higher than those observed in virgin animals in the mid-stage of pregnancy (Fig. 4.7).

Largely as a result of the marked changes in the E/T ratio during the last 5 days of pregnancy, the expressed activity of HMG-CoA reductase showed a rapid and marked decrease during the same period to a value approximating to 50% of that observed in unmated animals (Fig. 4.9). As the E/T ratio recovered during the first 2 days post-partum there was little change in total activity and consequently no significant increase in expressed activity. However, after the second day of lactation, both the E/T ratio and total activity increased simultaneously to the values observed on day-4 of lactation. Consequently the expressed activity increased approximately 3.3-fold over the same period such that at day-4, it was approximately 1.3-fold that observed during the mid-stage of pregnancy or in the unmated animals (Fig. 4.9).

During peak lactation (day-12), concomitant decreases in the E/T ratio and the total activity resulted in a depression of the expressed activity to a value slightly lower than those observed in unmated animals (Fig. 4.9).

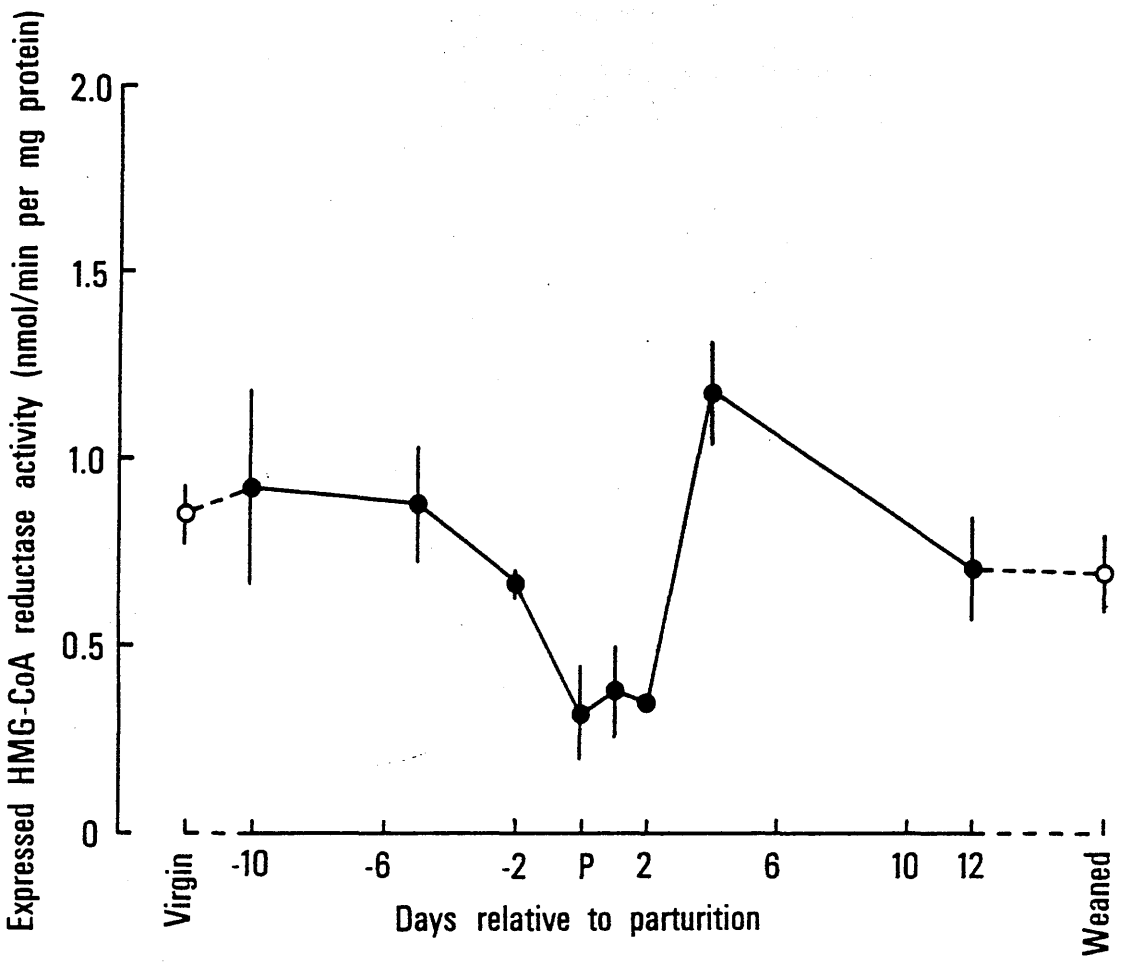


Fig. 4.9 Expressed activity of HMG-CoA reductase activity in isolated liver microsomes in virgin rats, rats at various stages of pregnancy and lactation and 24h-weaned rats

See legend to Fig. 4.8

The removal of pups from lactating dams 24h before sampling on day-12 of lactation induced opposing changes in total activity and in the E/T ratio of HMG-CoA reductase. The total activity decreased (from 1.939 ± 0.226 to 1.504 ± 0.108 nmoles/min per mg of microsomal protein; $P < 0.005$ paired 't' test; Fig. 4.7) whereas the E/T ratio tended to increase (39.3 ± 8.1% to 46.9 ± 9.6%; difference not statistically significant; Fig. 4.8). Because of these opposite responses the expressed activity was unchanged 24h after weaning (Fig. 4.9).

4.4.3 Discussion

Hepatic cholesterogenesis is required during pregnancy and lactation to maintain VLDL synthesis that accompanies the increased endogenous formation of triglycerides in these states (see Section 4.1). VLDL is required by the adipose tissue (pregnancy) and the mammary gland (lactation) as a source of fatty acids (Hamosh et al., 1970; Zinder et al., 1976) but is also possibly used by the mammary gland as a source of cholesterol for milk secretion (Zinder et al., 1976; Gibbons et al., 1983). Thus the observations from the present study that the expressed activity of HMG-CoA reductase was modestly increased compared with unmated animals during mid-pregnancy and lactation were consistent with these requirements. They also correlated well with previous determinations of the rate of hepatic cholesterogenesis in these conditions (when expressed as activity per wet wt. of tissue; Leoni et al., 1984; Gibbons et al., 1983). Taken in combination with the much increased liver weight during these states (Feingold et al., 1983; Campbell & Fell, 1976) the sustained

total activity and value of E/T ratio of HMG-CoA reductase would account for the increased rate of cholesterogenesis when expressed as activity per whole organ in pregnancy (Feingold et al., 1983) and lactation (Feingold & Moser, 1985).

The present study also demonstrated that the expressed activity of HMG-CoA reductase decreased markedly during the last days of pregnancy before recovering 2-4 days post-partum. Significantly, this decrease resulted almost exclusively from a marked decrease in the E/T ratio; there was no equivalent decrease in the total activity. (Thus the decrease in the E/T ratio accounted for about 80% of the decrease in expressed activity when late-pregnant animals are compared to virgin animals). Whilst this decrease in the expressed activity correlated closely with the decrease in the rate of cholesterogenesis and expressed activity of HMG-CoA reductase during the last days of the gestation period as shown by Leoni et al. (1984), the marked decrease in the E/T ratio that accompanied this decrease was not observed by these workers. This discrepancy, as discussed in Chapter 3, is likely to be the direct result of the conventional method of liver sampling used by these workers which must have resulted in artefactual data.

The different responses of the E/T ratio and total activity to the physiological changes occurring in the metabolism of the rat during late pregnancy demonstrates: (i) the physiological importance of changes in the E/T ratio in determining the value of the expressed activity while the animal maintains a relatively high concentration of enzyme protein. It also suggests (ii) that the mechanisms determining the values of total activity and the E/T ratio are

regulated independently. The decrease in expressed activity of HMG-CoA reductase coincided with a marked decrease in circulating insulin concentrations (Flint et al., 1979; Lorenzo et al., 1981; Benito et al., 1982) food intake (Walker & Hahn, 1981; Cripps & Williams, 1975) and hepatic lipogenesis (Lorenzo et al., 1983; Benito et al., 1982) as shown in Fig. 4.10. These concomitant changes suggest that the modulation of the phosphorylation state of HMG-CoA reductase enables the co-ordination of the rate of hepatic cholesterogenesis with the rate of lipogenesis such that the reduction in the amount of triglyceride formed is accompanied by decreased rates of cholesterol synthesized for VLDL formation and secretion. The physiological significance of the maintenance of this high level of enzyme protein may be to enable a very rapid response (increase) in the rate of hepatic cholesterogenesis on the resumption of feeding after parturition through an activation of the existing enzyme (increase in E/T ratio; see Fig. 4.10).

The suggestion that the values of the E/T ratio and total activity are determined by independently regulated mechanisms was supported by two further observations during lactation: (a) the two parameters show opposing responses (i.e. a decrease and a modest increase for total activity and the E/T ratio respectively) in the liver of mid-term lactating dams following the removal of pups 24h earlier and (b), the E/T ratio commenced a recovery two days prior to any increase in total activity at the onset of lactation.

In view of the proposed primary involvement of insulin in the generation of the diurnal rhythm of the E/T ratio of HMG-CoA reductase (see Sections 4.2, 4.3) it is plausible that

the decrease in this parameter during the last days of pregnancy is the direct consequence of the accompanying fall in the plasma concentration of insulin (Fig. 4.10). However, the involvement of other hormones cannot be excluded. Thus, there is a concomitant fall on plasma progesterone concentrations (Flint et al., 1979; Benito et al., 1982; Lorenzo et al., 1983) although there is no indication that this hormone modulates protein phosphorylation. The involvement of hormones other than insulin in affecting the E/T ratio is more obvious during lactation. The E/T ratio increased 2-fold during the first 4 days of lactation at a time when there is no increase in the plasma concentration of insulin (Fig. 4.10). Similarly, the large, transient increase in plasma insulin after weaning (Agius et al., 1979; Burnol et al., 1983) was accompanied by only a modest rise in the E/T ratio. The plasma concentration of a number of hormones is increased during lactation. They include progesterone corticosterone and prolactin (Flint et al., 1979; Flint et al., 1984; Amenomori et al., 1970). Although the acute effects of prolactin on liver metabolism has not been determined the marked increase in its concentration in the circulation during parturition may coincide with the commencement of the increase in the E/T ratio. Furthermore, the rapid decline in the levels of this hormone on weaning may be a mechanism by which the expected effect of insulin on the E/T ratio in these animals is counteracted. The phosphorylation state of HMG-CoA reductase during pregnancy and lactation may also be affected by the, as yet uninvestigated, variations in the rate of cholesterol uptake by the liver, animals (see below).

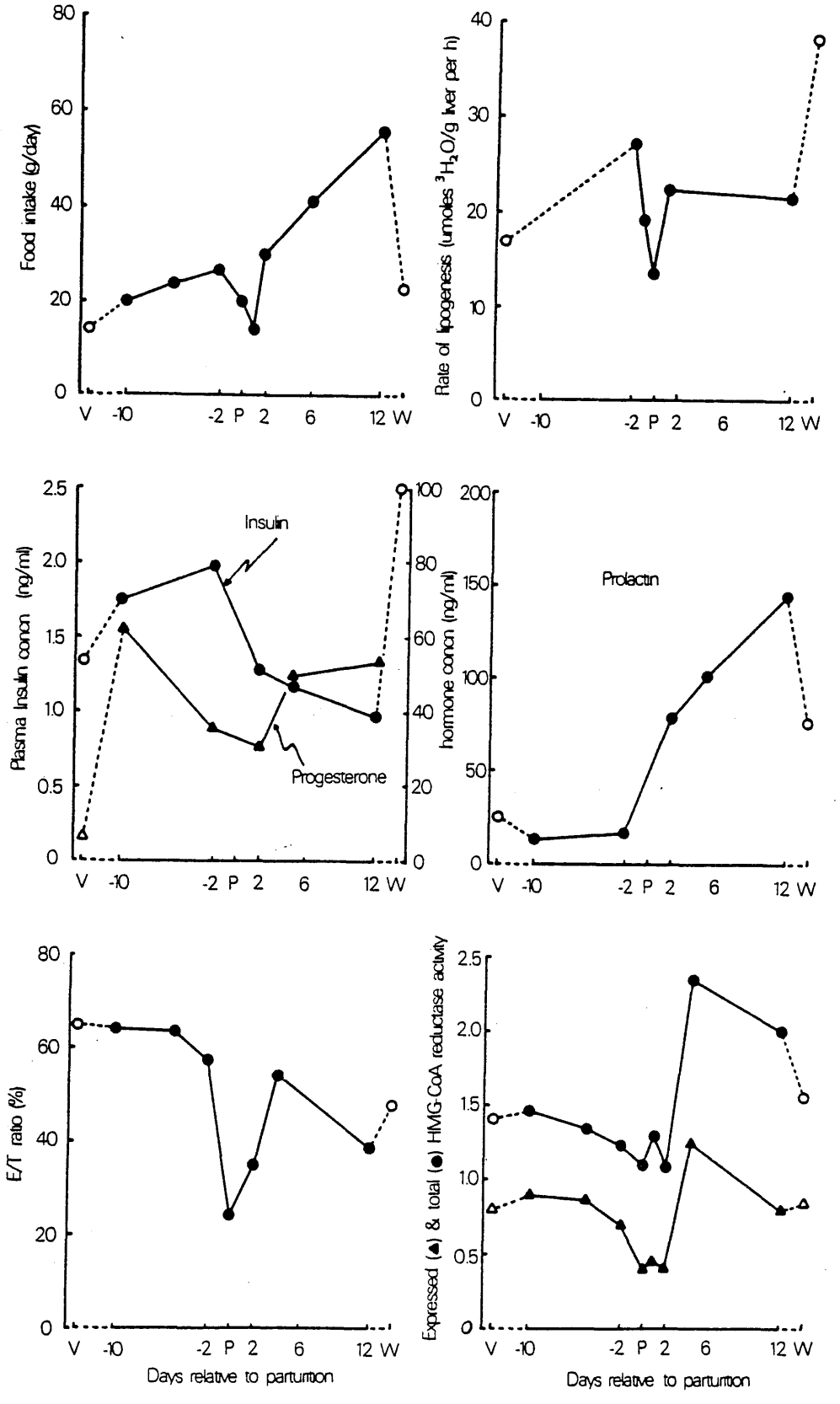


Fig. 4.10 Concomitant changes in food intake, plasma hormone concentrations and the rate of liver lipogenesis with changes in the parameters of HMG-CoA reductase activity

Source of references include Walker & Hahn (1981), Cripps & Williams (1975) (food intake); Flint et al. (1979), Agius et al. (1979) (hormone concentrations), Agius et al. (1979), Lorenzo et al. (1981) (lipogenesis).

The total activity of HMG-CoA reductase would also be determined by the result of the individual hormonal and/or nutritional changes on the turnover of the enzyme. The fact that the total activity was maintained at a high level during late pregnancy suggests that the decrease expected in this parameter due to the fall on plasma insulin concentrations (see above) was effectively counteracted by other factors. A likely candidate for this antagonism is the reduced delivery of cholesterol from chylomicron remnants to the liver during the relative hypophagia that occurs around parturition. The factors responsible for the increase observed in total activity during early lactation is unknown, although in view of possible involvement of glucocorticoids in the regulation of HMG-CoA reductase protein synthesis (see Section 4.3.3) it is noteworthy that lactation is a hypercorticoid condition. The increasing concentration of prolactin in the circulation during this phase of lactation may also be involved.

As indicated in the foregoing discussion, the increase observed in the expressed activity of HMG-CoA reductase during lactation is paradoxical, not only because of the low circulating concentration of insulin but also because of the gross hyperphagia of the lactating rat. Under these conditions both the total activity and E/T ratio of this enzyme would be expected to be reduced due to the increased delivery of dietary cholesterol to the liver via chylomicron remnants (see Section 1.3.1(i) and Chapter 6). Although hyperphagia, albeit less severe, is evident in rats during mid-pregnancy, its anticipated effects on the parameters of HMG-CoA reductase activity are possibly counteracted by the accompanying

hyperinsulinaemia of this state. During lactation, two possible mechanisms could be responsible for the apparent lack of feedback regulation of the activities of HMG-CoA reductase by the increased level of dietary intake of cholesterol:

(1) The rate of chylomicron remnant-mediated delivery of cholesterol to the liver may be restricted. This could be achieved by an increased depletion of the cholesterol content of these particles at the periphery. Evidence exists that during lipoprotein lipase action on chylomicrons (and VLDL) in the capillaries of mammary tissue, cholesterol (and cholesterol ester) is taken up by the mammary secretory cells (Zinder et al., 1976). Since the rates of cholesterogenesis within the mammary gland are relatively low an appreciable proportion of the cholesterol (approx. 16mg/day on Day-15 of lactation; Clarenburg & Chaikoff, 1966) secreted into the milk is obtained from the circulation (Connor & Lin, 1967; Gibbons et al., 1983).

(2) The delivery of cholesterol to the liver could be decreased by the attenuation of chylomicron remnant and LDL binding and internalization either by through a decrease in the number of surface receptors in the liver or by decreasing the affinity of these receptors for their ligands. Such an adaptation is known to occur under pathological conditions in which the normal response of hepatic HMG-CoA reductase to dietary cholesterol intake is lost or stunted (Barnard et al., 1984). It may be related to the altered composition (especially of apoproteins) of the lipoproteins (Levy et al., 1985). In lactation these effects could ensure a longer

residence time of chylomicron remnants and LDL in the circulation and consequently a more complete depletion of their cholesterol content by the mammary gland.

The mechanisms involved in the maintenance of a high expressed activity of HMG-CoA reductase in spite of hyperphagia provides scope for further study of the relationship between peripheral and hepatic lipoprotein metabolism. Feingold and Moser (1985) have recently reported that the feedback mechanism of dietary cholesterol on hepatic cholesterogenesis is intact during lactation. However, the increased requirement for bile and synthesis specifically induced during lactation and anticipated increased bile flow in response to the hyperphagia of this condition would be expected to produce major effects in the specific activity of HMG-CoA reductase. Similar effects may also occur during the diurnal cycle of feeding.

