CONTROL OF HEPATIC FATTY ACID SYNTHESIS IN RATS AND MICE

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Abstract

The rates of lipogenesis and the activities of acetyl-CoA carboxylase have been measured in perfused livers from lean and genetically obese (ob/ob) mice, in response to a number of hormones known to have catabolic actions on hepatic carbohydrate metabolism. Total rates of lipogenesis have been measured by following the incorporation of ^{3}H from $^{3}H_2O$ into fatty acids and cholesterol. The contribution of selected carbon sources was monitored simultaneously by the incorporation of ^{14}C from [^{14}C]-labelled precursors such as glucose or lactate, using a double label counting technique.

Vasopressin (4 mU/ml), previously reported to inhibit fatty acid synthesis in the perfused mouse liver, caused a significant decrease in the activity of acetyl-CoA carboxylase within 30 minutes. No such inhibition of acetyl-CoA carboxylase activity occurred in response to glucagon. Inhibition of lipogenesis did occur under these conditions, the effect being about 2 orders of magnitude less potent than the stimulation of glycogenolysis. Inhibition of lipogenesis and stimulation of glycogen breakdown by vasopressin, on the other hand, showed equipotency in the lean mouse liver. The inhibitory effect of glucagon on fatty acid synthesis is suggested to be secondary to effects on hepatic carbohydrate metabolism causing alterations in the substrate supply for lipogenesis.

Angiotensin II and adrenaline were found to inhibit lipogenesis and acetyl-CoA carboxylase activity in perfused

lean mouse livers within 40 min, at concentrations about an order of magnitude higher than those known to occur *in vivo*. The possible significance of these effects in the intact animal is discussed.

No significant inhibition of lipogenesis in response to glucagon, adrenaline or angiotensin II was found in perfused livers from genetically obese (*ob/ob*) mice. The resistance of hepatic fatty acid synthesis to inhibition by hormones is discussed as a possible cause of obesity.

The rate of fatty acid synthesis was found to be increased in the regenerating rat liver over the period 1-4 days post-op. Acetyl-CoA carboxylase activity was not found to be increased. The capacity of the regenerating rat liver for fatty acid synthesis was investigated in perfusion under a variety of substrate conditions; no difference between regenerating livers and those from sham-operated control rats could be found.

Rapid inhibition of liver acetyl-CoA carboxylase activity was found under conditions of ischaemia and anoxia both *in vivo* and in perfusion. Rapid sampling techniques were used to avoid this effect. Various nucleotides, known to increase in concentration in the liver in anoxia, were screened for inhibitory effects on acetyl-CoA carboxylase. AMP and ADP were found to have the most potent inhibitory effects. The inhibition by AMP was found to be partially competitive with ATP with a K_i of 4.6 mM.

The possible role of the factors investigated in this study in controlling hepatic lipogenesis are discussed.

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PUBLICATIONS

The following papers have been published in connection with the work appearing in this thesis:-

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ABBREVIATIONS

Acetyl-CoA carboxylase	- acetyl-CoA:CO_2 ligase (ADP) E.C. 6.4.1.2.
ADP	- adenosine 5'-diphosphate
AMP	- adenosine 5'-phosphate
ATP	- adenosine 5'-triphosphate
AVP	- 8-argenine vasopressin
Butyl-PBD	- 5-(4-bi-phenylyl)-2-(4-t-butyl-phenyl)-1-oxa-3-4 diazole
Ci	- Curie, 3.7×10^{10} desintegrations/sec
CoA	- coenzyme A
CPM	- counts per minute
DNA	- deoxyribonucleic acid
DTE	- dithioerythritol
D.R. ob/ob mice	- diet restricted ob/ob mice
EDTA	- ethylenediamine-tetra-acetic acid
FAME	- fatty acid methyl esters
FID	- flame ionization detector
GLC	- gas liquid chromatography
GMT	- Greenwich mean time
I.D.	- internal diameter
IMP	- inosine 5'-phosphate
IP	- intraperitoneal
IV	- intravenous
K_m	- Michaeli's constant, equals the substrate concentration at which velocity is equal to 1/2 V_{max}

K_I	- Inhibitor constant, which is the dissociation constant of the inhibitor enzyme complex, $K_I=\frac{[E][I]}{[EI]}$
NAD^+	- nicotinamide-adenine dinucleotide
NADH	- nicotinamide-adenine dinucleotide, reduced
NADPH	- nicotinamide-adenine dinucleotide phosphate, reduced
Nembutal	- sodium pentabarbitone
Obese (ob/ob) mice	- genetically obese mice, homozygous for the ob gene
O.D.	- optical density or outside diameter
Partial hepatectomy	- surgical removal of 70% of the liver mass
PCA	- perchloric acid
PEGA	- polythylene glycol adipate
Rf	- retention factor
RTA	- relative total activity
SRA	- specific radioactivity
TLC	- thin-layer chromatography
Tris	- 2-amino-2-hydroxymethyl-propane--1,3-diol
TUFA	- total unesterified fatty acids
UV	- ultra violet
Vasopressin	- 8-argenine vasopressin, synthetic grade VI
V_{max}	- maximum velocity of an enzyme reaction occurs when the enzyme is saturated with its substrate(s)
XMP	- xanthosine 5'-phosphate

CHAPTER 1

INTRODUCTION

1.1. Lipid synthesis and storage

1.1.1. The major sites of fatty acid synthesis

Liver and adipose tissue were identified as the two major sites of fatty acid synthesis by Barret et al (1938) and Stetten and Grail (1943) using the incorporation into long-chain fatty acids of deuterium from deuterium oxide (Schoenheimer and Rittenberg, 1937). Another important site of fatty acid synthesis in the female mammal is the lactating mammary gland.

The relative importance of liver and adipose tissue in synthesising long-chain fatty acids is still subject to doubt. Thus Jeanrenaud, in a review in 1968, concluded that in the rat and mouse, adipose tissue synthesised its entire store of fatty acids. This conclusion was based on the relatively high activity of the lipogenic enzymes and the extent of the insulin dependence of the process in the adipose tissue of these two species (Ball, 1966), later supported by Saggerson and Greenbaum, 1970 and Halestrap and Denton, 1973 . The findings of Hollenberg (1966) did not however endorse this view. Hollenberg found that the major products of fatty acid synthesis in adipose tissue in vivo were saturated fatty acids which were not desaturated and elongated to the extent required to produce the composition of depot fats

found in this organ. Hems *et al* (1975), using tritium oxide to measure total rates of fatty acid synthesis *in vivo*, concluded that the liver was the major site of *de novo* fatty acid synthesis in the fed lean mouse, with a significant contribution from adipose tissue. They considered that the rate of long-chain fatty acid synthesis in the liver had previously been underestimated due to the use of unsuitable precursors. Thus there could have been considerable dilution of [^{14}C]-acetyl-CoA derived from glucose owing to the minor significance of blood glucose as a carbon source for fatty acid synthesis in the liver (Salmon and Hems, 1974).

1.1.2. The importance of esterified long-chain fatty acids as a fuel store

In mammals the major fuel stores consist of depot fats. The neutral lipids which form the bulk of such stores are particularly suited to their role since they are hydrophobic and thus require no water for storage. They can therefore be stored in large amounts without causing detrimental effects on cells. Since carbohydrate polymers such as glycogen have a considerable amount of water of hydration associated with them, they form a less efficient fuel store (weight for weight) than neutral lipids. Oxidation of 1 g of fat releases over twice as much energy as the oxidation of 1 g carbohydrate. The main storage organ for depot fats is adipose tissue. Little esterified fatty acid is stored in the liver under normal conditions

(Section 6.4.), in contrast to the large amounts of glycogen which are found in the liver in the fed animal.

1.2. Physiological control of hepatic fatty acid synthesis

The cytoplasmic process of fatty acid synthesis (Stadtman and Barker, 1949; Brady and Gurin, 1952; Spencer et al, 1964) involving the conversion of acetyl-CoA to fatty-acyl-CoA can be divided into two parts, namely the carboxylation of acetyl-CoA to form malonyl-CoA, catalysed by the enzyme acetyl-CoA carboxylase (Wakil et al, 1958; Brady, 1958) and the conversion of one molecule of acetyl-CoA and seven molecules of malonyl-CoA to one molecule of palmitoyl-CoA (catalysed by the particulate enzyme complex fatty acid synthetase) with the concomitant oxidation of fourteen molecules of NADPH (Lin and Kumar, 1972; Brady et al, 1960; Hsu et al, 1965; Lynen, 1967).

The overall process of fatty acid synthesis is open to control in three ways: 1) through the supply of reducing equivalents; 2) by regulation of the pathway by which acetyl-CoA is made available and 3) by alterations in the activities of acetyl-CoA carboxylase and fatty acid synthetase. Veech and Guynn (1974) concluded that under normal circumstances the availability of reducing equivalents or acetyl-CoA would be unlikely ever to be limiting for fatty acid synthesis.

The regulation of enzyme activity is generally divided into short-term control and long-term control. Long-term regulation with a time scale of 3-48 hours involves a change in the quantity of an enzyme, brought about by a change in the rate of synthesis of the enzyme

protein, and sometimes also involving a change in the rate of degradation. Long-term regulation of enzyme activity occurs in response to nutritional and/or hormonal changes.