4.5 General Conclusions

The use of the cold-clamping technique has enabled the quantitation of the phosphorylation state of HMG-CoA reductase *in vivo*. The data presented in this chapter have demonstrated that this parameter varies considerably in different physiological conditions in response to acute hormonal and nutritional changes. These observations demonstrate that reversible phosphorylation has an important physiological role to play in the regulation of hepatic HMG-CoA reductase activity and cholesterogenesis *in vivo* and provides a mechanism by which this pathway can be regulated in concert with hepatic fatty acid synthesis and esterification (see Section 5.1; Fig. 5.1). Indeed, in the three conditions studied a change in the phosphorylation status of HMG-CoA reductase appeared to

be the primary mechanism through which the effective (expressed) activity of HMG-CoA reductase became rapidly adapted to the immediate needs of the animal. Thus in short- and medium-term starvation (up to 12h; i.e. the duration of starvation that is most physiologically relevant to the rat, see Section 4.3) the decrease in the E/T ratio accounted for approximately 50-75% of the changes observed in expressed activity. Moreover, these changes in the phosphorylation state of HMG-CoA reductase serve to co-ordinate more closely the expressed activity (i.e. cholesterol formation) with the feeding activity of the animal and the requirement for VLDL secretion by the liver (see Section 4.1 and 4.2). In these conditions, changes in the phosphorylation state amplify the effects of variations in HMG-CoA reductase activity produced by rapid changes in the rates of synthesis (and possibly degradation) of the enzyme. Similarly during late pregnancy, changes in the E/T ratio of HMG-CoA reductase appear to be the sole mechanism involved in mediating the decrease in the rate of hepatic cholesterogenesis during this period.

CHAPTER 5

EFFECTS OF HORMONAL TREATMENT OR DEFICIENCY ON HMG-COA REDUCTASE IN RAT LIVER IN VIVO

5.1 Introduction

In Chapter 4, it was demonstrated that the fraction of HMG-CoA reductase in the phosphorylated form varied markedly in a number of physiological conditions *in vitro*. Changes both in the phosphorylation status and in the concentration of the enzyme (due to the very short half-life) can occur in response to acute changes in the hormonal and nutritional status e.g. as demonstrated by the distinctive diurnal rhythms of the two parameters. Although changes in the phosphorylation state of the enzyme occur more rapidly than changes in enzyme concentration, nevertheless they may also persist in response to long-term changes in hormonal conditions (e.g. those induced by starvation or diabetes) due to chronic changes in the relative activities of protein kinases and phosphatases. Although the response to some hormones of the bicyclic cascade mechanism described for the regulation of HMG-CoA reductase has been described in isolated hepatocytes (see e.g. Ingebritsen, 1983) little work has been done to determine the types of hormones that could be involved in the regulation of this system *in vivo*.

In the present study the effect of acute hormonal treatment *in vivo* on the phosphorylation state of HMG-CoA reductase has been investigated. In particular, the effects of two types of hormone were studied: (a) the pancreatic hormones insulin and glucagon

because of the important role played by these hormones in the control of hepatic metabolism in relation to dietary intake and (b) the catecholamines, because of the apparently profound effects of stress on the phosphorylation state of this enzyme (see Chapter 3).

Insulin and glucagon are considered to be the main homeostatic hormones with respect to the synthesis and supply of substrates by the liver to extrahepatic tissues. Insulin stimulates glycogen and lipid synthesis whereas glucagon promotes fatty-acid oxidation, glycogen breakdown and gluconeogenesis. These antagonistic effects result in the homeostatic regulation of blood glucose as well as in changes in production and secretion of ketone bodies and lipoproteins by the liver under different physiological conditions. Their effects are known to be mediated through reversible phosphorylation of key enzymes in the pathways of carbohydrate and lipid metabolism. In conditions characterized by low glucagon, and high insulin concentrations, key enzymes (e.g. glycogen synthase, pyruvate kinase, pyruvate dehydrogenase, acetyl-CoA carboxylase) involved in anabolic processes are largely dephosphorylated and active (Fig. 5.1 for a review see Geelen et al., 1980). Under the inverse conditions (i.e. low insulin/glucagon ratio) these enzymes are phosphorylated and inactive whereas 'catabolic' enzymes (e.g. glycogen phosphorylase Fig. 5.1) are activated through phosphorylation. Because of this reciprocal relationship between the degree of phosphorylation and activity in the two sets of enzymes, conditions that favour the activity of protein kinases result in a catabolic state of the liver whereas those that favour protein phosphatase activity result in anabolic activity. Moreover, the same set of protein kinases and

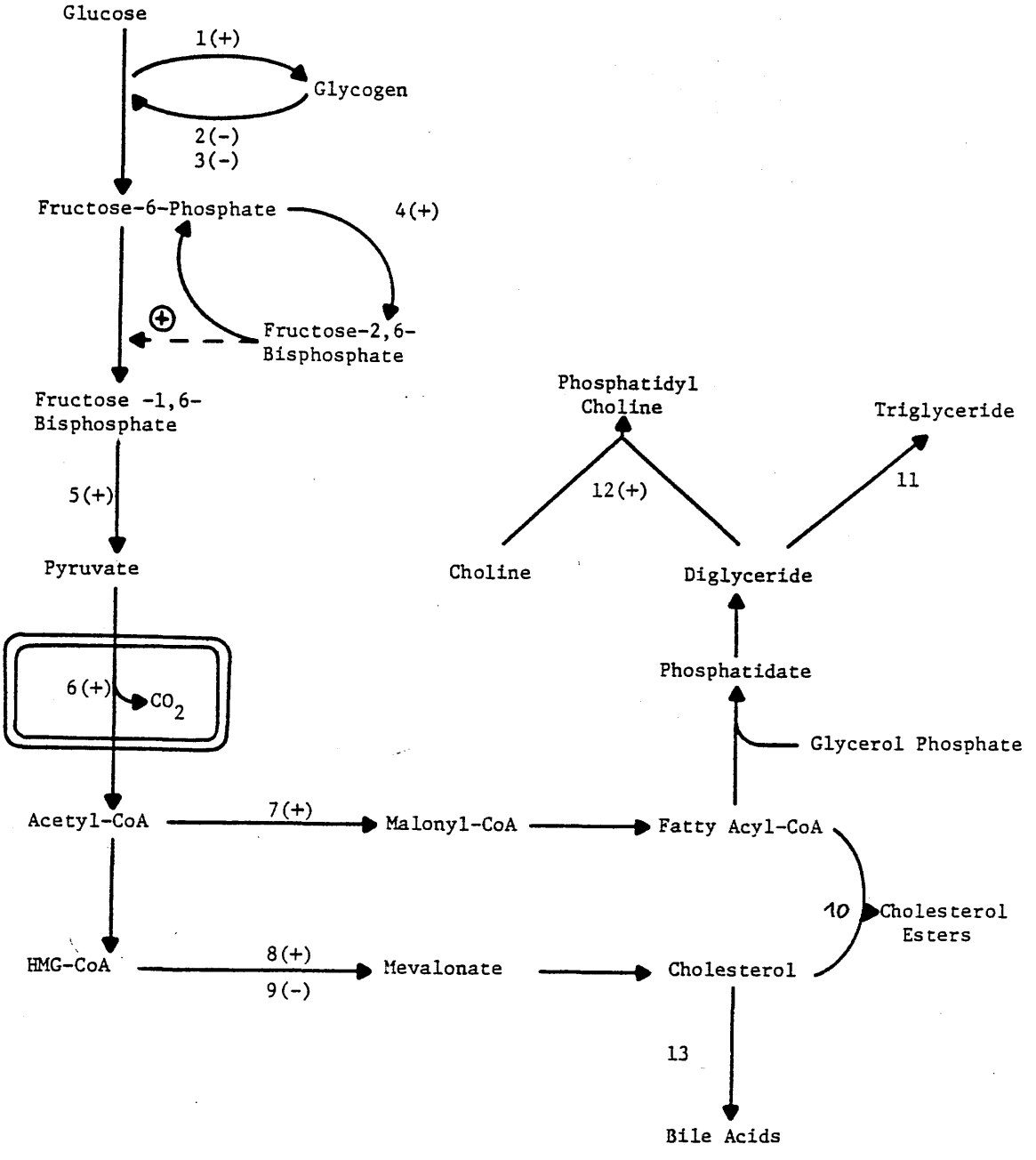


Fig. 5.1 Interconvertible enzymes of carbohydrate and lipid metabolism

Enzymes regulated by reversible phosphorylation are numbered and presented in the dephosphorylated form i.e. in conditions characterized by low glucagon, high insulin. (Increased (+) and decreased (-) enzyme activity.)

(1) glycogen synthase; (2) glycogen phosphorylase; (3) phosphorylase kinase; (4) 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; (5) pyruvate kinase; (6) pyruvate dehydrogenase; (7) acetyl-CoA carboxylase; (8) HMG-CoA reductase; (9) HMG-CoA reductase kinase; (10) acyl-CoA cholesterol acyltransferase; (11) diglyceride acyltransferase; (12) cytidylyl transferase; (13) cholesterol 7α-hydroxylase

phosphatases can potentially mediate both reciprocal processes and some cases of this are well established (e.g. glycogen metabolism; see Cohen, 1982).

In view of the early demonstrations that the phosphorylation state of HMG-CoA reductase can be modulated in isolated hepatocytes (by insulin and glucagon) and in intact animals by pharmacological doses of glucagon (see below) there are indications that these hormones may be primary factors involved in the modulation of the E/T ratio. For example, in the diurnal cycle (see Chapter 4) it is possible that the phosphorylation state of HMG-CoA reductase is regulated co-ordinately with that of other enzymes of hepatic lipogenesis.

Although, as indicated above, the effect of an intraperitoneal injection of glucagon to whole animals has been reported (see Beg et al., 1981; Section 1.5) these experiments were performed using classical liver sampling and fractionation techniques for the preparation of microsomes and therefore were susceptible to the same problems and artefacts outlined in Chapter 3. Indeed it can be calculated that the 'active' enzyme in the studies of Beg et al., represented only approximately 13% of the total activity of the enzyme. This low level of activation is possibly unphysiological because, as the results reported in the previous chapters indicate the E/T ratio never appears to fall below about 25% when measured in vivo. Consequently, a reappraisal of the effects of glucagon and an initial investigation of those of insulin on E/T ratios in vivo was desirable.

Modes of Action of Hormones

(i) Pancreatic hormones

The mode of action involved in the pleiotropic effects of insulin on cellular metabolism is not clearly understood in spite of numerous theories (for a review, see Fain, 1984). Insulin can induce both the phosphorylation and dephosphorylation of cellular proteins in vivo. The current emphasis, however, is on membrane protein kinases and phosphatases due to the recent discovery that the insulin receptor displays tyrosine kinase activity (Kasuga et al., 1982; Avruch et al., 1982). However, other than its autophosphorylation activity no other protein substrates in the cell have been identified for this kinase. Numerous second messengers including Ca^{2+} (Czech, 1977), peptides (see Larner et al., 1979; 1982), inositol trisphosphate (see Farese, 1984) and polyamines (see Cohen, 1985) have also been proposed to mediate insulin action.

In contrast, the cellular effects of glucagon are thought to be mediated solely by its increasing the intracellular concentration of cyclic AMP as the consequence of activating plasma membrane bound adenylate cyclase (Heyworth & Houslay, 1983). The increased cAMP concentration activates cyclic AMP-dependent protein kinase (type-A protein kinase) which is known to phosphorylate numerous intracellular proteins (see above; see Cohen, 1985) on serine or threonine residues (Krebs & Beavo, 1979).

(ii) Catecholamines

Epinephrine and nor-epinephrine regulate physiological processes via their interactions with a variety of plasma membrane receptors. These receptors are classified into 2 types, α and β that

are further subdivided into α_1, α_2 and β_1, β_2. This classification is based on the cellular effect elicited by the two hormones and by the relative potencies of their agonists (Table 5.1). The catecholamine α_1-adrenergic receptor system (for a review see Exton, 1985) exerts its action on target tissues via the increase in cytosolic Ca^{2+} concentration. Its effects are perhaps best characterized in smooth muscle (e.g. of blood vessels or intestine) in which occupancy of their receptor induces contraction or relaxation respectively. In the liver α_1-mediated effects include the activation of glycogenolysis through the increased activity of phosphorylase a (Charest et al., 1983; 1985) and increased gluconeogenic activity through effects at several sites including the phosphorylation and inactivation of pyruvate kinase (Garrison et al., 1979). α_2-(Bylund & U'Prichard, 1983) and β- (see Stiles et al., 1984) adrenergic receptors exert inhibitory or stimulatory effects on adenylate cyclase activity via the influence of GTPbinding proteins Ni and Ns, respectively. Thus the binding of agonist to α_2-receptors decreases, whereas that at β-receptors increases intracellular cyclic AMP concentrations (cf. for glucagon, see above). The liver possesses primarily β_2-adrenergic receptors (Minneman & Molinoff, 1980; Lacombe et al., 1976) which have a very much greater affinity for epinephrine than norepinephrine (Table 5.1). The β_1-adrenergic receptors which have an approximately equal affinity for epinephrine and norepinephrine are found primarily in the heart, adipose tissue and brain (Minneman & Molinoff, 1980; Table 5.1). Juvenile male rats possess a very active β-adrenergic system capable of mediating glycogen mobilization from the liver but ageing

TABLE 5.1

Adrenergic Receptor Subtypes, their Target Tissues and Mechanism of Action

Receptor	Agonists (order of potency)	Major Target Tissues	Mechanism of Action
α_1	EPI > Ne > ISO	Smooth muscle *Liver Heart Adipose tissue Kidney Sweat + Salivary glands Brain	Alter cellular Ca^{2+} fluxes
α_2	EPI > NE > ISO	Adrenergic nerve endings Liver Platelets Adipose tissue Endocrine pancreas Vascular smooth muscle Kidney Brain	Inhibit adenylate cyclase
β_1	ISO > NE $\simeq$ EPI	Heart Adipose tissue	Stimulate adenylate cyclase
β_2	ISO > EPI >> NE	Liver Skeletal muscle Smooth muscle Endocrine pancreas Salivary glands	Stimulate adenylate cyclase

EPI = epinephrine
NE = norepinephrine
ISO = isoproterenol
* only important in certain species e.g. rat; in humans β-adrenergic response predominates (Exton, 1985)

(Taken from Lefkowitz et al. (1983) and Exton (1985)

causes a progressive decline in this response such that in mature rats, glycogen mobilization is mediated primarily by the effects of α_1-adrenergic receptors (Morgan et al., 1983; Katz et al., 1985). Thus rats at the age used in the present study (i.e. 5-6 weeks, 160-200g) would be approaching the mid-point of this transition period (see Blair et al., 1979a,b; Morgan et al., 1983) and would have been expected to respond both to α and β effects of catecholamines.

5.2 Experimental

In each of the following studies, the expressed (E) and total (T) activities of HMG-CoA reductase and hence the E/T ratio were determined in the microsomal fraction isolated from liver samples obtained by the cold-clamping technique (see Chapter 3) as described in Methods (Section 2.8).

5.3 Results

5.3.1 Acute Alterations in Plasma Insulin Concentrations

The effects of acutely and chronically modifying the circulating insulin concentrations in rats on the total activity and E/T ratio of HMG-CoA reductase were studied. Several different approaches were adopted to achieve these alterations:

(i) Intraperitoneal administration of glucose into normal rats. This method was used in order to increase plasma insulin concentrations within a physiological range without inducing hypoglycaemia (see Stansbie et al., 1976b). (ii) Intravenous infusion of anti-insulin serum (raised in guinea-pigs) which was used

to acutely diminish the plasma insulin concentrations (Martin & Baldwin, 1971; Stansbie et al., 1976b). (iii) Streptozotocin treatment. This β-cell cytotoxic drug was used to produce chronic reduction in circulating insulin concentrations in rats on a longer-term basis. (iv) These animals were further used to investigate the effects of replenishment of circulating insulin concentrations by intravenous infusion and intraperitoneal injection respectively.

(i) Intraperitoneal injection of glucose into normal rats

Rats (160-200 g) were injected either with glucose (200 mg) or with an equivalent volume of saline (0.8 ml) 4h into the light period (L-4). This point in the normal diurnal cycle coincides with basal circulating concentrations of insulin (see e.g. Knox et al., 1979) and the nadir of the E/T ratio for HMG-CoA reductase (section 4.2). The treatment raised blood glucose by 150% after 10 min and normoglycaemia was restored after 50 min (Fig. 5.2). Although no direct measurements of plasma insulin concentrations were made, it is known from previous studies that a similar dose of glucose to rats results in moderately elevated circulating levels of this hormone (Burnol et al., 1983).