Short-term regulation with a time scale of 0-3 hours involves a change in the activity of an enzyme. This may be due to changes in the concentrations of substrates or products leading to an alteration in the activity of the enzyme. An alternative mechanism may involve the binding of allosteric modifiers to the enzyme causing an increase or decrease in the activity of the enzyme. These allosteric modifiers are usually low molecular weight compounds and often have no direct role in the pathway which they affect. A similar mechanism involves the binding of regulatory proteins to an enzyme to alter its activity. A final method of control is by reversible covalent modification of an enzyme. An example of such modification is the enzymatically controlled phosphorylation-dephosphorylation of pyruvate dehydrogenase. This may alter both the activity of the enzyme and/or its kinetic properties. Thus phosphorylation may cause an enzyme to be more or less susceptible to factors such as end-product inhibition.

1.2.1. Long-term regulation of fatty acid synthetase and acetyl-CoA carboxylase activities

The long-term regulation of hepatic acetyl-CoA carboxylase and fatty acid synthetase in response to changes in the nutritional state of rats and mice has been well documented (see reviews by Numa *et al*, 1965;

Vagelos, 1974; Veech and Guynn, 1974; Porter, 1978).

The activity of acetyl-CoA carboxylase is markedly depressed in rats fed a high fat diet, or fasted or made diabetic using alloxan and increased in rats fed or refed on a fat-free diet (Numa et al, 1961; Wieland et al, 1963; Allmann et al, 1965). These changes in the catalytic activity of the enzyme have been shown to be parallelled by changes in the amount of antibody-precipitable enzyme protein (Majerus and Kilburn, 1969; Nakanishi and Numa, 1970).

The long-term regulation of the activities of both acetyl-CoA carboxylase and fatty acid synthetase seems to be insulin-dependent. Thus the stimulation of the rate of synthesis of fatty acid synthetase following the refeeding of fasted diabetic rats was indistinguishable from that occurring in refed normal rats if the animals were maintained on insulin but absent when insulin was withheld (Lakshmanan et al, 1972). The stimulation of the rate of synthesis of fatty acid synthetase in response to refeeding could be reduced by glucagon injected into normal animals, the reduction being proportional to the glucagon dose (Lakshmanan et al, 1972).

Refeeding of fasted rats with a fat free diet leads to an almost immediate increase in the amount of immuno-precipitable hepatic acetyl-CoA carboxylase and fatty acid synthetase protein (Lakshmanan et al, 1975). No increase in the catalytic activity of either enzyme occurs for at

least 3 hours however (Yu and Burton, 1974; Lakshmanan et al, 1975). This lag between the formation of the enzyme protein and the acquisition of catalytic activity is due to the initial formation of the apoenzymes which are later converted to the active holoenzymes by the addition of the prosthetic groups, 4-phosphopantotheine and biotin to the inactive fatty acid synthetase and acetyl-CoA carboxylase respectively (Yu and Burton, 1974; Lakshmanan et al, 1975). These authors have suggested that enzymatic conversion between the holo- and apo-enzyme forms of the two enzymes may constitute one method for the short-term control of fatty acid synthesis. So far no experimental evidence for this hypothesis has been obtained (Porter, 1978).

1.2.2. Short-term regulation of fatty acid synthetase and acetyl-CoA carboxylase activities

At low concentrations the activity of fatty acid synthetase is proportional to the concentrations of the substrates acetyl-CoA, malonyl-CoA and NADPH (Katiyar and Porter, 1974). As the concentrations of acetyl-CoA and malonyl-CoA become higher, inhibition of the fatty acid synthetase occurs mainly due to competition of the two substrates for the 4-phosphopantotheine binding site. Since inhibition of the enzyme by acetyl-CoA is more potent than by malonyl-CoA the optimum ratio of the two substrates is 1:4 respectively (Katiyar and Porter, 1974). The true significance, of the substrate concentration-dependence of the activity of fatty acid synthetase, in vivo is however

obscure, since the cytosolic concentration of acetyl-CoA in the liver cell under differing hormonal and dieting conditions is unknown.

A complex pattern has emerged for the short-term regulation of acetyl-CoA carboxylase activity. Early work indicated that acetyl-CoA carboxylase had a much lower activity than fatty acid synthetase (Ganguly, 1960; Numa et al, 1961). Later work revealed that if the enzyme was first fully activated, its activity was comparable with that of fatty acid synthetase (Chang et al, 1967). From this it is evident that the degree of activation of acetyl-CoA carboxylase in vivo may determine the rate of fatty acid synthesis.

Brady and Gurin (1952) reported that fatty acid synthesis from acetate could be stimulated by citrate. This finding was explained when Martin and Vagelos (1962) and Vagelos et al (1963) reported that citrate and other tricarboxylic acid cycle intermediates stimulated polymerisation and activation of acetyl-CoA carboxylase from rat adipose tissue. Similar activation by citrate has been reported for the enzyme from rat liver (Matsuhashi et al, 1964b), avian liver (Gregolin et al, 1966a,b;Goto et al, 1967; Lane et al, 1970), bovine adipose tissue (Kleinschmidt et al, 1969) and rat mammary gland (Miller and Levy, 1969). In contrast to the above however no activation of acetyl-CoA carboxylase from E. coli (Alberts and Vagelos, 1968) or yeast (Matsuhashi et al, 1964a) occurs in response to treatment with citrate. It has been shown using the enzyme

from rat liver (Numa et al, 1965), avian liver (Gregolin et al, 1968; Ryder et al, 1967), and adipose tissue (Matsuhashi et al, 1964b)that the major effect of citrate-induced activation and polymerisation is to increase the V_{max} of the enzyme with very little effect on the K_m of the enzyme for its substrates. Since both partial reactions are stimulated by citrate (Matsuhashi et al, 1964b)it has been deduced that the prosthetic group is involved in conformational changes (Lane et al, 1970) leading to increased reactivity of the biotin group (Moss and Lane, 1972).

One of the most potent inhibitors of acetyl-CoA carboxylase is palmitoyl-CoA, the end product of fatty acid synthesis (Numa et al, 1965; Halestrap and Denton, 1973; Carlson and Kim, 1974 b). This inhibition by palmitoyl-CoA is competitive with citrate (Numa et al, 1965), with a K_i of 0.8-1.1μM for palmitoyl-CoA and a K_m of 2-6 mM for citrate. Thus the ratio of citrate concentration to palmitoyl-CoA concentration in the cytosol may be important in controlling the rate of fatty acid synthesis. Greenbaum et al (1971), have estimated the cytoplasmic concentration of citrate to be 0.1-0.2 mM. This would imply that acety-CoA carboxylase may not be fully activated in vivo; however the extent of citrate compartmentation in the cytosol is not known. The concentrations of long-chain acyl-CoA derivatives in the mammalian and avian liver vary over the range 15-140μM depending on the nutritional state of the animal (Bortz and

Lynen, 1963; Tubbs and Garland, 1964; Yeh and Leveille, 1971). This range of concentrations is well within those required to inhibit acetyl-CoA carboxylase, but the true concentrations to which the enzyme is exposed *in vivo* are not known.

Acetyl-CoA carboxylase from rat liver (Carlson and Kim, 1973; 1974 a; Lee and Kim, 1977) and rat adipose tissue (Brownsey *et al*, 1977; Denton *et al*, 1978) has recently been shown to exist in inactive, phosphorylated and active, dephosphorylated forms. The relationship between the reversible polymerisation (Gregolin *et al*, 1966 b) and reversible phosphorylation (Carlson and Kim, 1974 a) is not clear. Following treatment with citrate and magnesium for 30 minutes, maximal activity is attained, presumably because the enzyme is both polymerised and dephosphorylated.

Insulin has been shown to have a rapid short-term effect on the activity of acetyl-CoA carboxylase from rat white adipose tissue (Halestrap and Denton, 1973; 1974). This effect was on the initial activity of the enzyme and could be abolished by incubation of the extract with citrate. This effect of the hormone therefore seems likely to have been due to alterations in the state of polymerisation or phosphorylation of the enzyme.

Glucagon (Haugaard and Stadie, 1953; Akhtar and Bloxham, 1970; Bloxham and Akhtar, 1972; Allred and Roehrig, 1972; 1973; Bricker and Levey, 1972 a & b; Bricker and Marraccini, 1975; Meikle *et al*, 1974; Raskin *et al*, 1974;

Capuzzi et al, 1971; 1974; Harris, 1975; Edwards, 1975; Muller et al, 1976) and vasopressin (Ma and Hems, 1975; Hems and Ma, 1976) have been reported to inhibit fatty acid synthesis in the mammalian liver. In view of the finding of Halestrap and Denton (1973; 1974), that insulin affects the activity of acetyl-CoA carboxylase in isolated fat pads, the possibility exists that glucagon and vasopressin may alter the activity of this enzyme in liver. Since acetyl-CoA carboxylase is well established as the rate limiting enzyme for fatty acid synthesis (Numa et al, 1965), the effects of these and other hormones on the activity of the hepatic enzyme would seem to warrant further investigation. In this study such work has been undertaken together with an investigation of the effects of other variables such as hypoxia and nucleotides.