As shown in Fig. 5.2 the administration of glucose to animals produced a significant increase in the E/T ratio of HMG-CoA reductase after 30 min when compared to saline-injected animals (52.1 ± 2.4 v 33.9 ± 3.1; n = 3) and only appeared to last as long as the hyperglycaemia persisted. The rise in the E/T ratio was due to a marked increase in the expressed activity without any significant

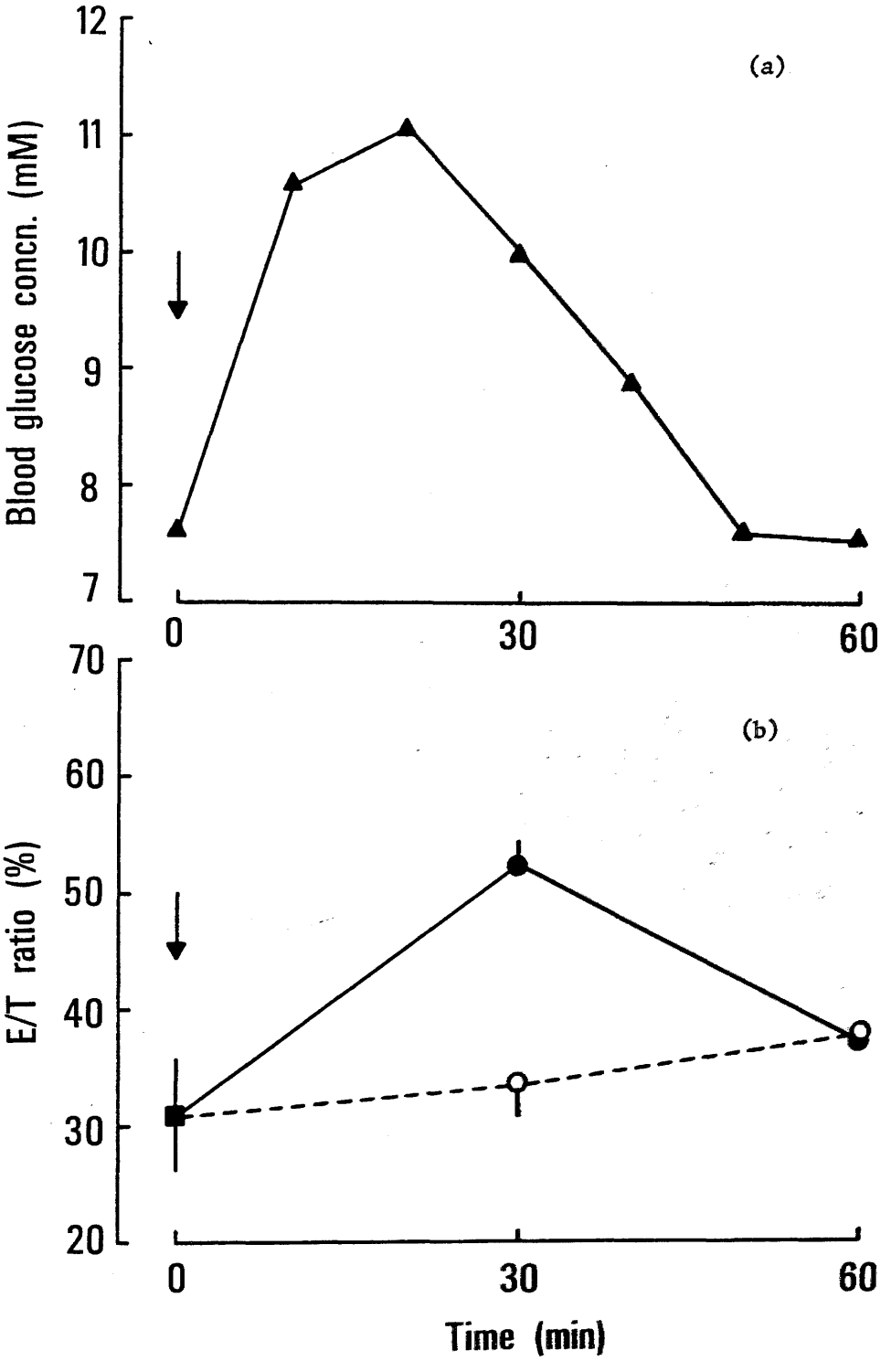


Fig. 5.2 Effect of intraperitoneal injection of glucose on (a) blood glucose concentration and (b) the E/T ratio of hepatic HMG-CoA reductase at L-4

(a) Glucose (200mg) was injected into rats at L-4 (arrow) Blood glucose concentration ( ) was determined in whole blood samples obtained from the tail of anaesthetized rats (see Section 2.11.1). (b) The E/T ratio for HMG-CoA reductase was determined in cold clamped liver samples simultaneous to, and at 15 and 30 min. subsequent to the injection (arrow) of glucose (●) or saline (○). Values are means ± S.E.M. except for 30 min. values which were averages of two separate determinations which gave very similar results.

variation in the total activity (results not shown). A much more gradual and modest increase in the E/T ratio was observed in control animals; this may have resulted from the normal increase in expressed activity that occurs after L-4 during the diurnal cycle (Section 4.2).

(ii) Intravenous infusion of anti-insulin serum (A.I.S.)

The effects of acutely diminishing the circulating insulin concentration on the E/T ratio of HMG-CoA reductase in the liver was investigated by intravenous infusion of an aliquot of guinea-pig serum containing an antibody raised against insulin (see Methods; section 2.13). In order to maximize the putative response of changes in E/T, this treatment was performed at D-3.5, a point in the diurnal cycle at which the circulating insulin levels are high and the E/T ratio is near-maximal (see Chapter 4). Futhermore, the total activity of HMG-CoA reductase at this time in the diurnal rhythm is relatively stable.

That the antibody in the serum was effective in decreasing insulin concentrations in the treated rats was ascertained by determining the concentration of glucose in the blood obtained from the tail subsequent to the intravenous infusion of 1 ml of A.I.S. Treatment of rats with A.I.S. resulted in a maximal increase in blood glucose of 2-fold after 30 min (Fig. 5.3) but the degree of effectiveness varied markedly between animals; the range of hyperglycaemia generated is demonstrated in Fig. 5.4. However, in each case, the return to normoglycaemia was as rapid as the initial rise in the blood glucose observed after the administration of

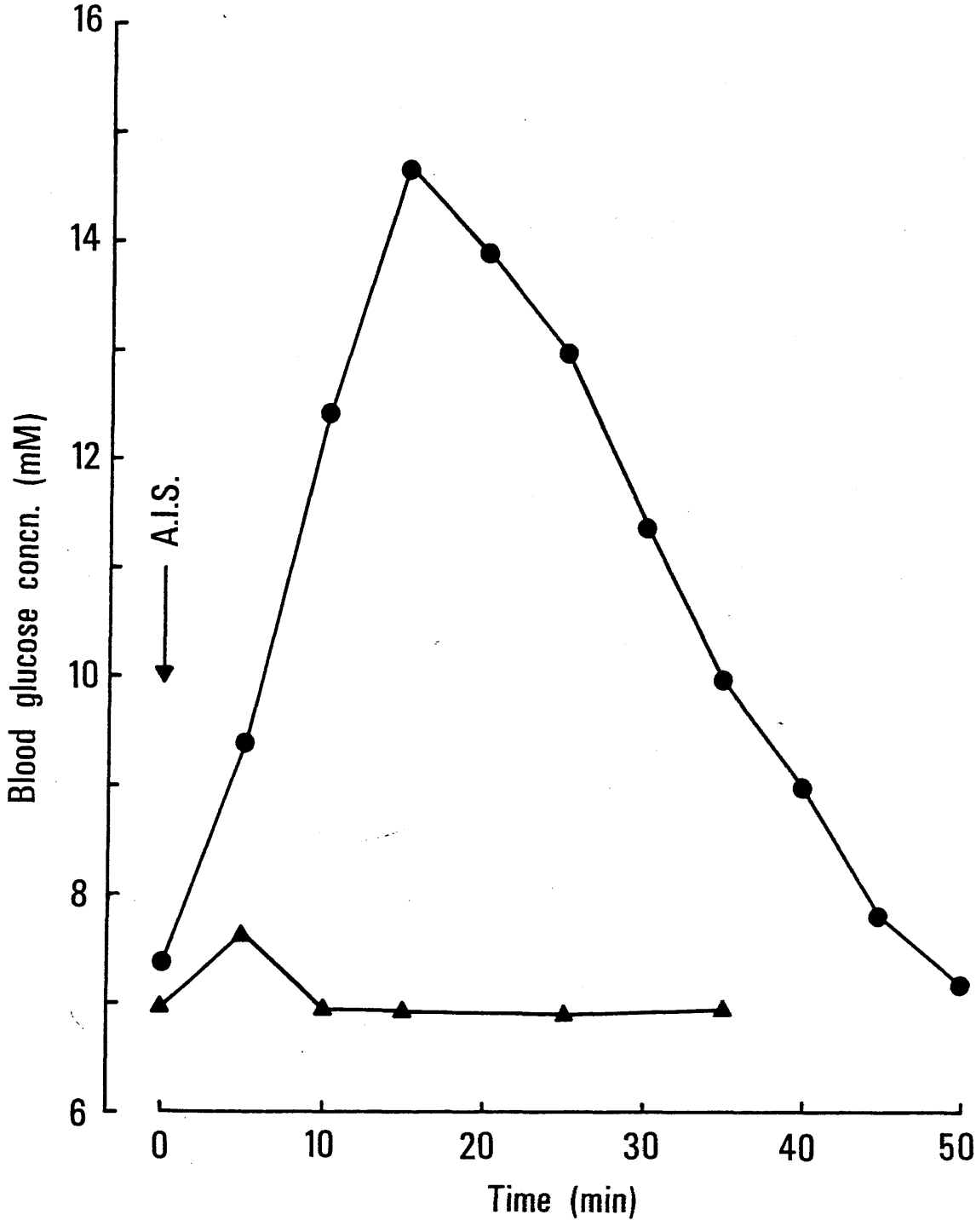


Fig. 5.3 Effect of intravenous infusion of anti-insulin serum (A.I.S.) on blood glucose concentration

A.I.S. (1ml) (●) or control serum (▲) were infused into anaesthetized rats at D-3.5 to D-4 and blood glucose concentration determined in whole blood samples obtained from tail at 10 min. intervals.

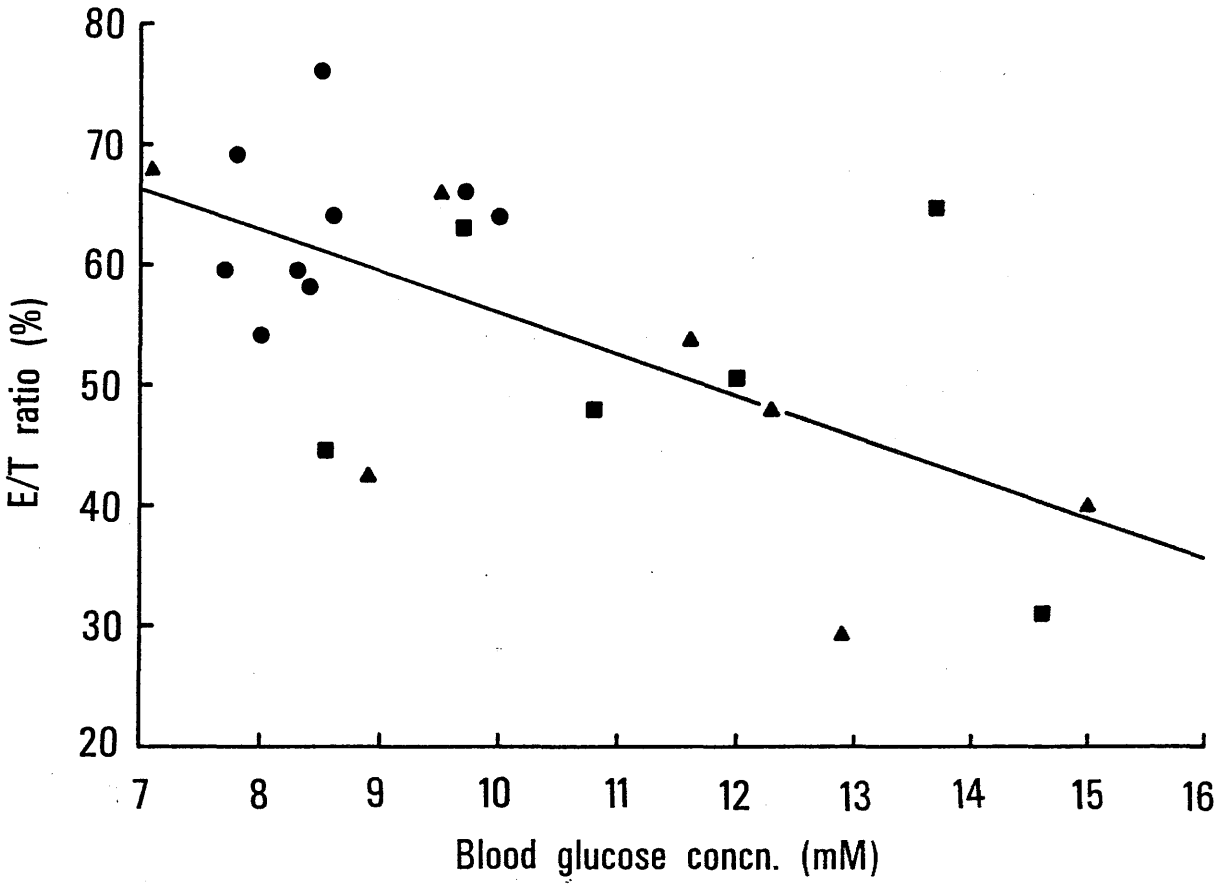


Fig. 5.4 Effects of different degrees of insulin deficiency on the E/T ratio of microsomal HMG-CoA reductase in animals treated with A.I.S.

Rats were intravenously infused with control serum (1ml; 15 and 30 min ●) or A.I.S. (1ml; 15 min ■ ; 30 min ▲). The straight line was obtained by performing linear regression analysis; the correlation coefficient was -0.61.

A.I.S. This was presumably due to an increased secretion of insulin from the pancreas in response to the increased blood glucose concentration with consequent titration of the limited amount of antibody infused.

Coincidental with the peak of glucose concentrations 15-30 min after the infusion of A.I.S. there was a significant decrease in expressed activity of the enzyme (1.035 ± 0.104 to 0.760 ± 0.169 nmoles/min per mg of microsomal protein) without any discernable loss of total activity (1.571 ± 0.176 v 1.621 ± 0.107 nmoles/min per mg of microsomal protein) (Table 5.2). Consequently the E/T ratio was markedly depressed from 63.0 ± 2.5 to 49.8 ± 6.9%. A similar fall in the E/T ratio was observed in animals that had been treated with A.I.S. for 15 mins (Table 5.2).

In control animals (to which serum from non-immunized guinea-pigs was administered) the total activity of HMG-CoA reductase and E/T ratio were not significantly different from those observed in other animals treated similarly but with saline at the same time of the diurnal cycle (e.g. E/T = 63.0 ± 2.5%, serum cf. 68.0 ± 1.7%, saline after 15 or 30 min).

There was an inverse correlation between the E/T ratio and the degree of hyperglycaemia induced by A.I.S. in individual animals. This suggests that the E/T ratio was directly affected by the concentration of insulin in the circulation.

(iii) Streptozotocin treatment

Long-term insulin deficiency was induced in rats in order to investigate (a) the effects of chronic insulin deficiency and (b)

TABLE 5.2

Effect of Acute Administration of A.I.S. on the Expressed and Total Activities of Microsomal HMG-CoA Reductase and the Fraction of Enzyme in the Active Form

Anti-insulin serum (A.I.S.; 1ml) or control serum (see text for details) were infused intravenously into normal rats at D-3.5 to D-4. After 15-30 min, the liver was cold-clamped and used for the measurement of expressed and total HMG-CoA reductase activities as described in Section 2.8. Activities are expressed as nmole/min per mg of microsomal protein at 37°C. The activities in samples obtained 15 or 30 min after the infusion of control serum were not significantly different and their values were pooled. Blood glucose concentrations were determined (see Section 2.11.1) in arterial blood obtained from the abdominal aorta immediately after liver sampling. Values are means (± S.E.M.) for the numbers of different determinations shown in parentheses. Those marked are significantly different (*, $P < 0.02$; **, $P < 0.10$; two sample 't' test) from controls.

	Blood Glucose (mM)	HMG-CoA Reductase Activity		
		Expressed (E)	Total (T)	E/T ratio
Control serum (15-30 min)	8.5 ± 0.3 (9)	1.035 ± 0.104 (9)	1.621 ± 0.107 (9)	63.0 ± 2.5 (9)
A.I.S. (15 min)	11.6 ± 1.0* (6)	1.110 ± 0.238 (4)	2.133 ± 0.399 (4)	50.6 ± 5.7* (6)
A.I.S. (30 min)	10.8 ± 1.3* (6)	0.760 ± 0.169** (6)	1.571 ± 0.176 (6)	49.8 ± 6.9* (6)

the acute and medium-term effects of insulin replacement on the E/T ratio of HMG-CoA reductase. In the case of (a) the livers of animals were sampled at D-2 whereas in (b) insulin replacement was commenced at D-2 and continued over the subsequent 4h period. This time period was chosen in order to demonstrate the effects of either deficiency or replacement of insulin at a time of day when the circulating concentration of the hormone is high in normal, untreated animals.

Streptozotocin (150mg/kg body wt.) was administered to rats by intraperitoneal injection. The animals were used 7-8 days later when the effect of insulin deficiency on HMG-CoA reductase activity was maximal (see Lakshmanan et al., 1973). This dose of streptozotocin was the highest routinely tolerated by the animals. However, it was effective to variable extents in inducing insulin deficiency in rats as indicated by the wide range of hyperglycaemia resulting in individual animals i.e. 10-38mM. In Fig. 5.5 and 5.6 the E/T ratio and total activity of HMG-CoA reductase in the livers of diabetic rats are plotted as a function of the respective concentrations of blood glucose achieved in the individual animals. It is evident that moderate insulin deficiency resulting in glycaemia equivalent to 20mM blood glucose or less had no significant effect on the E/T ratio (Fig. 5.5). Thus the ratio remained close to 80% as exhibited in the livers of normal animals at D-2. However, for animals that developed a glycaemia in excess of 20mM glucose, there was a distinct inverse relationship between the level of blood glucose and the value of the E/T ratio for HMG-CoA reductase. Therefore, if the degree of hyperglycaemia can be considered to be an inverse index of the degree of insulin deficiency, it is evident that the phosphorylation state of HMG-CoA reductase was only affected when the concentration of the hormone was decreased below a threshold value represented by a glycaemia of 20mM.

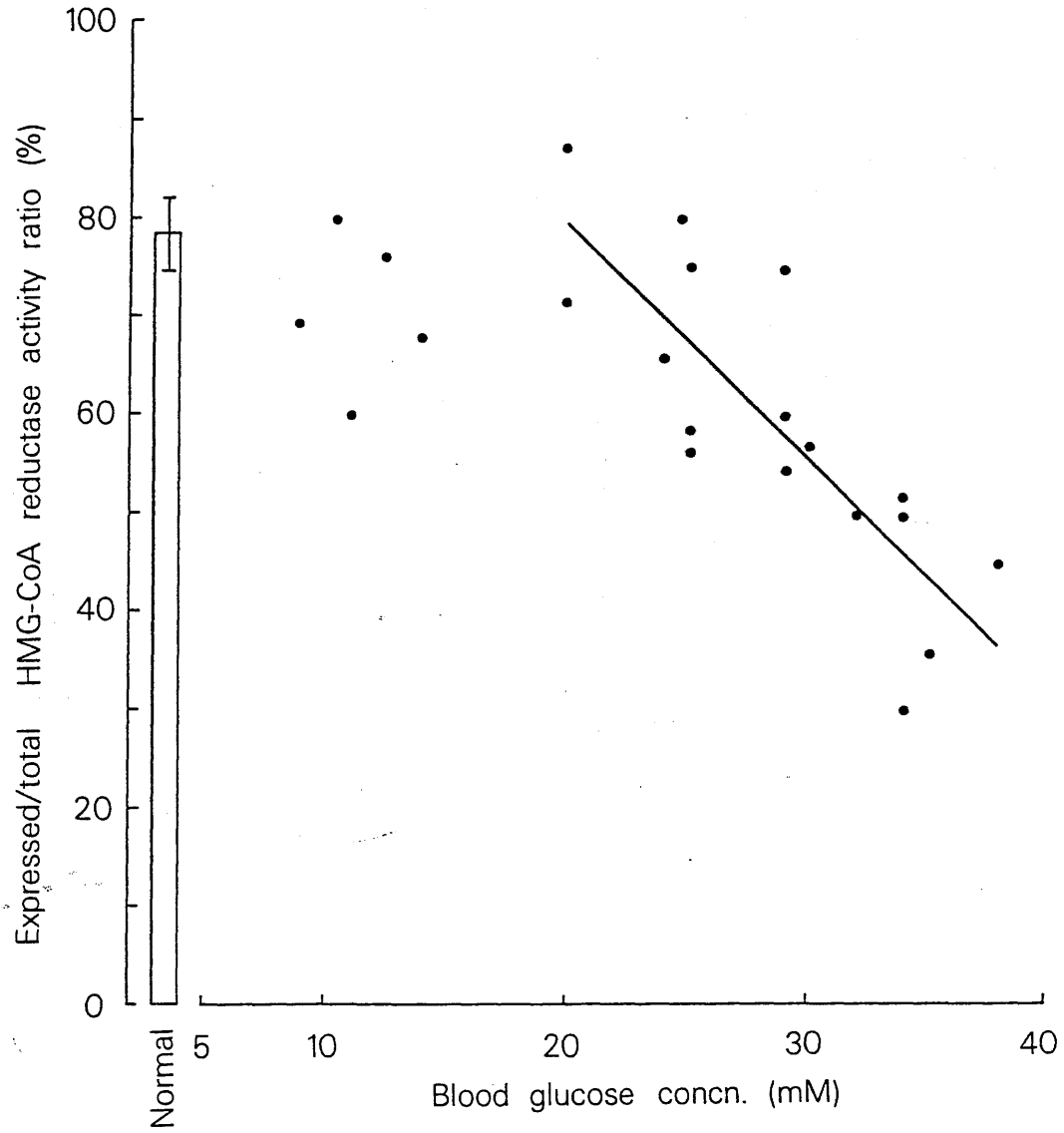


Fig. 5.5 Effects of different degrees of insulin deficiency on E/T ratio of HMG-CoA reductase in hepatic microsomal fractions from diabetic rat

The value given by the solid bar (± S.E.M.; n=4) relates to the E/T ratio in normal animals at 2h into the dark period (D-2). The straight line was obtained by performing linear regression analysis of the data obtained for rats which had blood glucose > 20mM. The correlation coefficient was -0.82.

The reason for the marked discrepancy between the effects of hyperglycaemia on the E/T ratio of HMG-CoA reductase produced by streptozotocin and A.I.S. treatment is unknown but may be a result of the different experimental conditions.

The total activity of HMG-CoA reductase also was affected only by more severe hypoinsulinaemia the effect being evident at a glycaemia level greater than 20mM (Fig. 5.6). Although this correlation was very similar to that observed for the E/T ratio it was less strong, at least on an empirical linear basis. This was reflected in a lower correlation coefficient ($r = -0.38$) and resulted partly from the variability in the absolute values of HMG-CoA reductase routinely encountered on assay of the enzyme as discussed previously. The E/T ratio being independent of the absolute specific activity of the enzyme was subject to a much lesser degree of variability between individual animals.

The consequence of the similar sensitivities of the two parameters of HMG-CoA reductase activity was that both the total activity and E/T ratio determined in liver extracts of severely insulin deficient animals (hyperglycaemia > 35mM) were very similar to those obtained at the nadir of the normal diurnal cycle (cf. Section 4.2).

(iv) Insulin administration to diabetic rats

For these experiments only diabetic rats that had blood glucose concentrations in excess of 30mM were used. Two experiments were performed: (1) insulin was (6U) administered acutely by intravenous infusion via the femoral vein into anaesthetized rats 4h into the dark period (D-4) and the liver was sampled 15-20 min later;

and (2) insulin (24U) was injected intraperitoneally into conscious rats every 60 mins for 4h, commencing 2h into the dark period; these rats were then anaesthetized 20 min before the sampling time during the period D2-D6. This latter procedure was adopted to produce a more gradual increase in circulating insulin concentrations such as would be expected to occur *in vivo*.

In the acute (intravenous infusion) experiments , the blood glucose concentration was decreased from 31.8 ± 2.1 to 22.2 ± 1.5mM (means ± S.E.M., n = 5) after 15-20 min. This treatment did not affect total activities of HMG-CoA reductase in liver microsomal fractions, but it increased the E/T ratio from 48.1 ± 2.5% (n = 11) to 98.0 ± 4.5% (n = 5), i.e. it resulted in the total dephosphorylation and activation of HMG-CoA reductase in the liver of the intact animals. In control animals infused with 0.9% NaCl, there was no change in the fraction of HMG-CoA reductase in the active form.

In the medium-term experiments (intraperitoneal injection of insulin) a very gradual decrease in blood glucose concentration was achieved, resulting in near-normoglycaemia after 3-4h (Fig. 5.7) of insulin treatment. Both the E/T ratio and total HMG-CoA reductase activity were increased by this treatment, such that for both parameters the values increased from those characteristic of diabetic animals to those observed at 2-6h into the dark period in normal animals. In these experiments the increase in E/T ratio was not as rapid or as extensive as that observed with intravenous infusion of insulin, but it was evident within the first 20 min after intraperitoneal injection of insulin and was maximal by 60 min, at

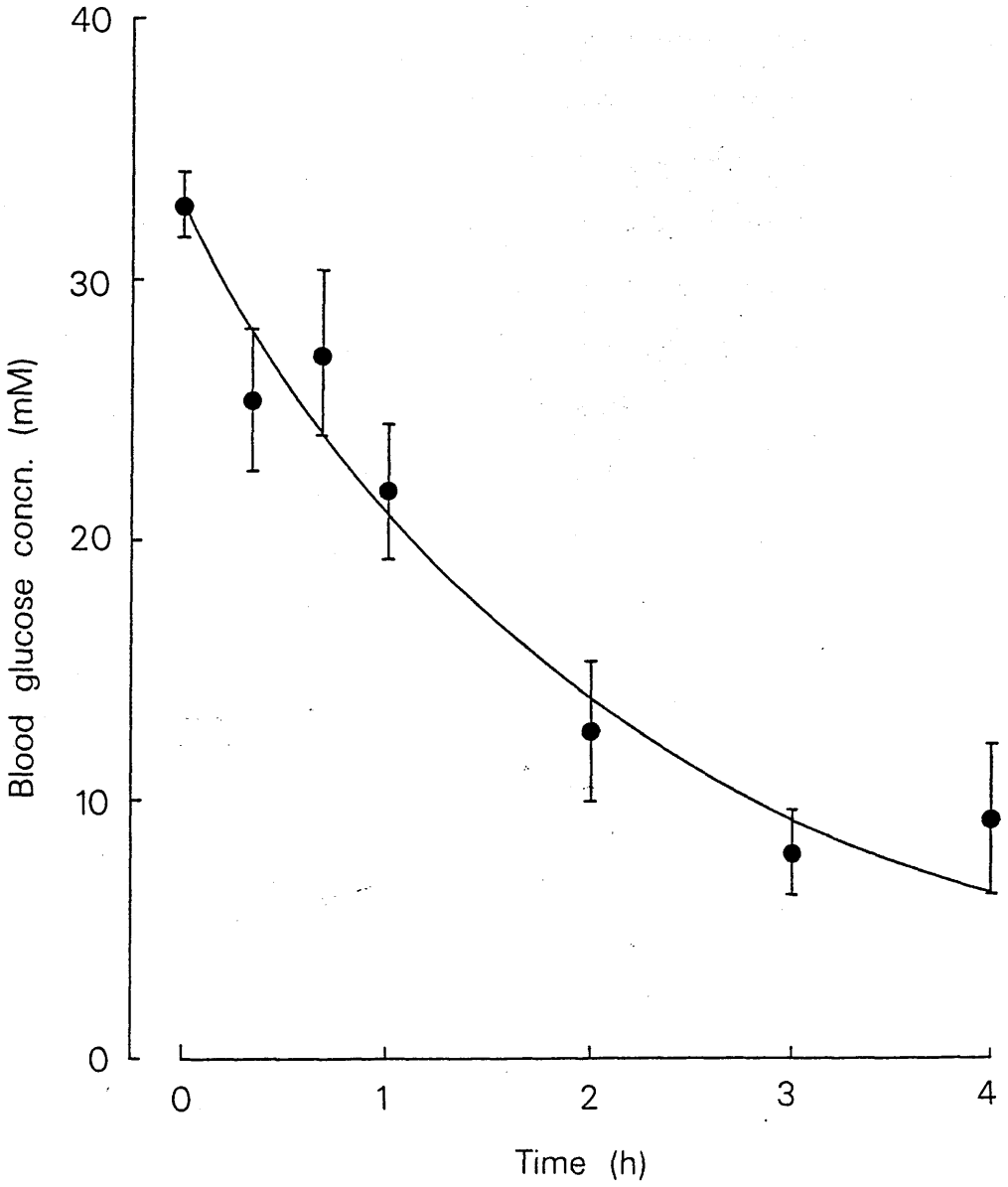


Fig. 5.7 Decrease in blood glucose concentration in diabetic rats injected hourly intraperitoneally with insulin

Values are means (± S.E.M.) for three to five determinations.

which time 80% of the enzyme was in the active state (Fig. 5.8a). Control diabetic animals injected with 0.9% NaCl showed a continuous small increase in the E/T ratio over this 4h period. This is presumed to have been due to residual insulin secretion in these animals, and suggests that even in relatively severe diabetes (30-40mM glucose in whole blood), diurnal changes in the fraction of HMG-CoA reductase in the active form may occur; however, the peak of this rhythm may be considerably later in diabetic animals than in normal rats. The effect was not studied further.

After intraperitoneal injection of insulin, the stimulatory effect of insulin on total HMG-CoA reductase activity occurred 2-3h later than that observed for the fraction of the enzyme in the active form (Fig. 5.8b). Thus, even after 2h of the commencement of insulin treatment there was no detectable increase in total HMG-CoA reductase activity, whereas at 3h the maximal effect had occurred (Fig. 5.8b). At its height, this effect resulted in total reductase activities similar to those observed in normal animals 6h into the dark period.

5.3.2 Intravenous Infusion of Glucagon

The livers of animals used for this experiment were sampled at D-3.5 for the reasons mentioned in Section 5.3.2(ii). Glucagon (1.25mg/kg body wt., in acidified saline) was infused intravenously (total volume of dose, 250µl) into animals via the femoral vein and the liver sampled 5 or 10 min later.

A marked inotropic effect of glucagon on the heart of the experimental animals was taken as indicative of the effectivenss of the infusion procedure. In addition, in order to ascertain that

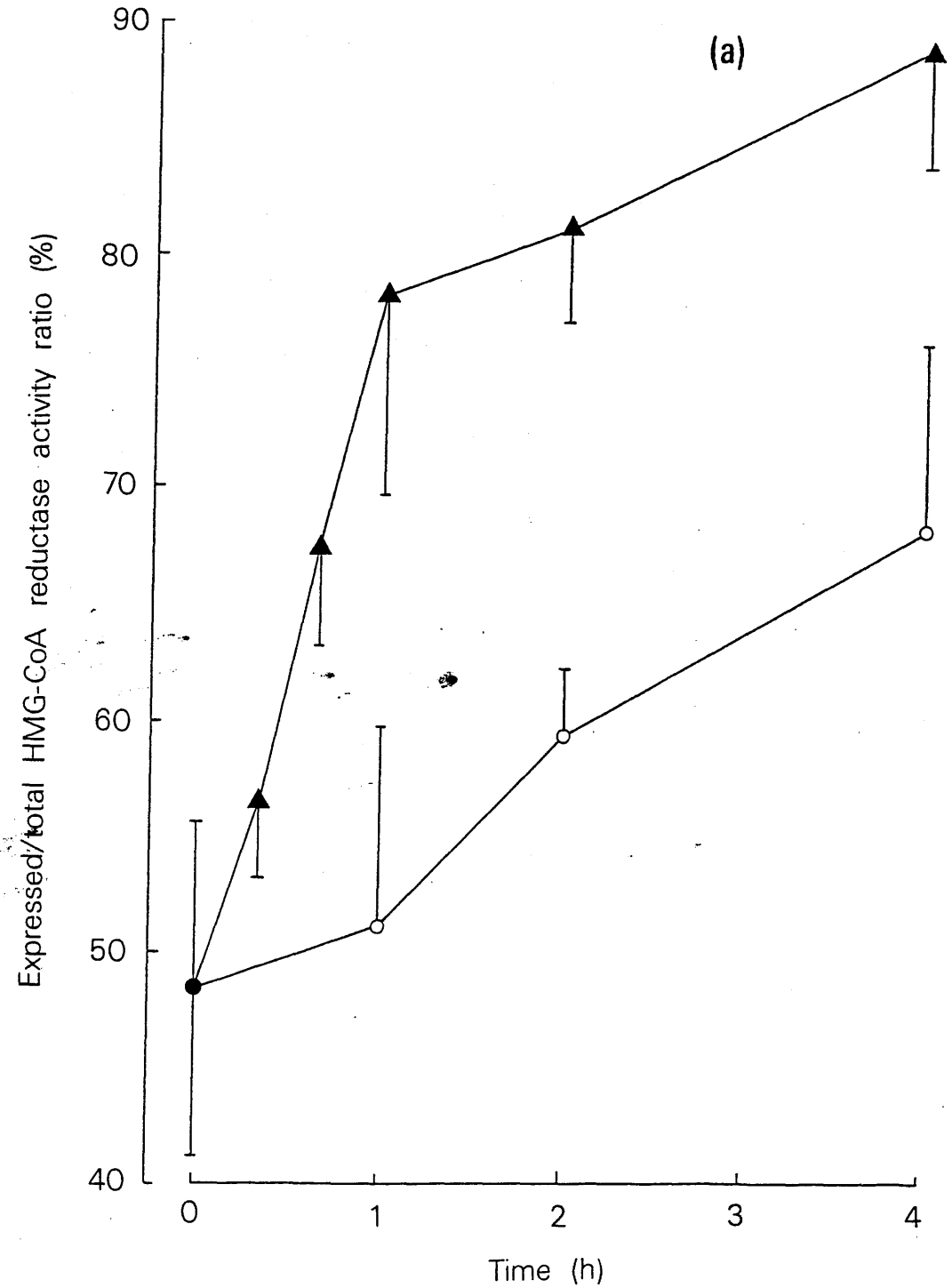


Fig. 5.8a Effect of intraperitoneal infusion of insulin on the expressed/total HMG-CoA reductase activity ratio.

Rats with blood glucose concentrations > 30mM were treated with insulin (▲). Control animals (△) were infused with 0.9% NaCl. Values are means (± S.E.M.) for three to five determinations.