1.3. Lipogenesis in states where there is a hypertrophic fatty liver

1.3.1. Lipogenesis in obesity

Obesity is the major dietary disease of the developed nations. By definition it is a disease of lipid metabolism and in particular fatty acid synthesis leading to excessive fat deposition. The disease appears to be associated with a predisposition to other diseases such as diabetes, vascular disease and hypertension. Slimming can lead to a fasting-refeeding cycle which may cause an increase in the deposition of fat. Such programmes of fasting-refeeding have been demonstrated to have just such an effect in laboratory animals (Szepesi et al, 1973). It is therefore important to understand the underlying biochemistry of obesity in order to devise more effective treatments. Experimental approaches to the problem in humans are limited on ethical grounds, so much work has been carried out using animal models. Obese rodents have been widely used for such investigations. One of the most studied models is the obese, hyperglycaemic mouse, which is homozygous for the autosomal, recessive gene ob (Ingalls et al, 1950; Beloff-Chain et al, 1975). These mice have increased rates of fat deposition, serum triacylglycerol turnover (though not concentration),fatty acid synthesis in both liver and adipose tissue, and also exhibit hyperglycaemia, hyperinsulinaemia and an increased rate of turnover of hepatic glycogen stores (Elliott et al, 1971; Salmon and Hems, 1973; Hems et al, 1975; Stigeta and Shreeve, 1964).

One of the characteristics of genetic obesity is that, following even relatively severe food deprivation, the body fat to protein ratio remains higher than in lean individuals of the same weight (Alonso and Maren, 1955). The abnormalities of carbohydrate metabolism are more or less reversed following diet restriction of obese (ob/ob) mice severe enough to ensure that their body weight remains similar to that of lean litter mates (Elliott et al, 1971). The alterations of lipid metabolism are less reversible by starvation however (Salmon and Hems, 1973; Volpe and Marasa, 1975b; Rath et al, 1974; Elliott et al, 1974).

Fat deposition in adipose tissue can be sustained by uptake of circulating neutral lipids, involving lipoprotein lipase, or by de novo synthesis within the adipose organ. In the obese (ob/ob) mouse there is both increased synthesis of fatty acids in adipose tissue (Hems et al, 1975) and an increase in the activity of lipoprotein lipase (Enser, 1972; Rath et al, 1974). Circulating neutral lipids originate by hepatic esterification of fatty acids absorbed from the diet, released from adipose tissue or newly synthesised in the liver. In laboratory animals fed a normal starch-based diet, fat deposition must be mainly sustained by de novo synthesis of fatty acids in liver and adipose tissue (see section 1.2.). In obese (ob/ob) mice adipose tissue is the major site of fatty acid synthesis (as determined using $^{3}H_2O$; Hems et al, 1975) even though the total rate of hepatic fatty acid synthesis is about twice as high as in lean mice, where the liver in the major site of synthesis.

The use of tritium oxide and [^{14}C]-labelled glucose (to simultaneously assess the total rates of fatty acid synthesis and the contribution of glucose carbon to the process), have revealed a difference in substrate preference between adipose tissue and liver (Hems et al, 1975). Blood borne glucose is the major carbon source for fatty acid synthesis in adipose tissue while lactate and glycogen appear to be the favoured precursors in liver (Salmon et al, 1974; Salmon and Hems, 1974; Hems et al, 1975). The rate of fatty acid synthesis in the obese (ob/ob) mouse liver seems to be less susceptible to alterations in the profile of substrates offered, as has been established in perfusion experiments (Salmon and Hems, 1974). This lack of a substrate effect on fatty acid synthesis in perfused livers from obese (ob/ob) mice may be because the process is already nearly maximally stimulated.

The rate limiting enzyme for fatty acid synthesis in both liver and adipose tissue is acetyl-CoA carboxylase (Numa et al, 1965; see section 1.2.2.). In the genetically obese (ob/ob) mouse the activities of both this enzyme and fatty acid synthetase are markedly raised (Nakanishi and Numa, 1971). This is also true, though to a lesser extent, for another genetically obese mouse the 'viable yellow obese' (A^{vy}/a) mouse (Yen et al, 1976).

Fatty acid synthesis in the obese (ob/ob) mouse liver shows resistance to inhibition by vasopressin (Hems and Ma, 1976) even after relatively severe food deprivation. The

processes of glycogenolysis and glucose output by the liver were, however, normally stimulated by vasopressin in diet restricted obese (ob/ob) mice (Hems and Ma, 1976). Evidence will be presented in this study that this resistance to inhibition of fatty acid synthesis in the obese (ob/ob) mouse liver is not restricted to vasopressin, but also includes angiotensin II and adrenaline. It will also be shown that inhibition of fatty acid synthesis in the perfused lean mouse liver by angiotensin II and adrenaline is associated with a decrease in the activity of acetyl-CoA carboxylase; such a decrease in the activity of this enzyme did not occur in the perfused livers of diet restricted obese (ob/ob) mice in response to these hormones.

1.3.2. Lipogenesis in the regenerating liver

In 1894 Valerian von Meister published a quantitative account of the process of liver regeneration after partial hepatectomy. By 1929 enough literature had accumulated to warrant a review of the subject (Fishback, 1929). In 1931 the paper of Higgins and Anderson promoted a wave of interest in the field of liver regeneration.

Resection of the liver immediately initiates the processes of regrowth, although the nature of the signal which elicits this response is still not known. Various proposals have been advanced but so far no single factor has emerged as being absolutely necessary for regrowth (see review by Bucher and Malt, 1971). One idea which gained wide support was that altered vascular flow within

the liver remnant was the stimulus for regrowth, but liver regeneration is still evident in animals following portal ligation, if the proper controls are carried out, (see Leduc, 1964; Bucher and Malt, 1971; Startzl and Putmam, 1975, Blumgart, 1978). Other ideas have centred around the involvement of blood-borne factors. These factors are generally thought either to be constantly present in serum or to be synthesised and released by the liver remnant. These latter substances are considered to stimulate growth (hepatotrophic) while the former may either be stimulatory (normally being removed and destroyed by the intact liver), or inhibitory to growth (i.e. they are synthesised continuously by the liver, and their concentration falls following resection) (see reviews by Bucher, 1963; 1975; Leduc, 1964; Bucher and Malt, 1971; Startzl and Putmam, 1975; Bucher et al, 1978; Startzl et al, 1978; Weinbren, 1978).

During regrowth of the liver there is a requirement for new tissue components. While there is a well established increase in the rates of DNA and protein synthesis, the production of new lipid components is not so well documented. Following partial hepatectomy there is usually an accretion of fat in the liver (fatty liver; Harkness,1952).

The accumulated lipid consists almost entirely of triacylglycerols (Glende and Morgan, 1968; this study), while phospholipid deposition almost exactly keeps pace with the growth of the liver remnant (Glende and Morgan, 1968; Tata, 1970; Fex, 1970 b; Bergelson et al, 1974;

Fex and Thorzell, 1975). The origin of the triacylglycerols and the reasons for their accumulation in the liver following partial hepatectomy have been studied extensively and more detailed consideration is given to these phenomena in the discussion (6.3.).

The de novo synthesis of fatty acids in rats following partial hepatectomy has been much less studied and there have been conflicting reports as to whether the process is stimulated or inhibited after the operation. Thus Johnson and Albert (1959, 1960), using [^{14}C]-acetate to measure hepatic lipogenesis, concluded that synthesis of fatty acids was low for the first few days but that cholesterol synthesis was stimulated to 2-3 times the normal rate by 18 hours post-operation. Neville et al (1970) found that incorporation of [^{14}C]-glucose into fatty acids was reduced after partial hepatectomy, while the incorporation of [^{14}C]-acetate reached a minimum 39 hours after the operation but then rose to a level above that in sham-operated controls (Neville et al, 1969). These workers also concluded that following partial hepatectomy rats derive a greater proportion of their energy from fat than do normal rats. Using liver slices Takeuchi et al (1976) found de novo synthesis of fatty acids from [^{14}C]-acetate was increased by about 3 days after partial hepatectomy. They also found that cholesterol synthesis from [^{14}C]-acetate was increased, but the process was still open to feedback control by dietary cholesterol.

In this study the total rates of fatty acid synthesis (from all carbon sources) have been measured using the incorporation of tritium into fatty acids from tritiated water (Lowenstein, 1971; Hems *et al*, 1975). These experiments have shown that *de novo* synthesis of fatty acids in the regenerating liver *in vivo* is increased over the period of most active liver growth (i.e. 1-3 days post-operation). This increase did not reflect an intrinsic change in the lipogenic capacity of the liver since the activity of acetyl-CoA carboxylase was not increased above that of sham-operated livers and the total rates of fatty acid synthesis in the isolated perfused regenerating liver were no different from those in livers from sham-operated rats.

1.4. Scope of the present work

In the present study an attempt has been made to gain some insight into the short-term control of hepatic fatty acid synthesis. Recently a number of peptide hormones, including vasopressin, glucagon, adrenaline and angiotensin II, have been shown to have short-term catabolic actions on carbohydrate metabolism in the liver. Inhibition of hepatic fatty acid synthesis in response to two of these hormones, vasopressin and glucagon has also been demonstrated (see section 1.2.2.). In this study two other peptide hormones, adrenaline and angiotensin II have been shown to inhibit fatty acid synthesis in the perfused lean mouse liver. The actions of glucagon, adrenaline and angiotensin II on fatty acid synthesis in the obese (ob/ob) mouse liver were investigated; resistance to these three hormones was found.