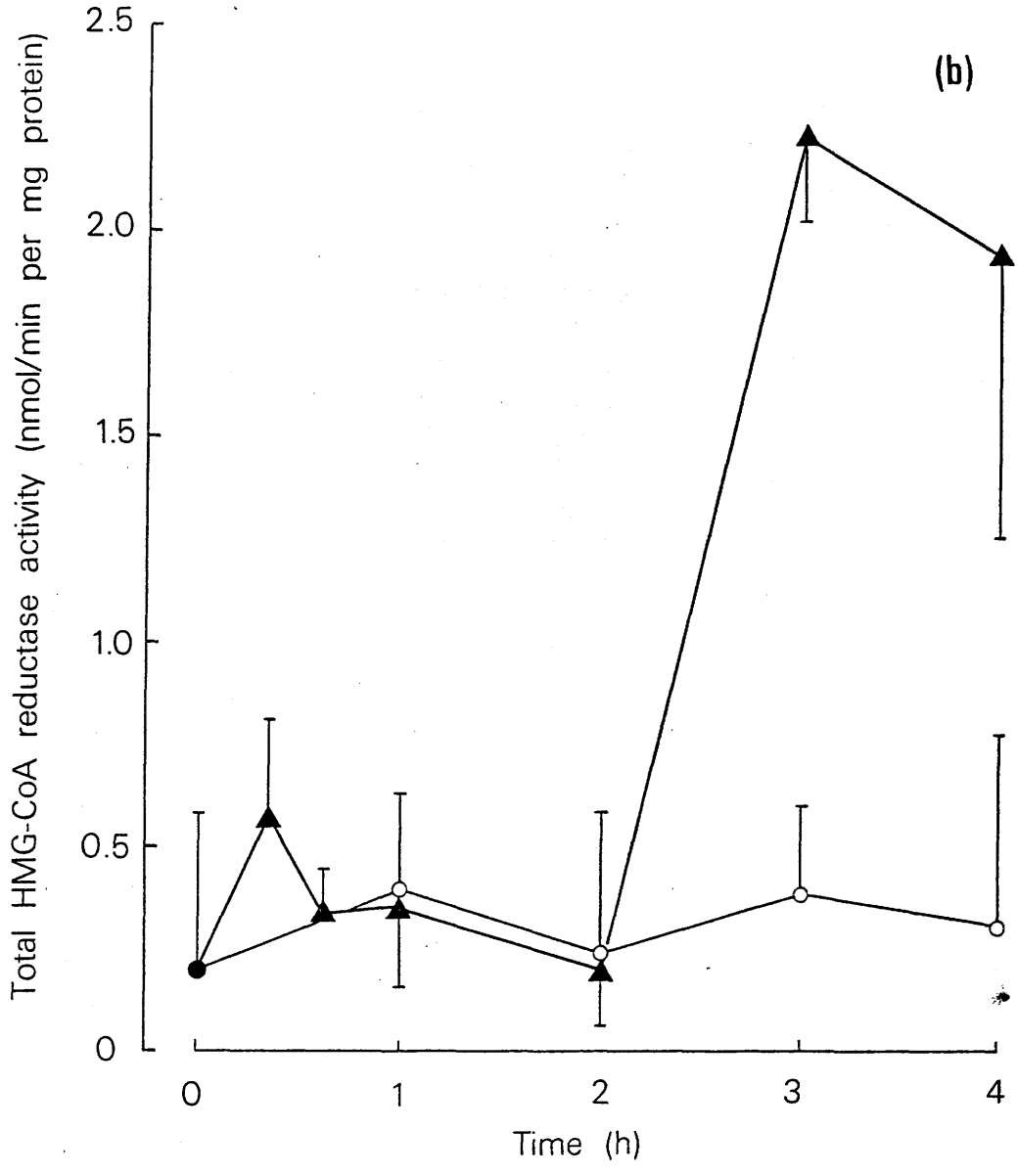


Fig. 5.8b Effect of intraperitoneal infusion of insulin on the total activity of the enzyme in liver microsomal fractions of diabetic rats in vivo

See legend to Fig. 5.8a for details.

glucagon was affecting hepatic metabolism specifically, the concentration of glucose in the blood and that of cyclic AMP in the liver were measured. Glucagon infusion (10 min) resulted in a 1.4-fold increase in blood glucose concentrations as would be expected from the well-documented effect of glucagon in increasing hepatic glycogenolysis (Whitton, 1981) and gluconeogenesis (Hue et al., 1981). As shown in Fig. 5.9 glucagon infusion induced a transient 10-fold increase in the concentration of cyclic AMP in the liver after 5 min which had returned to normal levels by 18 min.

The infusion of glucagon (5 or 10 min) produced a modest, but not statistically significant, decrease in the total activity of HMG-CoA reductase (i.e. 1.857 $\pm$ 0.329 to 1.359 $\pm$ 0.480 or 1.441 $\pm$ 0.218 for 5 and 10 min infusion respectively; Table 5.3). This effect could possibly have been due to an increased proteolytic degradation in response to the expected increased phosphorylation of the enzyme following glucagon treatment. However, the short period between hormone administration and sampling may suggest that the apparent decreases in total activity were the consequence of the natural variation encountered in the determination of this activity (see above).

The same treatment produced no significant effect on the E/T ratio (Table 5.3). This result was unexpected since Beg et al. (1979) had previously presented evidence to suggest that the subcutaneous administration of glucagon could effect an increased phosphorylation of HMG-CoA reductase concomitant with a decrease in activity (see Section 1.5). In addition, Ingebritsen et al. (1979) had reported similar changes in the E/T ratio of HMG-CoA reductase in

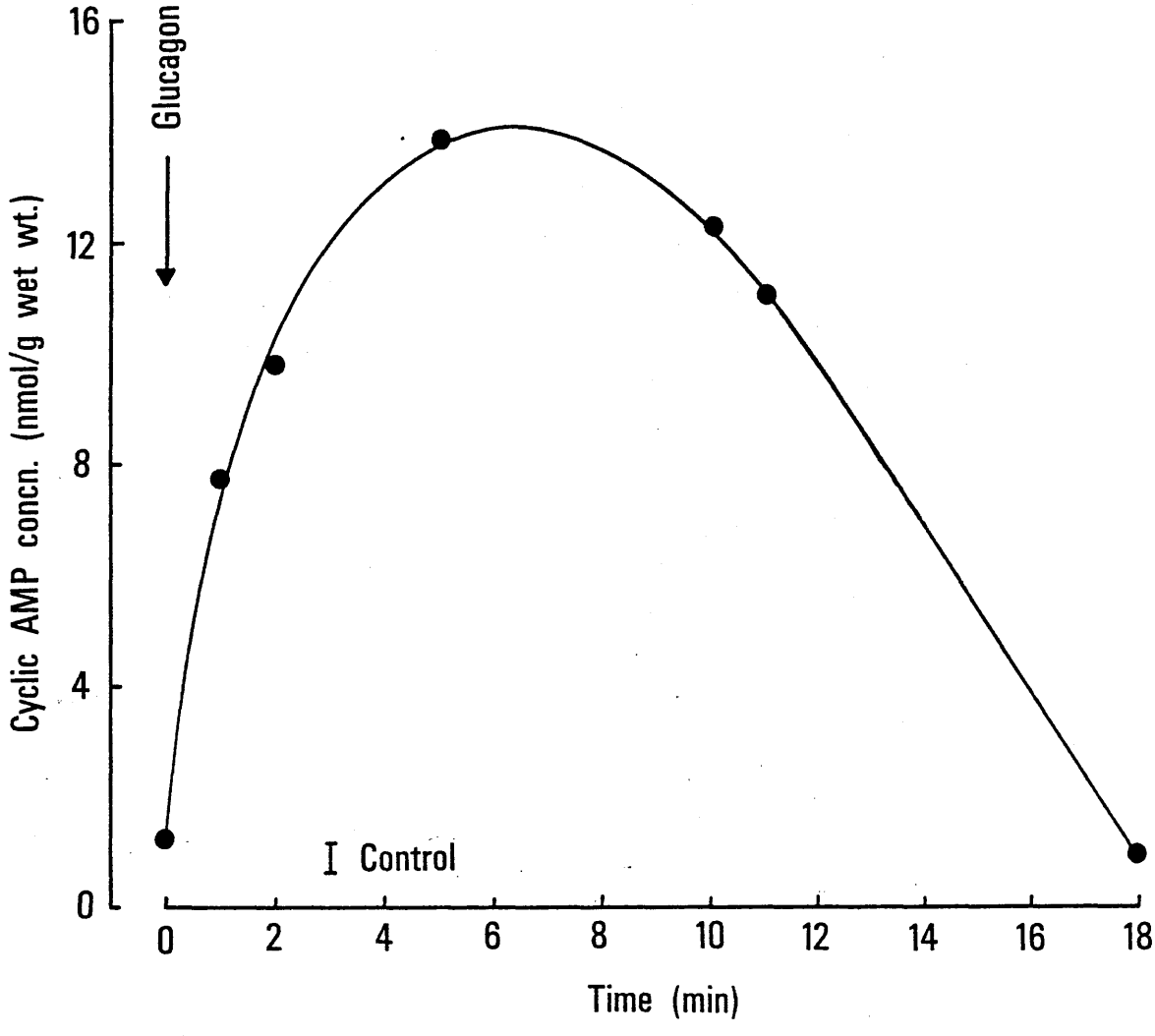


Fig. 5.9 Effect of glucagon on the concentration of cyclic AMP in liver

Glucagon was infused intravenously into anaesthetized rats and samples of liver taken by freeze-clamping at the intervals indicated and analyzed for cyclic AMP concentration. Range of liver cyclic AMP concentrations determined in normal, unstimulated rats are demonstrated by the bar ( ) (Data for control range taken from Steiner et al., 1972).

TABLE 5.3

Effect of Glucagon on the Expressed and Total Activities of Microsomal HMG-CoA Reductase and the Fraction in the Active Form

Livers of normal rats at D-3.5 and D-9.5 in the diurnal cycle and insulin-deficient (streptozotocin-treated) rats at D-3.5 were sampled by cold-clamping 5 or 10 min after glucagon (1.25mg/kg body wt.) or saline was administered by intravenous infusion. Expressed and total HMG-CoA reductase activities were determined (see legend to Table 5.2) and are expressed as nmole/min per mg of microsomal protein at 37°C. Blood glucose concentrations were determined in arterial blood obtained from the abdominal aorta immediately after the liver was sampled. Values are means (± S.E.M.) for the numbers of separate determinations shown in parentheses. Values marked (*) are significantly different ($P < 0.05$) from those for saline controls.

Animals	Treatment		Blood glucose (mM)	HMG-CoA Reductase Activity		
				Expressed (E)	Total (T)	E/T (%)
Normal (D-3.5)	Saline	(5)	7.3 ± 0.1	1.320 ± 0.298	1.857 ± 0.329	70.0 ± 4.8
	Glucagon (5 min)	(3)	8.0 ± 0.6	0.927 ± 0.360	1.359 ± 0.480	67.4 ± 4.0
	Glucagon (10 min)	(4)	11.4 ± 1.0	1.049 ± 0.193	1.441 ± 0.218	71.6 ± 3.0
Normal (D-9.5)	Saline	(5)	-	0.698 ± 0.173	0.961 ± 0.214	70.1 ± 10.7
	Glucagon (10 min)	(5)	-	0.470 ± 0.054	0.855 ± 0.138	57.1 ± 5.5
Insulin Deficient (D-3.5)	Saline	(7)	30.9 ± 4.6	0.372 ± 0.066	0.832 ± 0.186	49.6 ± 5.0
	Glucagon (10 min)	(4)	32.7 ± 3.9	0.269 ± 0.096	0.820 ± 0.186	30.3 ± 6.6*

isolated hepatocytes incubated with glucagon. Consequently it was necessary to investigate any possible reasons for these apparent discrepancies.

The present results suggested that the bicyclic phosphorylation system of HMG-CoA reductase was either insensitive to the glucagon-mediated increase in cyclic AMP concentrations *in vivo* or, alternatively, the effectiveness of cyclic AMP was counteracted in the liver by an antagonistic mechanism e.g. the activatory effect of insulin. The latter effect would tend to be exacerbated by increased insulin secretion in response to the hyperglycaemia caused by the increased circulating concentrations of glucagon.

To test this latter hypothesis the effect of glucagon on the E/T ratio of HMG-CoA reductase *in vivo* was investigated in three conditions designed to reduce the possible counter regulatory effect of insulin:

(i) *Simultaneous infusion of somatostatin*

In the first approach, cyclic somatostatin was infused intravenously at a constant rate (100-200μg/kg body wt. per h) for 10 min previous to and also during the 10 min period between the infusion of glucagon and cold-clamping. Somatostatin is a hormone that blocks the secretion of both insulin and glucagon from the islet cells of the pancreas (Guillemin & Gerich, 1976; Taborsky & Smith, 1980; Bone *et al.*, 1984) and hence will negate the increased secretion of insulin in response to the increased plasma concentration of glucagon.

The infusion of somatostatin alone (15-20 min) produced no significant change in the E/T ratio of HMG-CoA reductase when

compared to animals infused with saline (Table 5.4 cf. Table 5.3). However, when glucagon was infused simultaneously with somatostatin a marked, although not statistically significant decrease was observed in this ratio (Table 5.4). The concomitant and much larger increase in blood glucose (compared to controls, Table 5.4) suggested that this effect of glucagon on the E/T ratio was evident in the presence of somatostatin because of the inhibition of the compensatory surge of insulin secretion. Thus when the response of insulin to transient hyperglycaemia was prevented an effect of glucagon on the E/T ratio could be demonstrated. The fact that this effect was only modest may be the consequence of the substantial residual amount of insulin still remaining in the circulation after 10-20 min infusion of somatostatin (Chideckel et al., 1974).

(ii) Liver sampling at D-9.5

A second approach used to test for the possible counter regulatory effect of insulin on glucagon action was to sample the liver in animals during the declining phase of the diurnal cycle in plasma insulin concentrations (i.e. at D-9.5). At this point of the diurnal cycle both the total activity and the E/T ratio of HMG-CoA reductase are still relatively stable before the rapid decline observed over the D/L transition (see Section 4.2). By doing the experiments at this phase of the light-dark cycle it was anticipated that the basal level of insulin in the circulation would be sufficiently low to allow an effect of glucagon to become apparent (see e.g. Knox et al., 1979). At the same time the activities of HMG-CoA reductase would still be sufficiently high to allow accurate quantitative measurements to be made (see Section 4.2; Fig. 4.1).

TABLE 5.4

Effects of Simultaneous Infusion of Glucagon with Somatostatin on the Expressed and Total Activities of HMG-CoA Reductase and the Fraction of Enzyme in the Active Form

Glucagon (1.25 mg/kg body wt.) was intravenously administered to normal animals 10 min after a constant infusion of somatostatin (100-200μg/kg body wt. per h) was commenced. (This infusion was maintained during the remaining period of incubation). After 10 min a sample of the liver was obtained by cold-clamping and used for the assay of HMG-CoA reductase (see legend to Table 5.3). HMG-CoA reductase activities are expressed as nmole/min per mg of microsomal protein. Blood glucose concentrations were determined as indicated in Table 5.3. Values are means (± S.E.M.) for the number of separate determinations shown in parentheses. Values marked (*) are significantly different ($P < 0.10$ two sample 't' test) from controls.

		Blood glucose concentration	HMG-CoA reductase activity		
Treatment	(n)	(mM)	Expressed (E)	Total (T)	E/T (%)
Somatostatin	(4)	8.4 ± 1.0	1.416 ± 0.199	2.058 ± 0.333	69.3 ± 2.6
Somatostatin + Glucagon	(7)	12.7 ± 0.5	0.880 ± 0.135	1.452 ± 0.177	59.8 ± 3.5*

Again in contrast to the experiments in normal animals at D-3.5 the intravenous infusion of glucagon at D-9.5 caused a depression in the E/T ratio (70.1 ± 10.7% to 57.1 ± 5.5%) although this effect was not statistically significant. This effect was observed without any appreciable effect on total activity (Table 5.3) indicating that they were not artefacts arising from inadequate enzyme assays.

(iii) Use of diabetic animals

In a third series of experiments glucagon (1.25mg/kg body wt.) was intravenously infused into anaesthetized rats made severely hypoinsulinaemic by treatment with streptozotocin 7-8 days previously. Glucagon infusion produced a marked decrease in the E/T ratio (49.6 ± 5.0% to 30.3 ± 6.6%; Table 5.3) when compared to diabetic animals infused with saline and was due to a very marked (30%) fractional decrease in the expressed activity (0.372 ± 0.066 to 0.269 ± 0.096) in the absence of any change in total activity. These results again demonstrate that when the insulin response to hyperglycaemia was suppressed, the effect of glucagon on HMG-CoA reductase activity became apparent. These effects of glucagon were not accompanied by a further increase in the level of glycaemia as previously observed (Table 5.3) because of the depletion of liver glycogen which occurs in the first few days following streptozotocin treatment.

5.3.3 Adrenergic Agonists and Antagonists

Liver samples used for the determination of HMG-CoA reductase activities in this study were obtained from rats at D-3.5 to D-4.

All agents were administered intravenously via the femoral vein (see Methods, Section 2.4 & 2.5).

(i) Epinephrine

Epinephrine, whether administered in a single dose (0.63-1.50μg/kg body wt. in 100μM ascorbic acid; 250μl infused; 2.5 min) or by a constant infusion (1.60μg/kg body wt. per min; 5 min) produced no discernable effect on the E/T ratio or total activity of HMG-CoA reductase (Table 5.5). (Because no difference was observed between the methods of administration or the concentrations of hormone used the data were pooled and compared with animals infused with a similar volume of saline). The bolus infusion of epinephrine had to be executed with extreme care, especially at high concentrations to avoid fatal tachycardia.

The time course of the effect of epinephrine on liver cyclic AMP concentrations was monitored in freeze-clamped tissue as previously described for glucagon (Section 5.3.2). It showed a very rapid and transient 3-fold increase in the cyclic AMP concentration in liver which peaked after 1 min; it returned to basal levels after only 3 min, Fig. 5.10. In comparison to an equivalent treatment of glucagon, these effects were both more transient and less potent (Fig. 5.10 cf. Fig. 5.9). However, Foulkes & Cohen (1979) have shown that the intravenous infusion of epinephrine (although in very much greater quantity) will modulate the phosphorylation state of protein inhibitor-1 in rabbit skeletal muscle within a similar time scale.