Acetyl-CoA carboxylase is the first committed step of fatty acid synthesis and the work of Numa et al (1965) has shown that it can be rate limiting for the process. Recent work has shown that the enzyme can exist in an inactive monomeric form or an active polymerised form and also in inactive phosphorylated or active dephosphorylated forms (see section 1.2.2.). The activity of this enzyme in the liver was therefore measured in conditions where the rate of fatty acid synthesis is altered e.g. in obesity, in the regenerating liver and following treatment of the livers with hormones. From these studies it has emerged that parallel changes in the activity of acetyl-CoA carboxylase

and the rate of fatty acid synthesis, in the liver, usually occur.

The incorporation of ^{3}H from $^{3}H_2O$ into fatty acids has been used throughout to measure total rates of fatty acid synthesis, *in vivo* and in perfusions (Lowenstein, 1971; Hems *et al*, 1975 ; section 2.6.). During the course of this work, a variety of experimental approaches have been used. The perfused mouse liver was selected for studies of hormone action on fatty acid synthesis and acetyl-CoA carboxylase activity, since the small size of the liver and hence the low overall flow rate of medium through the liver allowed the use of flow-through or nonrecycling perfusion (section 2.4.1.). This had the advantage that the liver was subjected to constant concentrations of hormones, substrates and labelled precursors for the duration of the experiments. Another advantage of the use of mouse livers was that a parallel study of the obese (*ob/ob*) mouse model became possible. The total rate of fatty acid synthesis in the regenerating liver, was measured, *in vivo*, and in perfusion. Finally, high-speed supernatants of liver homogenates were used in investigations of the kinetic properties of acetyl-CoA carboxylase and in particular in studies in to the effects of adenine nucleotides on the activity of the enzyme.

CHAPTER 2

2. ANIMALS, MATERIALS AND METHODS

2.1. Animals

All experiments involving rats were carried out using male, albino, Sprague-Dawley rats of the CFY strain from Carworth, Europe. The male breeding stock was renewed every three months to ensure a close genetic relationship with the foundation stock. The rats were kept under controlled conditions with a twelve hour light-dark cycle (06.00 - 18.00 hours GMT light), a temperature range of 19 - 23^{o} and relative humidity of 55%. All rats were allowed free access to Thompson's formula cereal based, standard diet (Pilsbury's Ltd., Birmingham and Heygate & Sons, Northants.), and tap water at all times. Initially rats weighed between 190 g and 210 g, except for those donating blood for liver perfusion experiments which varied between 600 g and 900 g.

The lean and obese mice used in the present study were obtained from the random-bred, closed colony at Imperial College (Beloff-Chain *et al*, 1975). The autosomal, recessive gene *ob* arose spontaneously in the highly inbred strain of mice C57BL/6J at the Jackson Memorial Laboratory, Bar Harbor, U.S.A. (Ingalls *et al*, 1950) and was introduced into local mixed colonies at Edinburgh and Birmingham. The

original stocks of mice used to start the Imperial College mouse colony were obtained from these two colonies several years ago and have been bred as a closed colony ever since. The mice were housed in polycarbonate cages with sawdust bedding and had free access to tap water and Oxoid breeding diet (Oxoid Ltd., London, S.E.1.) until 6-8 weeks of age when the diet was changed to Thompson's formula cereal based, standard diet (Pilsbury's Ltd., Birmingham and Heygate & Sons, Northants).

Obese mice (homozygous for the ob gene), refered to as ob/ob, showed massive deposition of body fat and high plasma glucose and insulin concentrations when compared to their lean litter mates at the same age (either ob/ * or */ *). Obese mice fed ad libitum consumed 7-9 g food/day and had an average weight at 3 months and 6 months of 60 g and 90 g respectively compared to lean litter mates of 28 g and 33 g respectively.

Diet restricted ob/ob mice were fed Oxoid diet ad libitum from birth to 35 days, after which they were housed in separate cages with blotting paper bedding and given only 4 g of powdered Thompson's diet each morning with free access to tap water at all times. The diet restricted ob/ob mice (D.R. ob/ob) were kept under this regime for about 6 weeks before being used for experimental work and then weighed 25-35 g. While the weight and plasma glucose and insulin concentrations of the D.R. ob/ob mice are comparable with those of their lean litter mates of the same age (Abraham et al, 1971), they have a higher body fat content

and lower body protein content expressed as % total body weight, compared to the non-obese mice (Alonso and Maren, 1955).

The lean mice used throughout this study were homozygous for the lean alele. All mice were maintained under the same conditions as the rats (see above) until used for experiments at 10-11 weeks of age.

2.2. Chemicals and materials

Solvents, chemicals and reagents ('Analar' grade or of the highest purity available) were obtained from B.D.H. Chemicals Ltd., (Poole, Dorset, U.K.), Fisons Ltd., (Loughborough, Leics., U.K.), Hopkins and Williams (Romford, Essex, U.K.), or May and Baker (Dagenham, Essex, U.K.), unless otherwise stated. Alcohols were from James Burrough Ltd., (London, S.E. 11, U.K.). Standard lipids (used for chromatography), oleic acid, acetyl-CoA, adenine nucleotides, L-lactate, 8-argenine vasopressin (synthetic grade VI), adrenaline bitartrate and dithioerythritol were from Sigma (London) Chemicals Co. Ltd. (London, S.W.6., U.K.).

The activity of each batch of 8-argenine vasopressin was determined by bioassay of its antidiuretic action, by Dr. M. Forsling (Dept. of Physiology, Middlesex Hospital, London, U.K.). The activity was consistently about 90% of that stated by Sigma (London) Chemical Co. Ltd., so the true activity of each batch was calculated using this factor.

Enzymes and substrates for glycerol determination were from Boehringer Corp., (London) Ltd., (London, W.5., U.K.). Radioactive isotope-labelled compounds were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Glucagon (crystalline, insulin free) was kindly donated by Eli-Lilly Ltd., (Indianopolis, U.S.A.). Heparin was from Evans Medical Supplies Ltd., (Liverpool, U.K.), and 'Nembutal' (sodium pentabarbitone) from Abbott Labs. Ltd., (Queenborough, Kent, U.K.).

Silica gel G for TLC was from Anderman Ltd., (London, S.E.1., U.K.) and was manufactured by Merck Ltd.. Liquid and stationary phases for GLC were from Pye Unicam (Cambridge, U.K.).

Surgical instruments and Michel clips were purchased from Holborn Surgical Instrument Co. Ltd., (London, E.C.1., U.K.) and John Weiss and Sons Ltd., (London, W.1., U.K.). Metal intravenous cannulae (West Middlesex Hospital pattern) for rat liver perfusions were from Downs Surgical Ltd., (London, W.1., U.K.) or A.C. Daniels Ltd., (London, W.1., U.K.). Plastic cannulae for mouse liver perfusions were made from 'Portex' plastic intravenous cannulae, obtained from Portland Plastics Ltd., (Hythe, Kent, U.K.). Silicone rubber tubing ('Silescol') was from Esco, (Rubber) Ltd., (London, W.1., U.K.).

Bovine serum albumin ('Pentex' fraction V) was obtained from Miles Laboratories Ltd., (Stoke Poges, U.K.). As required, albumin was defatted by a slightly modified version of the method of Chen (1967). Prewashed Norit SX-1 charcoal (Hopkins and Williams Ltd., Essex, U.K.) was stirred into a 10% (w/v) solution of bovine serum albumin at the rate of 0.5 g charcoal to each 1.0 g of albumin. The pH of the mixture was brought to 3.0 by the addition of 0.2 M HCl and the mixture was then stirred for 1 hour at 4^{o}. Most of the charcoal was removed by centrifuging at 18,000 xg and 4^{o} for 30 minutes. The remaining charcoal was removed by centrifuging at 20,200 xg for 20 minutes at 4^{o} in a Beckman L2-65 ultracentrifuge using a swing-out rotor. The clear

supernatant was adjusted to pH 7.0 with 0.2 M NaOH and dialysed for 2 days against 4 changes of distilled water, to remove the NaCl, before being freeze-dried. The resultant powder was stored dry at 4^{o}.

2.3. Partial hepatectomy procedure

2.3.1. Operation

Operations were performed between 11.00 H and 13.00 H GMT and all sampling was carried out at the same time of day, up to 6 days after operation, to minimise the effects of the diurnal variations reported to affect liver regeneration (Bonney et al, 1973).

Partial hepatectomies were performed essentially as described by Higgins and Anderson (1931).

Rats were anaesthetised with diethyl ether/air mixture to an even medium-deep anaesthesia. The abdomen was opened by a longitudinal incision, about 75 mm long, slightly to the left of the mid-ventral line and extending up to the xiphisternum. The edges of the wound were retracted and the liver ejected by gentle pressure applied with the tips of the fingers below and lateral to the opening. Both the median and left lateral lobes of the liver were carefully drawn out and dissected free of connective tissue. The branches of the hepatic artery and vein supplying the lobes were tied off tightly with double suture thread (size 2/0) just above the lobes which were then trimmed off as close as possible to the tie. The abdomen was inspected for haemorrhage and the muscle layer closed with about four stitches. The skin was closed with 11 mm Michel clips. The operation was generally of about 8 minutes duration, and rats were fully conscious within 10 minutes of completion of the operation (Fig. 1).

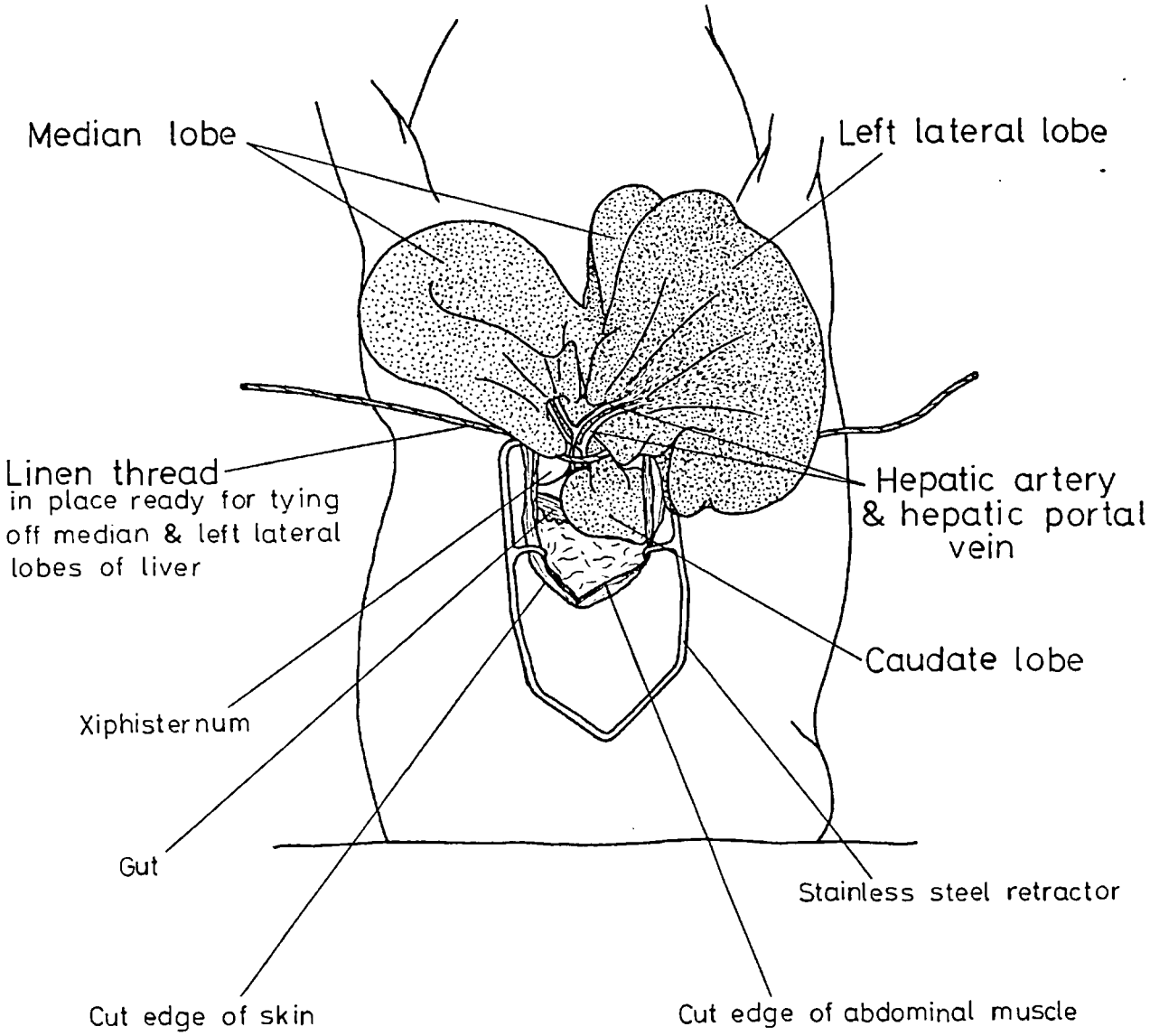

Fig. 1 Partial hepatectomy operation in the rat.

Sham operated rats received the same treatment as the partially hepatectomised rats except that following the freeing of the median and left lateral lobes from connective tissue and the handling thus caused, the lobe was carefully returned to its usual position in the abdomen.

A high degree of cleanliness rather than total sterility was observed during the operative procedures, however only one case of abdominal infection occured. No mortality occured subsequent to the operation.

Batches of 4, 6 or 8 rats were used, with equal numbers of partially hepatectomised and sham operated rats in each group. Partial hepatectomies and sham operations were alternated to reduce variations.

Non-operated control rats were free of all surgical or anaesthetic procedures. Rats were kept under observation for about 2 hours after the operation and then returned to the animal house holding room. The rats were inspected at 24 hour intervals, after operation, to make sure that the clips and stitches were still in place. Following inspection the rats were weighed and returned to their cages. Body weights were measured between 11.00 hours and 13.00 hours G M T. Changes in body weight were calculated using the post-operation body weight to eliminate differences due to removal of liver tissue from the partially hepatectomised rats.

Free access to food and water was allowed at all times before and after operation.

To determine food intake, groups of three partially hepatectomised, sham operated or non-operated rats were placed in a cage with a known weight of food in the hopper. The food was reweighed at 24 hour intervals and made up to the original weight. The weight of food consumed was then divided by three to obtain the average food intake per rat per 24 hour.

2.3.2. Sampling procedures

Total rates of lipogenesis *in vivo* were measured using the incorporation of tritium from tritium oxide into fatty acids and cholesterol. The rats were injected intra-peritoneally or intra-venously with a 0.2 ml dose containing 10 mCi $^{3}H_2O$ and 12 μCi D-[U-^{14}C] glucose made up in saline. Exactly one hour later the rats were stunned by a blow on the head and a blood sample (approx. 1-2 ml) taken by cardiac puncture, using a clean disposable syringe. The abdomen was immediately opened and a 1-2 g liver sample frozen in liquid nitrogen. Finally a piece of epididymal fat pad was removed and frozen in liquid nitrogen. The total sampling time was usually less than one minute.

Serum was prepared from the clotted blood by centrifuging at 5,000 xg for 10 minutes. The serum was stored at -20^{o} or used immediately for the determination of specific radio-activity and free fatty acid and triacyl-glycerol concentrations.

Frozen liver and adipose tissue were broken into small fragments and kept in liquid nitrogen until extraction of lipids and glycogen could be started.

Liver tissue required for the assay of acetyl-CoA carboxylase activity was freeze-clamped between aluminium tongs chilled in liquid nitrogen, within 10 seconds of stunning the rats (Wollenberger et al, 1960).

2.4. Liver perfusion

2.4.1. Apparatus

Liver perfusions were carried out using apparatus essentially the same as that described by Hems *et al*, (1966) which was developed from that of Miller *et al*, (1951) and Schimassek (1963). The apparatus used for mouse liver perfusion has been slightly modified by Elliot *et al*, (1971) and Salmon *et al*, (1974).

The apparatus was enclosed in a heated cabinet thermostatically maintained at 36°. The front of the cabinet consisted of a counter-balanced 'Perspex' window which could slide vertically to provide access. The heater in the roof of the cabinet was equiped with a fan to circulate the warm air, this circulation was augmented by a separate fan inside the cabinet. Perfusion medium within the main reservoir was continuously mixed by a magnetic stirrer located under the floor of the cabinet. The entire perfusion apparatus and liver preparation were thus maintained at a constant temperature within the cabinet.

The apparatus consisted of a large reservoir (capacity approx. 100 ml) from which the perfusion medium was pumped by a MHRE flow inducer (Watson-Marlow Ltd., Cornwall, U.K.) through a plastic screen filter (from a disposable blood transfusion set) to the top of a vertical gas exchanger (see Fig. 2). The medium flowed down over the inside surface of the gas exchanger while gas mixture (generally O_2:CO_2 ; 95%:5%) flowed in the opposite direction. Excessive