TABLE 5.5

Effects of Epinephrine Infusion on Hepatic Microsomal HMG-CoA Reductase Activity and the Fraction of Enzyme in the Active Form

Microsomal HMG-CoA reductase (expressed and total) were determined in cold-clamped samples of liver from animals intravenously infused with epinephrine (0.63-1.50μg/kg body wt., see text) or saline, at D-3.5. HMG-CoA reductase activity is expressed as nmole/min per mg of microsomal protein at 37°C. Values are means (± S.E.M.) for the numbers of separate determinations shown in parenthesis.

		HMG-CoA Reductase Activity		
Treatment		Expressed (E)	Total (T)	E/T (%)
Saline	(4)	1.349 ± 0.179	1.987 ± 0.269	68.0 ± 1.7
Epinephrine	(12)	1.105 ± 0.96	1.701 ± 0.100	64.7 ± 3.4

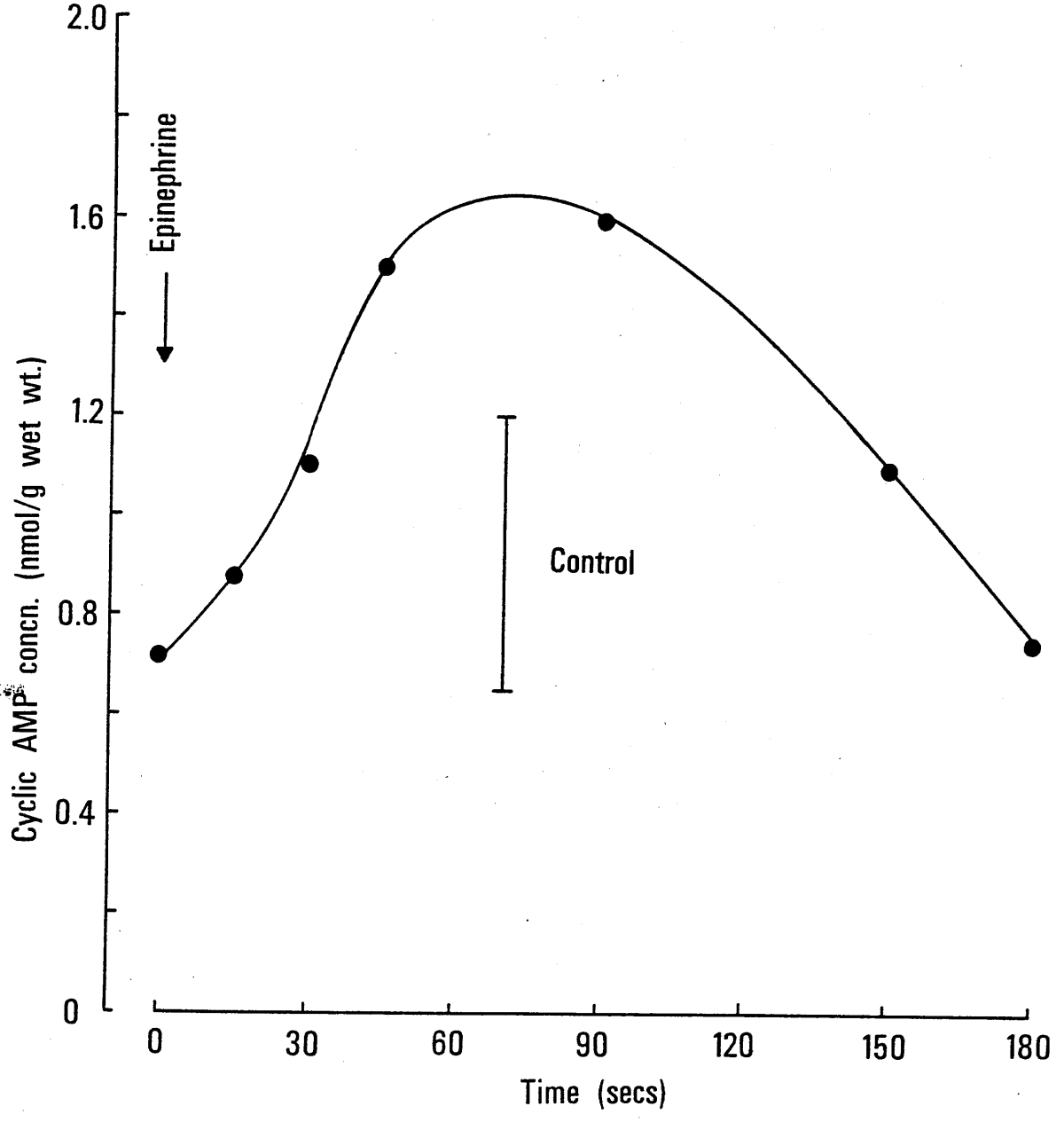


Fig. 5.10 Time course of the effect of epinephrine on the concentration of cyclic AMP in liver

Determinations were made as described in legend to Fig. 5.9. Bar indicates range of liver cyclic AMP concentrations recorded in normal, unstimulated rats (Data for control range taken from Steiner et al., 1972).

(ii) β-adrenergic antagonist

A general β-adrenergic antagonist (propranolol; 125μg/kg body wt.) was infused intravenously 1h prior to the sampling of the liver by cold-clamping. It was evident that this treatment had no discernable effect on the E/T ratio or total activity of HMG-CoA reductase when compared to untreated animals at the same time of the diurnal cycle (see Table 5.6).

(iii) α_1-adrenergic antagonist

A specific α_1-adrenergic antagonist (prazosin; 50μg/kg body wt. in 0.8mM lactic acid) was infused intravenously into normal rats 1h prior to the sampling of the liver by cold-clamping at D-3.5. This treatment produced a modest increase in the E/T ratio compared to control animals infused with carrier only (81.1 ± 2.7% to 91.1 ± 5.2%; Table 5.6) without any significant change in the total activity (1.715 ± 0.483 vs 1.815 ± 0.675 nmoles/min per mg of microsomal protein).

5.4 Discussion

The most striking observation from the present study was that insulin appeared to be pre-eminent in the control of the phosphorylation state of HMG-CoA reductase in the intact animal *in vivo*. It is evident that in normal animals under physiological conditions only treatments in which the circulating insulin concentrations were increased (administration of glucose) or acutely decreased (anti-insulin serum) could respective increases or decreases in the E/T ratio be observed. By contrast, when glucagon

TABLE 5.6

Effects of β- and α_1-Adrenergic Antagonist Infusions on Hepatic Microsomal HMG-CoA Reductase Activities and the Fraction of Enzymes in the Active Form

Prazosin (α_1-antagonist; 50μg/kg body wt. in 0.08M lactic acid) or propranolol (general β-antagonist; 125μg/kg body wt.) in physiological saline were administered intravenously into rats under ether anaesthetia. After 1h, a sample of the liver was obtained by cold-clamping and used for the determination of expressed and total activities of HMG-CoA reductase. Animals were sampled at D-4. HMG-CoA reductase is expressed as nmoles/min per mg of microsomal protein at 37°C. Values are means (± S.E.M.) for the numbers of separate determinations shown in parenthesis.

		HMG-CoA Reductase Activities		
Treatment		Expressed (E)	Total (T)	E/T (%)
Control	(3)	1.713 ± 0.329	2.216 ± 0.275	76.8 ± 6.9
Propranolol	(5)	1.879 ± 0.296	2.372 ± 0.358	78.7 ± 3.0
Control	(3)	1.507 ± 0.608	1.815 ± 0.675	81.1 ± 2.7
Prazosin	(4)	1.532 ± 0.395	1.715 ± 0.483	91.1 ± 8.2

or epinephrine (agents that are known to produce antagonistic effects to those of insulin in the liver e.g. on glycogen metabolism, see Whitton, 1981) were administered to whole animals between D-3 and D-4 of the diurnal cycle the E/T ratio was not reduced. During this phase of the light-dark cycle the circulating insulin concentrations are high. This observation was unexpected because previous studies of the effects of glucagon on rat hepatocytes *in vitro* (Ingebritsen *et al.*, 1979; Henneberg & Rodwell, 1981; 1985) or glucagon administration to animals *in vivo* (Beg *et al.*, 1979) had indicated that they result in increased phosphorylation of HMG-CoA reductase (see Section 1.5). However, the physiological significance of this effect of glucagon *in vivo* is doubted because of the artefactual increase in the phosphorylation state that would occur in the method of sampling used in this study (see Chapter 3;). In the present study, glucagon tended to produce an inhibitory effect on the expressed activity of HMG-CoA reductase only under conditions in which insulin secretion was suppressed either in the course of the normal diurnal cycle (by using animals at D-9.5 in the diurnal cycle; see Section 5.3.2(ii)) or pharmacologically (by using chronically insulin-deficient animals or the simultaneous infusion of somatostatin, see Section 5.3.2.(i)). These observations suggest that when the concentrations of insulin were high they were sufficient to antagonize any effects these agents had in decreasing the E/T ratio of HMG-CoA reductase in the liver of these animals. Such insulin concentrations appeared to prevail physiologically at D-4 but not at D-9.5 and also in response to hyperglycaemia induced by hormones that mobilize liver glycogen. These observations are therefore consistent

with the reported effects of glucagon on the E/T ratio in hepatocytes which were studied in the absence of any antagonizing effect of insulin.

The apparent dominance of insulin in the control of the phosphorylation state of HMG-CoA reductase suggests that insulin may play the major role in establishing the diurnal rhythm observed in the E/T ratio of hepatic HMG-CoA reductase (see Section 4.2; Fig. 4.2). This conclusion is drawn from a number of observations made in the present study which confirm the observations made in the previous chapter.

(1) The E/T ratio was markedly depressed in the microsomes obtained from the liver of insulin-deficient animals at a time in the diurnal cycle that coincides with the peak of the diurnal rhythm of the ratio in normal animals (Fig. 5.8a cf. Fig. 4.2). This suggests that the diurnal peak in the E/T ratio is depressed in these animals. That this was the direct result of insulin deficiency was supported by the observation that the reintroduction of insulin in such a way as to gradually restore normoglycaemia (i.e. intraperitoneal injection of insulin) restored (within 40 min) the E/T ratio to values observed in normal animals at a similar time of day (Fig. 5.8a).

Furthermore, if a high circulating concentration of insulin was maintained on these animals by the repeated intraperitoneal administration of insulin, the E/T ratio continued to increase gradually over D-4 to D-6 when, in normal animals this ratio would have normally started the decline from a peak at D-4 (Fig. 5.8a cf. Fig. 4.2). This observation, in combination with the observed fall

and rapid activation of HMG-CoA reductase effected by intravenous infusion of insulin (Section 5.3.1.(iv)) demonstrates that the hyperinsulinaemia induced in these conditions overcomes any counter-regulatory effect that may be provoked by the accompanying hyperglycaemia.

(2) Acute removal of insulin from the circulation by the infusion of anti-insulin serum at a time approximating the attainment of the normal diurnal peak in the E/T ratio produced a marked decrease in this ratio. Since under these experimental conditions the pancreatic response would be to increase insulin secretion in response to the increased blood glucose concentration the observed decrease in the E/T ratio could not have been due to the secretion of a counter-regulatory hormone.

(3) The E/T ratio was increased by the administration of glucose at a time in the light:dark cycle when both the E/T ratio and plasma insulin concentrations were at their lowest. The close correlation observed between the plasma glucose concentration and the E/T ratio suggests that it is primarily caused by the concomitant increase in plasma insulin concentrations (see Stansbie et al., 1976b). It seems, therefore, that the value of the E/T ratio is largely determined by the circulating concentrations of insulin and, in support of this, a strong correlation was observed between the relative hyperglycaemia of a series of diabetic rats and the resulting E/T ratio of HMG-CoA reductase on the liver (Fig. 5.5).

The present study investigating the effect of insulin administration to diabetic animals also demonstrated that insulin is required for the full expression of the total activity of HMG-CoA

reductase (i.e. synthesis of enzyme protein). This response of HMG-CoA reductase is well documented (e.g. Lakshmanan et al., 1973; Huber et al., 1973; Uchida et al., 1979; Young et al., 1982; see Section 1.3.3) but it is of interest that this effect lagged 2-3h behind the rapid effect observed in the E/T ratio (see above; Fig. 5.8a&b). Not only is the magnitude of this lag period consistent with the expected requirement for the induction of protein synthesis but it is also similar to the phase separation observed between the respective diurnal rhythms of the E/T ratio of HMG-CoA reductase and the total activity in vivo (see Section 4.2.2). It was also evident that both parameters sensitive to the same range of insulin concentrations (Fig. 5.5 & 5.6.). In both instances moderate hypoinsulinaemia (blood glucose < 20mM) had little discernable effect both on the E/T ratio and total activity. In more severely diabetic animals (blood glucose 20-38mM) there was a negative correlation between the severity of hyperglycaemia and both total activity of HMG-CoA reductase and the E/T ratio. These observations are consistent with the important role of insulin in the generation of the diurnal rhythm for both total activity (although probably not the sole determinant, see Section 4.3.3) and the E/T ratio. Furthermore, they may contribute to the apparent variable effect of streptozotocin-induced insulin deficiency on the rate of hepatic cholesterogenesis (Feingold et al., 1982; Nakayama & Nakagawa, 1977) and the fraction of HMG-CoA reductase in the active form (see Goodman et al., 1982; Young et al., 1982) reported in the literature. Only animals in which the extent of hypoinsulinaemia induced experimentally is sufficiently severe to increase blood glucose above

20mM would exhibit any significant depression of expressed HMG-CoA reductase and cholesterogenesis *in vivo*.

The mode of action of insulin in promoting the dephosphorylation of HMG-CoA reductase is, as yet, unknown. This is, in part, due to the lack of understanding of the mechanism of insulin action (see introduction to this chapter). The mechanism could involve the inhibition of kinases and/or the activation of the phosphatases constituting the bicyclic system operation on HMG-CoA reductase (see Section 1.4.2). The mechanisms by which the activities of reductase kinase and reductase kinase kinase are regulated have not been identified (except for the activation of reductase kinase by ADP; see Sections 1.4.3.1 and 3.4). However, by analogy with the effects of insulin on protein phosphatase activities involved in glycogen metabolism (see Cohen *et al.*, 1985; Cohen, 1985) it is possible that the effects of insulin HMG-CoA reductase are mediated primarily through similar mechanisms.

HMG-CoA reductase and reductase kinase are substrates for three of the four protein phosphatase activities characterized in the control of carbohydrate and fat metabolism, namely protein phosphatase-1, -2A and -2C (by the terminology of Ingebritsen & Cohen, 1983). In spite of the very high specific activity of protein phosphatase-2C towards HMG-CoA reductase there is no evidence that this phosphatase activity is regulated within the cell. However, evidence is emerging that insulin may influence the activities of protein phosphatases-1 and -2A. For example, the observation that spermine (a polyamine) increases the specific activities of protein phosphatase-1 and in particularly phosphatase-2A towards a number of

cellular substrates in rabbit skeletal muscle (Tung et al., 1985) has been linked with the proposed second messenger activity of polyamines for insulin (see Cohen, 1985). Although protein phosphatase may have an important role to play in the regulation of cytosolic reductase kinase, protein phosphatase-1 is likely to have the major HMG-CoA reductase phosphatase activity by virtue of its partial association with the microsomal membrane of cell homogenates (Ingebritsen et al., 1983b; Section. 1.4.3.2). In chronic insulin deficiency the effects on protein phosphatase activities may include not only effects on the specific activity of protein phosphatase-1 but also on the concentration of this enzyme. Thus the activity of protein phosphatase-1 is decreased by 40-50% in chronically insulin-deficient animals (Foulkes & Jefferson, 1984; Dragland-Meserve et al., 1985). Moreover, the activation of HMG-CoA reductase (i.e. the increase in the E/T ratio) induced by insulin administration (intravenous or intraperitoneal injection) to diabetic animals could be mediated by the concomitant, rapid (5 min) activation of protein phosphatase-1 by this hormone as demonstrated by Shahed et al. (1980) and Dragland-Meserve et al. (1985).

In Chapter 1, two mechanisms were described by which protein phosphatase-1 activity could be regulated, namely via the putative effects of protein inhibitors-1 and -2. Although Cohen (1980) has proposed inhibitor-2 as a target for insulin action its involvement in the regulation of HMG-CoA reductase would seem unlikely on two accounts: (1) the phosphorylation of inhibitor-2, through which dissociation and activation of protein phosphatase-1 are produced (see Section 1.4.3.2) is achieved by a cyclic AMP-independent protein

kinase activity on a serine-threonine residue (Ballou et al., 1983; Hemmings et al., 1982). The kinase activity associated with the event of insulin binding, however, is specific for tyrosine residues (Fain, 1984). (2) The protein phosphatase species with which inhibitor-2 interacts is found exclusively in the cytosol (Cohen, 1985). As such, this activity will not readily effect the phosphorylation of microsomal bound HMG-CoA reductase although it is possible that its limited activity may contribute to the dephosphorylation and activation of cytosolic reductase kinase.

The phosphorylation state and hence activity of inhibitor-1 has been shown to be modulated by insulin in both skeletal muscle (Foulkes et al., 1980; 1982) and adipose tissue (Nemenoff et al., 1983) in vivo and as such has been proposed as a primary mechanism underlying the effects of insulin on cellular metabolism in these tissues. A protein of similar properties, has been found in the rat liver (Khandelwal et al., 1977; Goris, 1978). Significantly the specific acitivity of this protein inhibitor is found to be greater in the liver of diabetic animals and therefore, may account for the apparent depressed activity of protein phosphatase-1 in these animals (Khandelwal et al., 1977). The action of insulin on protein inhibition-1 could therefore facilitate the rapid increase in E/T ratio of HMG-CoA reductase observed after the injection of insulin to diabetic animals (see above; Section 5.3.1.(iv); Fig. 5.8a). It is not known, however, how insulin mediates its effects on inhibitor-1.