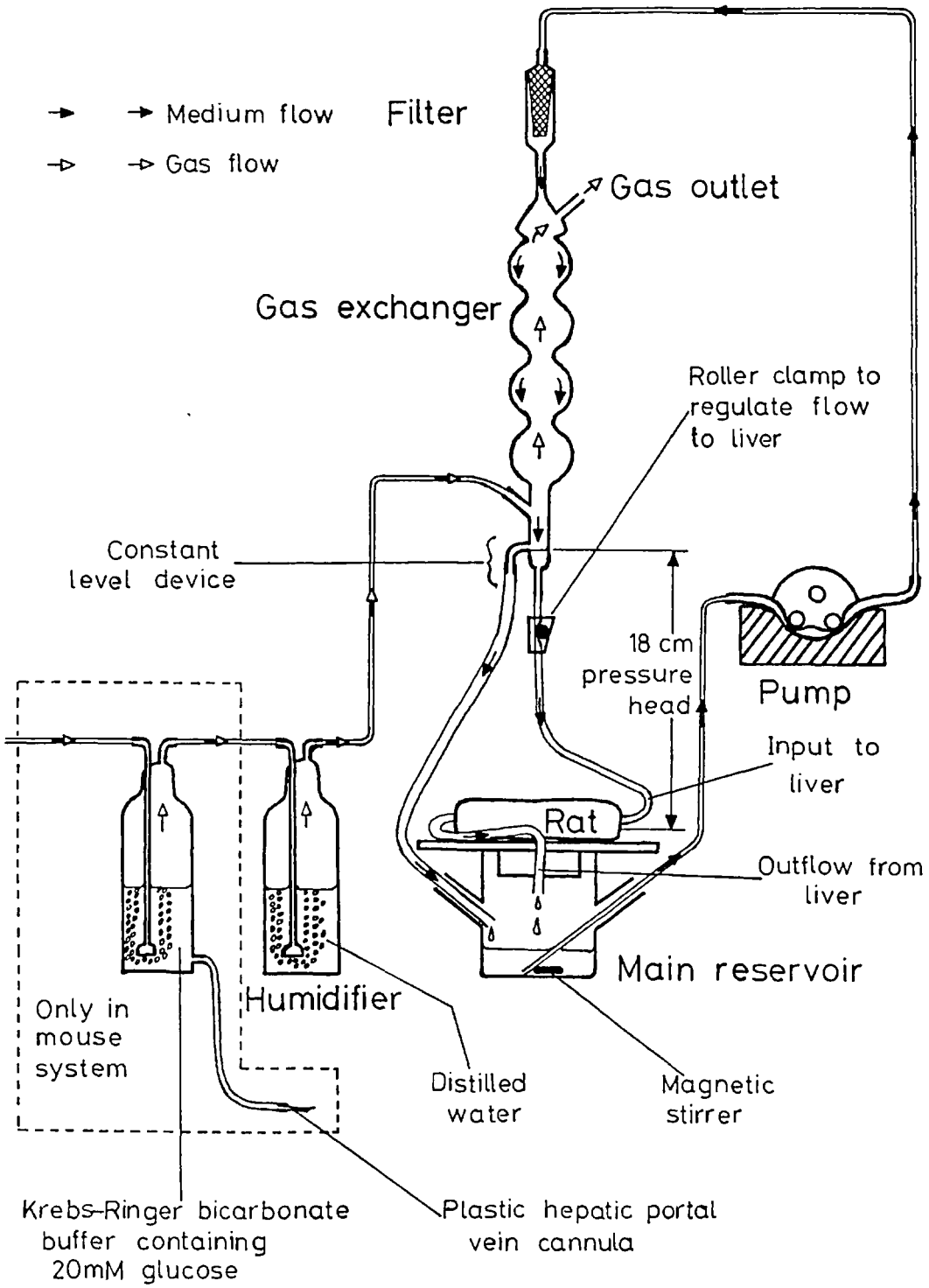

Fig. 2 Apparatus for perfusion of the liver.

evaporation of the perfusate was prevented by saturating the gas with water by bubbling it through distilled water, immediately before it entered the gas exchanger.

At the bottom of the gas exchanger a constant head of perfusate was maintained by an overflow tube leading back to the main reservoir which allowed a small reservoir to remain constantly full. At the bottom of the small reservoir was an outlet, connected to the liver by a length of silicon rubber tubing. The hydrostatic pressure head thus provided was maintained at 18 cm above the liver, but the flow of perfusate to the liver was regulated by a roller clamp to prevent swelling of the organ. The effluent medium from the liver was allowed to flow back into the main reservoir in recirculating perfusions, or collected in a separate container in single pass (or flow through) perfusions.

2.4.2. Liver perfusion medium

The basic medium used in all rat liver perfusion experiments consisted of 60 ml Krebs-Ringer bicarbonate buffer (Krebs and Henseleit, 1932) and 12 ml 15% (w/v) bovine serum albumin, with 10-15 ml packed washed rat red blood cells, added after the liver was connected into the system, giving 10-15% haematocrit.

The bovine serum albumin ('Pentex' fraction V from Miles Labs.) was dialysed at 4^{o} for three days against four changes of Krebs-Ringer bicarbonate and then kept at

-20° until used. Dialysis tubing (Visking tubing (HMC) 36/32") was boiled for at least 8 hours with three changes of distilled water and kept at 4° in distilled water until used.

Washed rat erythrocytes were prepared from whole blood obtained by bleeding large fed donor rats, anaesthetised with diethyl-ether, from the aorta. The blood was defibrinated on glass beads in siliconised flasks(Baron and Roberts, 1963) by gentle shaking in a circular motion until a clot had formed. The flask was covered and left to stand for ½ hour to allow for contraction of the clot to maximise the yield of red cells. The defibrinated blood was then decanted and washed twice by centrifugation with 20 volumes of Krebs-Ringer bicarbonate.

The pH of the medium, tested before, during and after perfusion varied between 7.35 and 7.55.

The perfusion medium used for the mouse liver consisted of 40 ml Krebs-Ringer bicarbonate buffer containing 10 ml 15% (w/v) bovine serum albumin (dialysed as for rat liver perfusions) and 20 ml packed human erythrocytes added about 10 minutes before the start of the perfusion to give a haematocrit of 25-30%

Aged human erythrocytes, which still retain almost their full oxygen carrying capacity but lose their glycolytic activity, were prepared from aged whole human blood. The blood had been stored for 4-5 weeks in A.C.D. anticoagulant medium at 4°. A.C.D. anticoagulant medium consists of a 2% aqueous solution of disodium citrate containing 3% D-glucose and is mixed 1:4.4 v/v with the

whole blood. The aged whole blood in A.C.D. medium was expired blood-bank blood and was kindly donated by St. George's Hospital, (Hyde Park Corner, London, S.W.1.). The packed red cells were prepared by centrifuging the A.C.D. blood three times at 10,000 xg in four volumes of Krebs-Ringer bicarbonate buffer and discarding the supernatant. Although human erythrocytes have approximately twice the volume of mouse erythrocytes there is little difference in the diameter (Altman and Dittmer, 1964) which is 7.5 and 6.0 μ respectively. Glucose was present in the perfusate from the start of all perfusions at the concentrations indicated for each set of experiments.

Lactate when present was added as a single initial dose immediately after the completion of the operation. In rat liver perfusions the correct concentration of C_3-substrates was maintained by infusion of a solution of lactate, glycerol and pyruvate (molar ratio 3:2:1 respectively, total concentration 0.33 M) at the rate of 3 ml per hour. In perfusions lasting up to 3 hours, the perfused mouse liver did not take up sufficient lactate to cause a significant fall in the concentration.

Sodium oleate was added as a warm solution, to the perfusion medium containing albumin, immediately after the operation was completed. 20 mM sodium oleate pH approximately 8.5 was added slowly to produce a final concentration of 0.1 mM.

Hormones were added as a single dose to the perfusate in single pass (or flow-through) perfusions to produce the

required concentration. The dose was added at the start of the flow-through phase of the perfusion, i.e. after the 1 hour stabilizing period of recycling perfusion thus ensuring that the liver was exposed to a constant concentration of hormone throughout the experimental period.

In recyling perfusions the hormones were added in serial doses at 15 minute intervals, each dose being sufficient to produce the concentration indicated for each group of experiments.

2.4.3. Perfusion operations

The operation in rats was carried out essentially as described by Hems et al, (1966). The rats were anaesthetised to a medium deep anaesthesia using diethyl ether - air mixture. The anaesthetised rat was attached to a perfusion platform using masking tape and the abdomen opened by a transverse incision approximately 15 mm below the xiphysternum. The intestines were deflected to the left onto a damp tissue.0.1 ml of heparin solution (1,000 U/ml) was injected into the inferior vena cava below the point of entry of the right renal vein. A damp tissue was placed over the injection site to prevent haemorrhage. Ties were placed loosely round the inferior vena cava and hepatic portal veins in the positions shown in the diagram (see Fig. 3). The hepatic portal vein was then cannulated