The mechanism of acute glucagon-mediated inhibition (at low insulin concentrations in vivo) of HMG-CoA reductase could be achieved indirectly through the increased phosphorylation of

inhibitor-1 (the activities of both reductase kinase and reductase kinase kinase are cyclic AMP-independent see Section 1.4.3.1). However, the fact that the activation state of HMG-CoA reductase is unaffected by glucagon under conditions of high, but physiological, insulin concentrations in spite of a large increase in the intracellular concentration of cyclic AMP (Fig. 5.9) suggests that even if insulin does not mediate its effects via the suppression of the levels of the metabolite it can antagonize its effects on the activity of HMG-CoA reductase. One mechanism by which insulin could achieve this was described by Larner et al. (1979; 1982) who demonstrated that insulin could directly inhibit cyclic AMP-dependent protein kinase activity (Fig. 5.11). Alternatively, insulin could act directly on protein phosphatase-1 activity in such a way as to ovecome any increased effect of inhibitor-1 on protein phosphatase-1 under conditions of increased levels of cyclic AMP (Fig. 5.11).

It is significant that even under conditions in which insulin secretion was suppressed (i.e. either by using normal animals at D-9.5 or by infusing somatostatin) the administration of large, pharmacological doses of glucagon produced only modest decreases in the E/T ratio of HMG-CoA reductase (Tables 5.3 & 5.4). By contrast when severely hypoinsulinaemic (diabetic) animals were used glucagon produced a much more marked decrease in the fraction of HMG-CoA reductase in the active form. It is noteworthy that the exposure of rat hepatocytes to glucagon (10^{-9}M) does not completely antagonize the effects of insulin (10^{-10}-10^{-9}M) on the E/T ratio of HMG-CoA reductase (Parker et al., 1981). These observations raise the question as to whether it is the insulin concentration to which

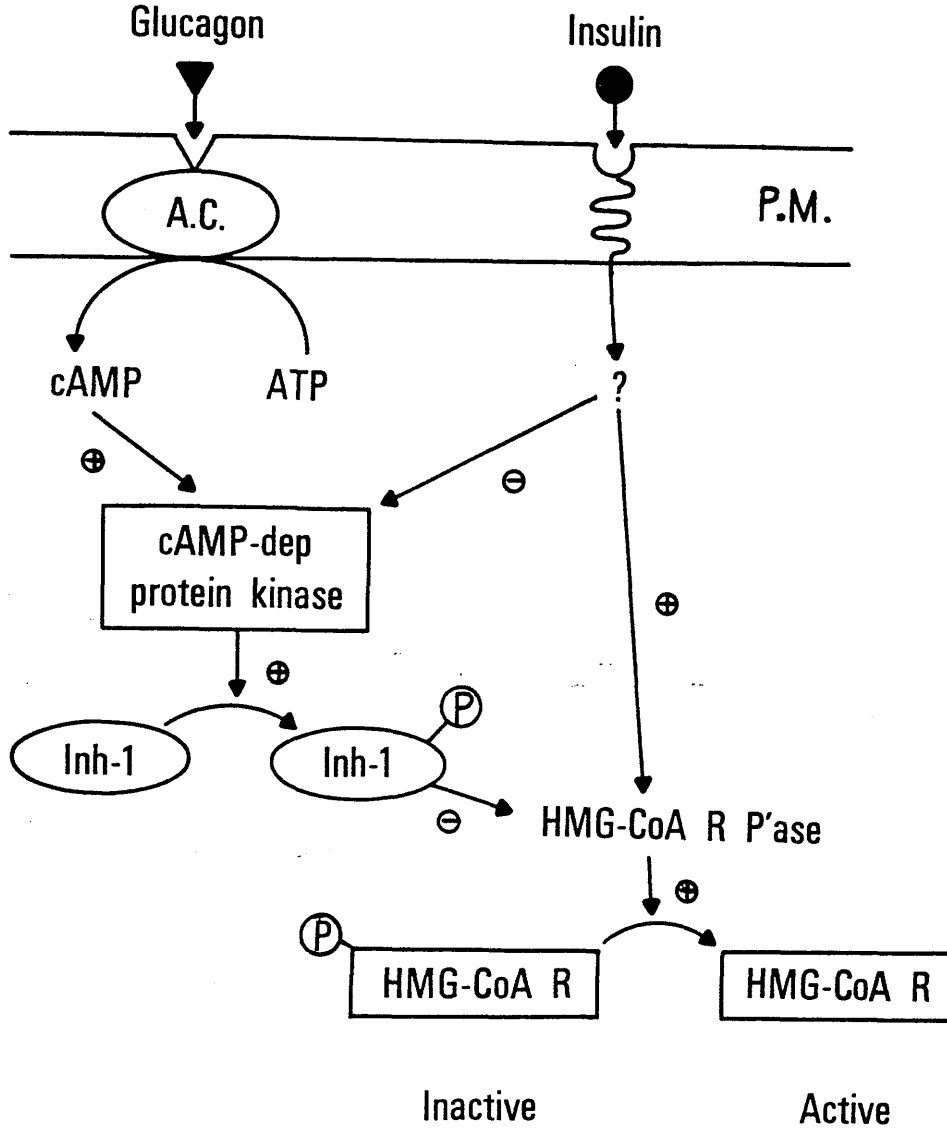


Fig. 5.11 Hypothetical mechanism for the dominant effect of insulin on the phosphorylation state of HMG-CoA reductase (HMG-CoA R) via HMG-CoA reductase phosphatase (HMG-CoA R Phase)

Insulin (via an unknown intermediate; ?) could activate directly HMG-CoA reductase phosphatase and simultaneously inhibit the action of glucagon on the same enzyme (via inhibitor-1, Inh-1) through an inhibition of cyclic AMP-dependent (cAMP-dep) protein kinase. (A.C., adenylate cyclase; P.M., plasma membrane).

hepatocytes are exposed in vivo or the insulin:glucagon ratio that is important in the regulation of the E/T ratio of HMG-CoA reductase. On the present evidence it would appear that the absolute concentration of insulin is more important at high insulin concentrations but that the insulin:glucagon ratio becomes important at very low insulin concentrations. Cherrington & Steiner (1982) reached a similar conclusion in a study investigating the effects of these hormones on hepatic glycogen metabolism. They found that under conditions in which the absolute concentrations of insulin and glucagon were both increased the effects of insulin (i.e. decreased glucose production) clearly dominated. Relatively low concentrations of insulin could antagonize the effects of glucagon on the rates of glycogenolysis and gluconeogenesis.

From the foregoing discussion, the effects of epinephrine through the interaction with β-adrenergic receptors (through increased concentrations of cyclic AMP) would not be expected to be detectable at least in the presence of normal insulin concentrations. Epinephrine produced a more transient, less potent (3-fold; Fig. 5.10) increase in the concentration of cyclic AMP in comparison with equivalent measurements made following the administration of glucagon (i.e. 10-fold; Fig. 5.9). This difference may be a reflection on the concomitant interaction of epinephrine with the α_2-receptor which acts to lower the concentration of cyclic AMP (see Section 5.1) or alternatively due to the limited amount of hormone that could be administered in vivo without precipitating fatal tachycardia (see

Section 5.3.3(i)). Nevertheless, epinephrine will also interact with α_1-adrenergic receptors (see Table 5.1) and thus increase the cytosolic Ca^{2+} concentration. In view of the recent demonstration that two Ca^{2+}-dependent kinases i.e. Ca^{2+}-phospholipid-dependent protein kinase-C and Ca^{2+}-calmodulin-dependent protein kinase phosphorylate HMG-CoA reductase *in vitro* (Beg *et al.*, 1985a&b; see Section 1.4.3.1) the modulation of cytosolic Ca^{2+} concentrations of the liver *in vivo* would be expected to induce a decrease in the E/T ratio. Therefore it is suggested that physiological concentrations of insulin also antagonize the effects of α_1-receptor occupancy. Such an antagonism between insulin and α_1-receptor-mediated effects on enzymes of glycogen metabolism in rat hepatocytes has been reported previously (Massague & Guinovart, 1978; Blackmore *et al.*, 1979; Thomas *et al.*, 1985).

In contrast to the insensitivity of the E/T ratio of hepatic HMG-CoA reductase to general β-adrenergic receptor blockade (which is consistent with the proposed insignificance of changes in intracellular levels of cyclic AMP on the enzyme in the presence of high concentrations of insulin), the specific blockade of α_1-adrenergic receptors *in vivo* did produce an increase in the E/T ratio (Table 5.6). This observation suggests that under normal physiological conditions, the occupancy of α_1-adrenergic receptors antagonizes the effects of insulin such that when these effects are blocked, insulin is able to produce a greater increase in the E/T ratio than is seen at a similar time in the diurnal cycle in normal animals. Since the circulating levels of catecholamines increase during the activity of feeding (Barrand & Callingham, 1983) it is

possible that such an antagonism between α_1-adrenergic receptor occupancy and insulin binding on the E/T ratio of HMG-CoA reductase on the liver could account for the less than total (i.e. 80-85%) dephosphorylation of the enzyme at the peak of the diurnal cycle (see Section 4.2).

It is evident therefore that the intravenous infusion of epinephrine under the present conditions in vivo will not mimic the profound decrease observed in this parameter as the result of severe stress i.e. as encountered in the conventional method of killing an animal by a stunning blow to the head and cervical dislocation (see Chapter 3). However it has been noted previously that the intracellular cyclic AMP concentrations were only modestly increased following the administration of epinephrine. It may therefore be that the increase in plasma catecholamine concentration achieved by the intravenous infusion was much lower than that achieved physiologically in animals as the result of stress (see Kvetnansky et al., 1978; 1980) and therefore not able to produce the same effect i.e. in opposition to the high insulin concentrations. In support of this mechanism, Foulkes et al. (1982) demonstrated that isoproterenol, a potent β-adrenergic agonist could only antagonize the effects of insulin on the phosphorylation state of protein inhibitor-1 in perfused skeletal muscle when its concentration was increased to values equivalent to those expected for catecholamines in the circulation as the result of severe stress. Futhermore, it has been demonstrated that epinephrine at suitably high concentrations can block the insulin induced-rise in the E/T ratio in isolated rat hepatocyte preparations (Henneberg & Rodwell, 1981). In

view of the apparent relative importance of α_1-adrenergic-mediated effects in the regulation of the phosphorylation state of HMG-CoA reductase, it is possible that the acute effects of stress are controlled primarily via the sympathetic innervation such that the effective concentration of norepinephrine, which will act on the liver only through α_1- and α_2-adrenergic receptors, is very high in the vicinity of the cell. However, it has to be appreciated that when rats are killed by cervical fracture other factors, such as transient anoxia, can affect liver metabolism (and the phosphorylation state of HMG-CoA reductase) in the period between killing the animal and sampling of the liver (see Chapter 3). Under conditions of severe stress, it is possible that the combined effects of the increased intracellular concentrations of cyclic AMP and Ca^{2+} produce a greater change in the phosphorylation state of HMG-CoA reductase than can be achieved by either alone. It has recently been shown that the α_1-adrenergic responses of rat liver glycogen metabolism are potentiated by a cyclic AMP-dependent mechanism (Morgan *et al.*, 1984; see also Jakobs *et al.*, 1985).

In conclusion, the present data have demonstrated that the effects of insulin dominate in the regulation of the phosphorylation state of hepatic HMG-CoA reductase *in vivo*. These observations highlight the difficulties encountered in studying the effects of specific hormones *in vivo* as well as the problems inherent in the interpretation of the results obtained *in vitro* by exposure of hepatocytes to individual hormones.

ACUTE REGULATION OF THE ACTIVITY OF HMG-CoA REDUCTASE BY DIETARY CONSTITUENTS

CHAPTER 6

6.1 Introduction

In previous chapters it was demonstrated that the expressed activity of HMG-CoA reductase is closely related to the food intake of the animal (see e.g. Section 4.2 & 4.3). It is possible that, in addition, the phosphorylation state of HMG-CoA reductase could be acutely modulated through the direct effects of individual dietary constituents. In this respect, previous work has indicated that cholesterol or cholesterol precursors (e.g. mevalonate) can lower the fraction of enzyme in the active form when administered to whole animals *in vivo* (Beg *et al.*, 1981; 1982; 1984b; Arebalo *et al.*, 1981; Erickson *et al.*, 1980) or, to isolated hepatocytes *in vitro* (Parker *et al.*, 1983). These effects, which have been shown to involve the increased incorporation of [^{32}P]-phosphate from [^{32}P]-ATP:Mg^{2+} into HMG-CoA reductase protein (Beg & Brown, 1981; 1982) and reversed by the addition of protein phosphatase (see e.g. Erickson *et al.*, 1980; Arebalo *et al.*, 1980; 1981), appear to occur before any changes occur in the concentration of enzyme in the cell. Although this latter effect is generally thought to be the consequence of an increased rate of enzyme degradation (Hardgrave *et al.*, 1979; Arebalo *et al.*, 1981) there is evidence that this could be the result of irreversible inactivation of existing enzyme (Arebalo *et al.*, 1982).

The ability of dietary constituents (e.g. cholesterol) to modulate the activity of the existing enzyme by covalent phosphorylation *in vivo* is, however, not universally accepted (see Kleinsek *et al.*, 1980; Jenke *et al.*, 1981; Dugan *et al.*, 1982). Using immunotitration techniques, these workers concluded that the changes in the enzyme activity of HMG-CoA reductase observed after short-term cholesterol-feeding were the consequence of changes in the quantity of enzyme rather than to the modulation of catalytic activity. Therefore it was necessary to use the cold-clamping technique to resolve the controversy as to whether the administration of such factors could acutely alter the E/T ratio of HMG-CoA reductase *in vivo*.

A number of other dietary components are known to modulate hepatic HMG-CoA reductase and cholesterogenesis *in vivo*. For example, in rats, the feeding of unsaturated fats induces an increase in the activity of HMG-CoA reductase* (Goldfarb & Pitot, 1972; Goh & Heimberg, 1979). Recently, in studies in man, emphasis has been placed on the correlation between dietary components and the control of serum cholesterol levels mainly in response to the suggestion of the strong correlation between serum cholesterol levels and atherosclerotic diseases (see Grundy, 1978; Brown & Goldstein, 1978). One component of the diet in particular, milk, has received appreciable attention following the observation that East African (Masai) tribesman have low serum cholesterol concentrations in spite of the consumption of large quantities (4-5 litres/day) of fermented whole milk (Mann & Spoerry, 1974). This observation was paradoxical because the high saturated fat content of milk and especially the

*although the significance of these effects is controversial.

large proportion of cholesterol (0.22-0.41 wt. percent of total lipids in bovine milk; 3mg/g milk fat; Christie, 1978, Renner, 1983) would have been expected to produce hypercholesterolaemia. Therefore it was suggested that milk contained a hypocholesterolaemic factor (Mann & Spoerry, 1974). Subsequent observations indicated that sufficient amounts of yoghurt and unfermented milk (whole, low-fat, skim) also exhibited a hypocholesterolaemic effect both in man and animals (Mann, 1977a,b; Howard & Marks, 1977; Nair & Mann, 1977; Kritchevsky et al., 1979; Richardson, 1978). Several substances have been suggested as candidates for this 'milk factor' and include HMG-acid (Mann, 1977b), lactose (Helms, 1977) Ca^{2+} (Howard, 1977), orotic acid (bovine milk; Ahmed et al., 1979; Bernstein et al., 1976;1977; Dull et al., 1983), uric acid (human milk; Ward et al., 1982) and factors contained in the milk fat globule, particularly the fat globule membrane (Antila et al., 1980; National Dairy Council, Digest, 1982). Other reports have suggested that fermented milk had a greater ability to produce a hypocholesterolaemic effect than non-fermented milk (Rao et al., 1981; Hussi et al., 1981). The presence of this factor in milk is, however, controversial since several investigators have failed to observe hypocholesterolaemic effect of milk (e.g. Hepner et al., 1979; Massey, 1981; Roberts et al., 1982). Further work indicated that the lack of control of dietary intake during the experimental period when the milk was used to supplement the diet contributes to the inconsistency of the results (Roberts et al., 1982; see Howard & Marks, 1982). Consequently it was suggested that the hypocholesterolaemic effect observed in the above experiments may be, in part, due to the variability in the dietary lipid intake.

Nevertheless, a number of these proposed factors e.g. orotic acid have been shown directly to modulate the total activity of HMG-CoA reductase *in vivo* (see Richardson, 1978; Marlett *et al.*, 1981). It is possible that the same factors, or others present in the milk could have a more acute effect on the rates of liver cholesterogenesis. Therefore, various fractions of milk were tested for their ability, when administered orally, to affect acutely the E/T ratio of hepatic HMG-CoA reductase. These effects could be directly compared with the effects of mevalonolactone elicited in the same series of experiments.

6.2 Experimental

Rats were lightly anaesthetized and intubated with mevalonolactone (750-780mg/kg body wt.; total volume, 0.3ml), various spray-dried milk fractions (whole milk, skim milk, buttermilk or whey proteins; 1.8g/kg body wt.; total volume, 0.8ml) or distilled water (control) as described in Methods (Section 2.3). After 20 min (mevalonolactone) or 30 min (milk factions) a sample of the liver was taken using the cold-clamping technique and used to determine expressed and total microsomal HMG-CoA reductase activities as described in Methods (Section 2.8). Cold-clamped samples of liver were obtained 4h into the dark period (i.e. at D-4).

Compositional analysis of the milk fractions for total solids, fat, crude proteins (see Section 6.3.3) was performed by the methods described in Section 2.14.2.

TABLE 6.1

Effects of Oral Administration of Mevalonolactone on Expressed and Total Activities of HMG-CoA Reductase and the Fraction of Enzyme in the Active Form

Expressed and total HMG-CoA reductase activities were measured in microsomes isolated from cold-clamped samples of liver obtained from rats intubated with mevalonolactone (750-780mg/kg body wt., total volume 0.8ml), or distilled water, 20 min earlier (see Section 2.3). The experiments were performed 4h into the dark period (D-4). Activities of HMG-CoA reductase are expressed as nmole/min per mg of microsomal protein at 37°C. Values are means (± S.E.M.) for the numbers of separate determinations shown in parentheses. Those marked (*) are significantly different ($P < 0.01$; two sample 't' test) from controls.

Treatment		Activity of HMG-CoA Reductase		
		Expressed (E)	Total (T)	E/T (%)
Control	(6)	1.543 ± 0.227	1.939 ± 0.239	78.9 ± 2.9
Mevalonolactone	(10)	0.725 ± 0.124*	1.525 ± 0.266	50.5 ± 5.0*

6.3 Results

6.3.1 Administration of Mevalonolactone

The oral administration of mevalonolactone produced a marked decrease in the E/T ratio from 78.9 ± 2.9% to 50.5 ± 5.0% after 20 min (Table 6.1). This decrease was accompanied by a modest decrease (approximately 21%) in the total activity (Table 6.1) but this was not statistically significant and may have resulted from natural variation routinely encountered in the determination of total activity (see Sections 6.3.2 & 5.3). The combined effects of these individual changes in the E/T ratio and total activity resulted in a decrease (approximately 53%) in the expressed (i.e. from 1.543 ± 0.227 to 0.725 ± 0.124 nmoles/min per mg of microsomal protein at 37°C; Table 6.1). This effect was primarily accounted for (i.e. about 78%) by the changes in the E/T ratio. The administration of distilled water (as a control) into normal animals by stomach intubation produced very little effects on these parameters of the enzyme activity which were very similar to values determined in untreated rats at the same time in the diurnal cycle (cf. Section 4.2).

6.3.2 Administration of Milk Fractions

When compared to the administration of distilled water (control) only buttermilk of the four milk fractions administered showed any detectable effects on the E/T ratio of HMG-CoA reductase (Table 6.2). Administration of this fraction provoked a statistically significant ($P < 0.05$) increase in this parameter (from 72.1 ± 3.1%

TABLE 6.2

Effect of Oral Administration of Various Fractions of Milk on the Expressed and Total Activity of Hepatic Microsomal HMG-CoA Reductase and Fraction of Enzyme in the Active Form

Expressed and total activities of HMG-CoA reductase were determined as described in legend to Table 6.3. Animals were intubated 30 min before sampling 4h into the dark period (D-4). Activities of HMG-CoA reductase are displayed as nmole/min per mg of microsomal protein at 37°C. Values are mean (± S.E.M.) for the numbers of separate determinations shown in parentheses. Those marked (*) were significantly different ($P < 0.05$, two sample 't' test) from controls.

	Activity of HMG-CoA Reductase		
Milk fraction	Expressed (E)	Total (T)	E/T (%)
Control	1.096 ± 0.109 (7)	1.531 ± 0.154 (7)	72.1 ± 3.1 (7)
Whole milk	1.321 ± 0.249 (5)	1.739 ± 0.245 (5)	74.4 ± 5.0 (5)
Buttermilk	1.359 ± 0.164 (4)	1.539 ± 0.222 (4)	88.9 ± 4.4* (4)
Whey proteins	1.518 ± 0.498 (3)	1.796 ± 0.549 (3)	79.2 ± 5.4 (7)
Skim milk	1.574 ± 0.203 (4)	2.112 ± 0.287 (4)	74.8 ± 5.4 (4)

to 88.9 ± 4.4%; Table 6.2). By contrast, the E/T ratio determined in liver samples obtained from rats treated with other fractions i.e. whole milk and skim milk were very similar to those observed in animals treated with distilled water only (i.e. range 72.1% to 74.8%; Table 6.2) although the administration of whey protein fraction induced a modest and statistically insignificant increase in this parameter (i.e. from 72.1 ± 3.1 to 79.2 ± 5.4%, Table 6.2).

The total activities for HMG-CoA reductase determined in these experiments showed considerable variation. This was assumed to be due to the variation routinely encountered in the determination of this parameter (see also Section 5.3).

6.4 Discussion

The marked decrease observed in the E/T ratio of hepatic HMG-CoA reductase in rats *in vivo* as the result of the administration of a large dose of mevalonolactone by intragastric intubation was consistent with previous studies, both *in vitro* and *in vivo* (see Section 6.1). It was confirmed that the initial changes observed in the expressed activity of the enzyme are due to rapid phosphorylation and inactivation of existing enzyme (see Beg & Brewer, 1981).

The employment of the cold-clamping technique has further enabled the quantitation of the mevalonolactone-induced phosphorylation of HMG-CoA reductase (i.e. the variation from normal). Thus after 20 min the E/T ratio was decreased by approximately 40% (i.e. from 78.9 ± 2.5% of normal animals to 50.5 ±

5.0% of treated animals). This effect was therefore very similar to the previously determined effects of acute (i.e. A.I.S., or chronic insulin-deficiency (see chapter 5). It was also similar to, although less extensive than, the effects of the incubation of rat hepatocytes with mevalonolactone (see Parker et al., 1983). This suggests that mevalonolactone may be rapidly absorbed from the gut and elicits acute effects on liver metabolism.

The mechanism by which mevalonolactone mediates its effect on the phosphorylation state of HMG-CoA reductase is unknown although there is evidence to suggest that both reductase kinase(s) and phosphatase(s) may be involved (mevalonate addition to microsomes will not directly modulate the activity of HMG-CoA reductase; Erickson et al., 1980). Gibson et al. (1981) and Parker et al. (1983) who used isolated hepatocytes in vitro and Erickson et al. (1980) and Beg et al., (1984b) who worked on rats in vivo. have demonstrated that mevalonolactone treatment produces a rapid and marked (40-70%) inhibition of reductase phosphatase activity. These effects were not the consequence of a direct action of mevalonate (or mevalonolactone) on the phosphatases but rather were suggested to be mediated by the action of derivatives formed in the subsequent metabolism of mevalonate in the cell. Among these, inorganic pyrophosphate (released during squalene synthesis), mevalonate pyrophosphate and isopentenyl pyrophosphate inhibit reductase phosphatase activity in vitro (Parker et al., 1983, see Fig. 6.1).

Beg et al. (1984b) have presented evidence that the treatment of whole animals with mevalonolactone results in the stimulation in vivo of both reductase kinase (2- to 3-fold) and reductase kinase

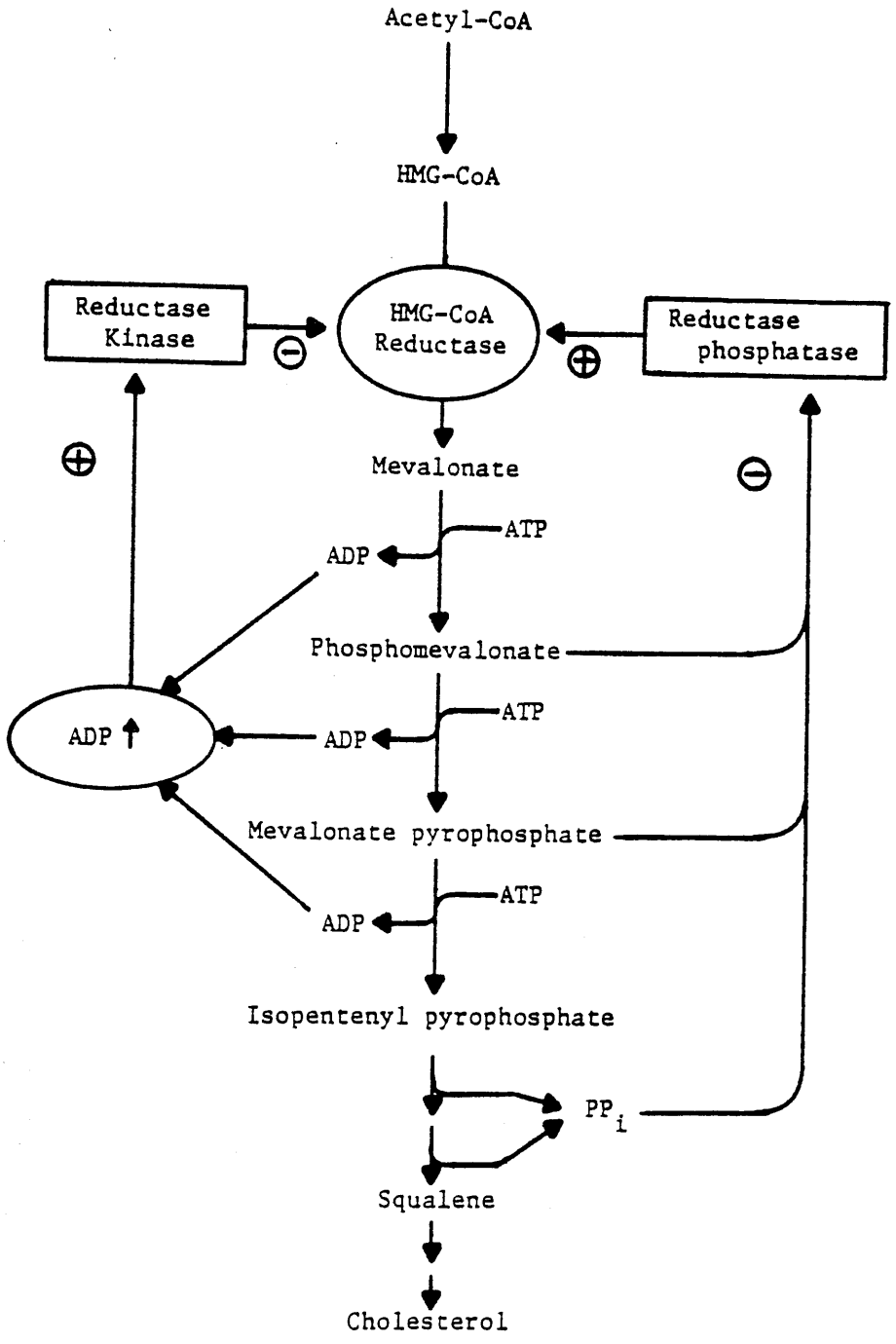


Fig. 6.1 Possible effects of Mevalonate Metabolism on the effective activities of HMG-CoA reductase kinase and phosphatase.

The increased mevalonate concentration in the hepatocyte due to mevalonolactone-feeding could produce both an activation of reductase kinase activity and a reduction in reductase phosphatase activity as shown.

kinase (4-5-fold). These effects could be elicted by the formation of derivative of mevalonate (see Beg et al., 1984b; see above). However, it is possible that these activations were induced by the previously characterized stimulatory effect (at least on reductase kinase) of an increased intracellular concentration of ADP (see Harwood et al., 1984) which may occur as a consequence of the increased metabolism of mevalonate in the hepatocyte in vivo. Mevalonate is the substrate for mevalonate kinase and this, together with the subsequent two enzymes in the cholesterogenic pathway, involves the hydrolysis of ATP and consequent generation of ADP (Fig. 6.1). Thus, under conditions of excessive loading of the liver (or isolated hepatocytes) with pharmacological doses of mevalonolactone, partial depletion of cellular ATP concentrations could occur with a consequent rise in intracellular ADP. Such an effect is known to be produced by fructose loading of the liver (Phillips & Davies, 1985). Therefore, it would be of interest in future experiments to determine the effects of the administration of mevalonolactone in vivo on the intracellular concentration of ADP (and ATP).

The increased metabolism of mevalonate in hepatocytes would also be expected to result in an increased concentration of cholesterol and non-sterol metabolites of mevalonate in the liver cell. These may contribute to the modulation of the phosphorylation state of HMG-CoA reduc although neither have been shown to influence the activity of reductase kinases or phosphatase(s). Nevertheless, it has been reported to facilitate the phosphorylation of HMG-CoA reductase by increasing the rigidity of the endoplasmic reticulum and making the enzyme a better substrate for the action of the reductase kinases (see Gibson &

Parker, 1985). Consequently, the rapid decrease observed in the phosphorylation state of HMG-CoA reductase may be the result of the combined effects of mevalonate (or a derivative thereof) and cholesterol.

The fact that mevalonolactone produces this marked decrease in the E/T ratio 4h into the dark period (i.e. D-4) was very significant because it represented a factor that could reverse the apparently dominant effects of the high plasma insulin concentrations characterizing this time of the light/dark cycle (i.e. D-4; see Chapters 4 & 5). It will be recalled that the administration of glucagon at a similar time was unable to decrease the E/T ratio except when insulin secretion was simultaneously suppressed (see Section 5.3). This may suggest that mevalonate mediates its effects on the E/T ratio of HMG-CoA reductase via a mechanism completely distinct from the modulation of reductase kinase and phosphatase activities, and may therefore involve changes in membrane fluidity (see above). However the more marked decrease in the E/T ratio produced by mevalonolactone treatment in comparison to the effects of glucagon may merely reflect the fact that the effect of this metabolite was not counteracted by a compensatory increased secretion of insulin. It is possible, therefore, that if physiological changes occur in the liver concentration of mevalonate or a metabolite thereof (e.g. oxysterols), this would represent a major mechanism for the counter-regulation of the effects of insulin. If this suggestion is valid it would favour the direct involvement of mevalonate (or its metabolites) in the regulation of HMG-CoA reductase phosphorylation independently of any effect on reductase

phosphatase activities. This is because, as described elsewhere (see Chapter 5), such effects appear to be effectively dominated by insulin. This is particularly interesting in the case of the diurnal rhythm in the E/T ratio observed in normal rats since an increased intracellular concentration of mevalonate may result from the diurnal peak in flux through the HMG-CoA reductase reaction at D4 and thus may signal the decline from the peak between D-4 and L-2 (see Section 4.2).

The significance of the decrease in the total activity observed 20 min after the oral administration of mevalonolactone is difficult to assess (Table 6.1) because of the large variation routinely observed in the determination of this parameter (see Section 5.3). Nevertheless, it is possible that this decrease corresponded to previously observed decreases in the total activity following the exposure of hepatocytes to mevalonolactone *in vitro* (Parker *et al.*, 1983) as well as mevalonolactone treatment *in vivo* (Arebalo *et al.*, 1980; 1982). Although further studies are required, the present observations lend support to the recent proposition that the increased phosphorylation of HMG-CoA reductase (i.e. decreased E/T ratio) results in the increased degradation of the enzyme (see Orci *et al.*,1984; Gibson & Parker, 1985).

Of the various milk fractions administered to rats only buttermilk had any appreciable effect on the E/T ratio of hepatic HMG-CoA reductase. Buttermilk consists of the aqueous phase remaining after the removal of butter-fat from cream by churning and contains most of the milk fat globule membrane. Consequently, the observation that this fraction induced an increase in the E/T ratio

TABLE 6.3

Compositional Analyses of Spray-Dried Fractions of Milk

Four fractions of milk (whole milk, buttermilk, whey proteins and skim milk) were analysed for total solid, fat, crude protein and lactose content by methods described in Section 2.14.2. Each component is expressed as g/100g of milk.

Milk Fraction	Composition (g/100g)			
	Total Solid	Fat	Crude Protein	Lactose
Whole milk	9.76	3.08	2.42	3.63
Milk whey	9.36	0.10	1.38	6.54
Buttermilk	9.43	0.80	3.01	4.80
Skim milk	9.42	-	3.46	5.01

of HMG-CoA reductase was unexpected because (i) of the enriched membrane composition and hence large cholesterol content of this fraction, and (ii) the possibility that a hypocholesterolaemic factor resides in this membrane (see Section 6.1). In human milk, cholesterol accounts for 0.6-0.7% of total lipids in the fat globule membrane compared to 0.2-0.3% of total lipids in the intact milk fat globule (Jensen et al., 1980; Renner, 1983). As indicated above, the increased dietary intake of cholesterol-enriched food would have been expected to induce a decrease in the E/T ratio. It is appreciated that the present short-term study may not be directly comparable with the long-term manipulations used to identify the initial effects of a hypocholesterolaemic factor and also that, besides, the presence of such a factor is controversial.

The milk fat globule membrane is also a rich source of phospholipids. They account for approximately 60% of total milk phospholipids (Jensen et al., 1980). The phospholipid content of buttermilk represents an enrichment of approximately 30 to 35-fold in comparison to the total lipid content of intact milk fat globules. Consequently, the cholesterol/phospholipid ratio is markedly decreased in the fat globule membrane and, therefore, in the buttermilk fraction. It is possible, therefore, that the increased dietary intake of phospholipids directly or in relation to cholesterol induced the increase in the E/T ratio of HMG-CoA reductase observed following the administration of buttermilk to rats (Table 6.2) in spite of the concomitant cholesterol intake.

Alternatively, this fraction may have produced the observed increase in the E/T ratio via an increased stimulation secretion of

insulin (i.e. to a greater extent) than other fractions. This however, is improbable since the factor most likely to produce such an effect, i.e. lactose and casein are present in other sub-fractions of milk (Table 6.3) but eachh induced no similar increase in the E/T ratio (Table 6.2).

In conclusion, the marked decrease observed in the E/T ratio of hepatic HMG-CoA reductase following the administration of mevalonolactone together with the effect of buttermilk, demonstrates that the phosphorylation state of the enzyme can be modulated acutely by dietary manipulation. In the case of mevalonolactone it is suggested that this is the effect of the direct effect of the dietary factor on the hepatocyte in vivo and therefore may play an important role in the diurnal rhythm of the phosphorylation state of HMG-CoA reductase that occurs in response to feeding. The reasons for the changes in the E/T ratio induced by buttermilk are less clear although it will be of interest to determine the effects of e.g. the oral administration of phospholipids, particularly phosphatidylcholine, phosphatidylethanolamine or sphingomyelin which are the most prominent phospholipids of bovine milk.

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