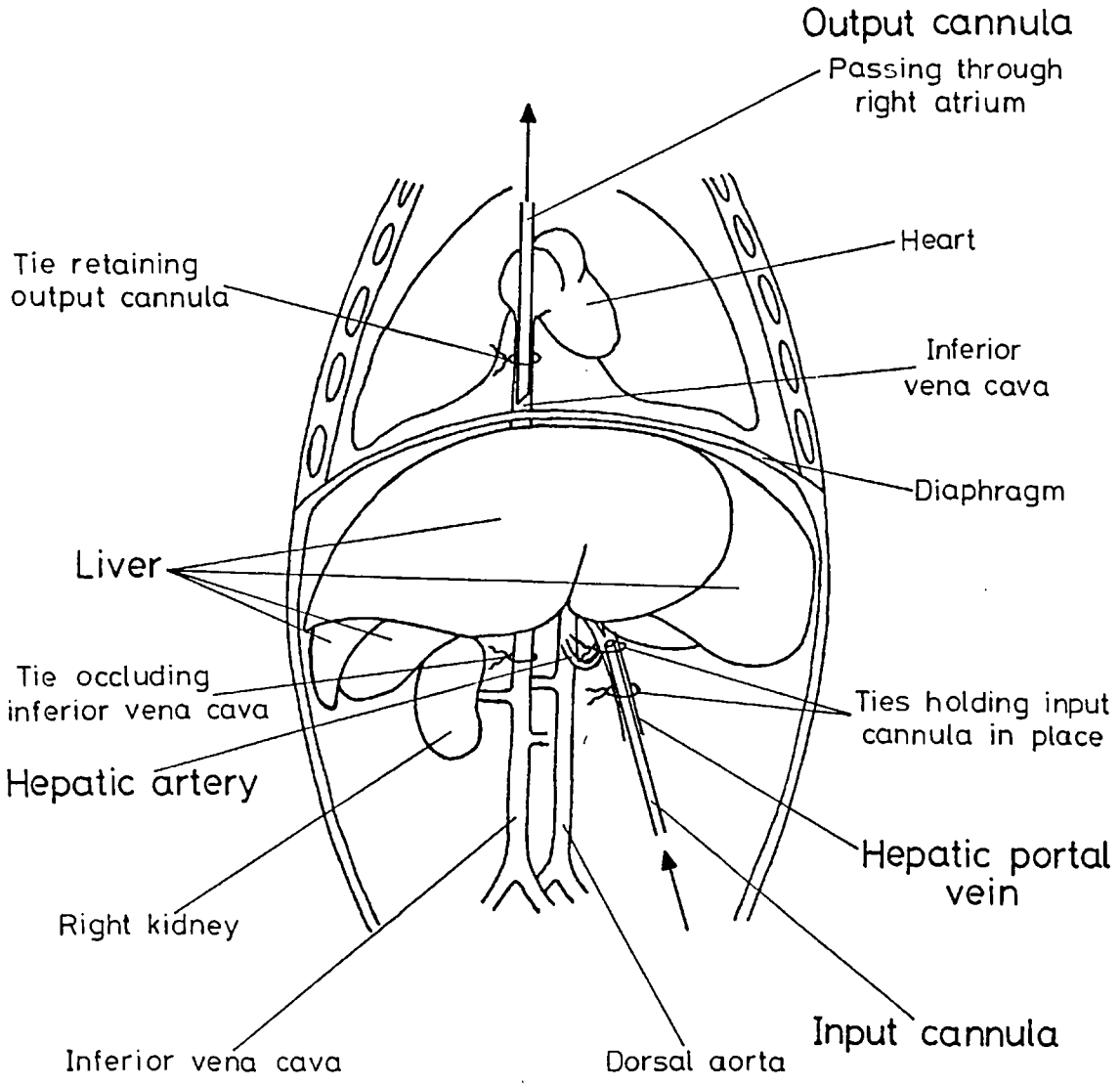

Fig. 3 Liver perfusion operation in the rat.

using a 17 S.W.G. stainless steel, intravenous cannula (West Middlesex Hospital pattern). The needle was removed and the cannula tied into place using the proximal tie. Backflow of blood through the cannula was generally immediate. In the absence of backflow of blood (only occurring after a difficult cannulation) the cannula was filled with Krebs-Ringer bicarbonate buffer, from a disposable syringe, to prevent air entering the liver. The thoracic cavity was then opened to expose the heart and inferior vena cava; a flap of skin being left in situ in the region of the diaphragm,to cover the liver and keep it warm and moist. A loose tie was inserted round the inferior vena cava just below the heart. A cannula made from rigid plastic tubing ('Portex' tubing size 3.00 mm x 2.00 mm/2.42 mm x 1.67 mm, drawn out to a taper and cut to a bevelled point), was inserted into the inferior vena cava through the right atrium and pushed down until the end was level with the diaphragm. The tie was then tightened round the inferior vena cava to hold the plastic output cannula in place.

The liver was left in position in the carcass and connected into the perfusion system. The outlet from the constant level device at the bottom of the gas exchanger was connected to the metal input cannula in the hepatic portal vein, and the clamp opened to allow red cell free perfusion medium to flow into the liver. The effluent medium from the liver was allowed to run to waste until the liver had been cleared of stagnant blood and had assumed a

light olive-brown colour. While the liver was clearing, the tie round the inferior vena cava above the right kidney, and the second tie round the hepatic portal vein and hepatic artery were tied off isolating the liver completely from the rest of the carcass. Generally about 20 ml of medium was required to fully clear the liver. Once cleared the output from the liver was allowed to flow back into the perfusion reservoir, so that the perfusion medium recycled. At this point the washed rat erythrocytes were gradually added to the medium. The flow rate through the liver was adjusted to approximately 35 drops/10 seconds (approx. 17 ml/minute) by altering the flow to the liver using the roller clamp. The preparation was then covered with tissues soaked in Krebs-Ringer bicarbonate buffer and supported clear of the liver by a wire cage.

The successfulness of the operation was assessed from the rapidity with which the liver cleared to an olive-brown colour with no remaining areas of stagnant blood, and the evenness of the red-brown colour assumed by the liver following addition of the red blood cells.

The liver was allowed to warm up again inside the cabinet for about 5 minutes, before the dudenum was cannulated with plastic tubing ('Portex' size 3.00 mm x 4.00 mm) to allow the free flow of bile from the liver.

The operative procedure used for mouse liver perfusion was based on that used for the rat (Hems et al, 1966) but

modified slightly by Elliott et al, (1971), Salmon et al, (1974) and Ma and Hems (1975).

The mice were anaesthetised with 0.5 ml sodium pentobarbitone ('Nembutal') injected intraperitoneally. Once the abdomen had been opened and the intestines deflected, only two ties were inserted, one round the inferior vena cava just above the junction with the right renal vein and one round the hepatic portal vein. No heparin was injected as the hepatic portal vein was cannulated with a plastic cannula ('Portex' intravenous cannula, 0.75 mm O.D. x 0.5 mm I.D. shortened to about 15 mm and cut to a bevelled point) through which warmed gassed Krebs-Ringer bicarbonate was continuously flowing. This technique ensured that as soon as cannulation was completed clearing of the liver commenced. Immediately after cannulation of the hepatic portal vein, the inferior vena cava was cut through well below the right kidney to allow the excess buffer to flow out. The inferior vena cava was cannulated from the heart end in a similar manner to that used in the rat operation except that a second 'Portex' cannula (1.02 mm O.D. x 0.6 mm I.D. shortened to 15 mm and cut to a bevelled point) was used as the output. Following insertion of the output cannula, the tie round the inferior vena cava just above the right kidney was tied off, so that the effluent buffer had to leave the liver through the output cannula.

All other details of the operation were similar to that of the rat except that a separate reservoir (containing Krebs-Ringer bicarbonate buffer at 37°and 20 mM glucose to prevent loss of glycogen from the liver) was used to clear the liver of stagnant blood (see Fig. 2).

2.4.4. Preparation of samples from perfusions

In recyling perfusions samples of medium were removed from the main perfusion reservoir and mixed with an equal volume of 6% perchloric acid to precipitate the protein. After centrifuging (5,000 g for 5 minutes) the supernatant was used for the determination of glucose or lactate concentration.

In flow-through perfusions or for the determination of glucose output in the mouse liver (by the difference in concentration of glucose in the medium leaving the liver, and that entering) separate samples of medium were taken simultaneously, from the main reservoir, and from the output tube draining the liver.

Samples for the determination of medium specific radioactivity were removed from the main reservoir. The samples were centrifuged at 10,000 xg for 10 minutes, after which the supernatant was transfered to a clean stoppered tube. Supernatants were either processed immediately or stored at -20°.

Samples of liver intended for acetyl-CoA carboxylase assay were freeze-clamped between aluminium tongs which

had been chilled in liquid nitrogen (Wollenberger et al, 1960). The samples were cut and freeze-clamped within 1-2 seconds and then kept below -20° until assayed. Samples were never stored for longer than 7 days before assay.

During the study of the effects of anoxia on the activity of acetyl-CoA carboxylase, serial samples were taken from the perfused rat livers. The first sample was cut from part of the median lobe and freeze-clamped immediately prior to the rest of the lobe being ligatured, with size 3/0 linen thread, to prevent the escape of perfusion medium from the cut surface. The second sample was taken from the left lateral lobe.

Ischaemic samples of perfused rat liver were prepared by clamping off the supply of perfusate to the liver immediately after the first sample (zero-time) had been freeze-clamped. Ischaemic samples were then taken at 0.5, 1 and 2 minute intervals from each liver.

Serial samples were not taken from perfused mouse livers during hormone studies, since it was found in control perfusions, that samples taken after the initial sample had lowered acetyl-CoA carboxylase activity. Time courses of hormone effects in perfused mouse livers were therefore obtained by carrying out one perfusion for each observation.

Liver samples taken for all other purposes were cut and dropped into liquid nitrogen, and then stored below -20°.

2.5. Extraction and separation of lipids from tissues

2.5.1. Extraction methods (see Fig. 4)

Total lipids were extracted from tissues by the method of Folch *et al* (1957), as follows.

Frozen liver and adipose tissue samples were weighed and homogenised in glass-stoppered tubes with 20 volumes (w/v) of chloroform:methanol (2:1, v/v) using a 'Vortex' mixer. The homogenate was allowed to stand overnight at room-temperature before being filtered under vacuum. The filtrate was shaken with 0.2 volumes of 0.1 M KCl and then centrifuged at 1,000 xg for 5 minutes to aid separation into two clear phases. The upper phase was discarded and the lower phase was washed with 0.2 volumes of synthetic upper phase (chloroform:methanol:0.1 M KCl:water, 3:48:47:1 by volume). After separation into two clear phases, the upper phase was discarded and the lower chloroform phase was evaporated to dryness, under a constant stream of nitrogen, on a sand bath at 70-80°. The dry total lipid extract was used immediately or stored in chloroform at 4° in the dark to reduce oxidation of polyunsaturated fatty acids.

2.5.2. Separation of lipid classes by thin-layer chromatography

Lipid classes were separated by thin-layer chromatography according to the method of Freeman and West (1966). Lipid extracts (up to 250 mg) prepared by the method of

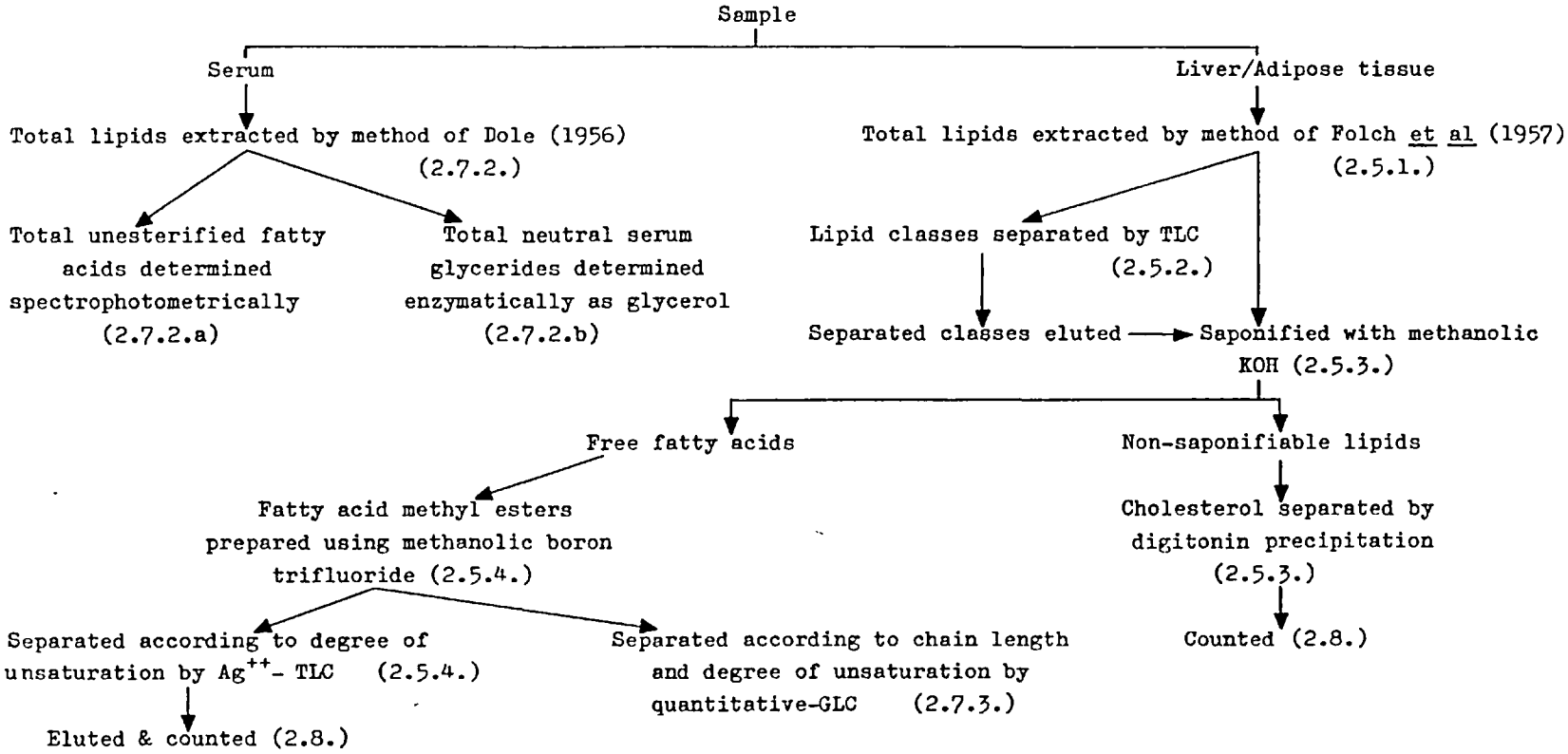

Fig. 4 Extraction and analysis of lipids from tissues.

Folch et al (1957) (see 2.5.1.), were dissolved in chloroform:methanol (2:1, v/v) and applied as a line about 2 cm from the bottom edge of 20 cm square, glass TLC plates spread with a 0.25 mm thin-layer of silica gel 'G', pre-activated by heating for 60 minutes at 105°. The plates were developed by ascending chromatography in a double solvent system. The plates were allowed to run in the first solvent system (diethyl ether:toluene:ethanol: glacial acetic acid, 50:40:2:0.2 by volume) until the solvent front had risen to about 15 cms above the bottom of the plate. The plates were air dried and developed in the second solvent system (n-hexane:toluene, 94:4, v/v) in the same direction until the solvent front was within 1 cm of the top of the plate. The plate was air dried and lightly sprayed with 0.1% (w/v) rhodamine 6 G in methanol, the lipid bands were then identified under U.V. light at 254 nm. The phospholipids were eluted from the origin with chloroform:methanol:water (100:50:1, by volume), all other lipids were eluted with petroleum ether (boiling range 40-60°):diethyl ether:formic acid (50:50:1, by volume) (Bickerstaffe and Annison, 1970). Eluted lipids were either saponified and the fatty acids counted for radio-activity or further separated by other techniques (see Fig. 4).

Recovery of radio-activity by this procedure was at least 85% of that present in total fatty acids counted directly after saponification.

2.5.3. Saponification of lipid classes and separation of cholesterol

Esterified long-chain fatty acids in dried lipid extracts of tissues were hydrolysed in alkaline solution to release their constituent fatty acids. To less than 50 mg dry lipid extract (equivalent to about 0.5 g fresh liver) in a greased, glass stoppered tube,was added 2 ml 30% NaOH and 1 ml ethanol. After heating at 80-90% for 3 hours in a water bath, 5 ml of distilled water was added and the saponified extract allowed to cool. The non-saponifiable lipid fraction, comprising mainly free sterols, was extracted with three washes of 8 ml petroleum ether (boiling range 40-60°). The development of two phases was assisted by centrifugation at 1,000 xg for 5 minutes. The upper petroleum ether washes were pooled in a clean tube ready for separation of cholesterol. The lower phase which contained the fatty acid soaps was acidified with concentrated HCl in the presence of phenolphthalein indicator and the fatty acids so released were extracted with 3 washes of 8 ml petroleum ether (boiling range 40-60°). The petroleum ether extract was transfered to a clean tube after centrifuging as above. The petroleum ether extracts were evaporated to dryness,either under a constant stream of nitrogen,on a sand bath,at 70-80°,or under vacuum on a rotary evaporator at 30-40°.

The dry fatty acid fraction was dissolved directly in 10 ml liquid scintillation fluid (8 g Butyl-PBD in 1 litre toluene) for counting of radio-activity or further

separated by argentation-TLC or GLC (see sections 2.5.4. and 2.7.3.).

3-β-hydroxysterols were separated from other non-saponifiable lipids by digitonin precipitation using the method of Sperry and Webb (1950) and Brunengraber et al (1973). The dried non-saponifiable lipid extract from up to 1 g fresh liver was dissolved in 5 ml acetone:ethanol (1:1, v/v), acidified with 1 drop 10% (v/v) acetic acid and precipitated with 2 ml 0.5% (w/v) digitonin (BDH Chemicals Ltd., Poole, Dorset, U.K.) in 50% (v/v) ethanol. At least 4 hours at room-temperature was allowed for complete precipitation of the sterols, after which the precipitate was sedimented by centrifuging at 10,000 xg for 10 minutes and washed with 6 ml acetone:diethyl ether (1:2, v/v) followed by 6 ml diethyl ether. The washed precipitate was dried, taken up in 1 ml methanol and counted in 10 ml liquid scintillation fluid. Contamination of the cholesterol by radioactive fatty acids with this method is of the order of 1.0% (Brunengraber et al, 1973).

2.5.4. Preparation and separation of fatty acid methyl esters

Free fatty acids were methylated with methanolic boron trifluoride to produce volatile fatty acid methyl esters (FAME).

Free fatty acid extracts were dried under vacuum in glass-stoppered tubes at 30-40°. The fatty acids were methylated with 1 ml 15% (w/v) boron trifluoride in

methanol at 90° for 5 minutes. When cool 5 ml water was added to each tube and the FAME were extracted 3 times with 8 ml petroleum ether (boiling range 40-60°). The petroleum ether extracts were washed twice with water and then dried with anhydrous Na_2SO_4. The petroleum ether was removed under a continuous stream of nitrogen with gentle heating and the FAME were taken up in a known volume of diethyl ether. FAME were separated by gas-liquid chromatography (see section 2.7.3.) and by argentation- TLC as follows.

Argentation- TLC

FAME were separated by argentation-TLC according to their degree of unsaturation (Malins, 1966 and Dunn and Robson, 1965).

Silica gel G was made up in 10% (w/v) aqueous $AgNO_3$ and spread in 0.25 mm thick layers on 20 cm square, glass TLC plates. The plates were dried in the dark, activated at 110° for 90 minutes, and pre-run in diethyl ether before use. The plates were kept in the dark as much as possible to reduce silver oxide formation. FAME were spread on the plates in a narrow band approximately 2 cm above the bottom of the plate and the plates were developed in toluene:n-hexane (1:1 v/v). The plates were air dried and lightly sprayed with 0.1% rhodamine 6 G in methanol. The bands were quickly identified under U.V. light at 254 nm by comparison with standard spots. At least four bands were usually identifiable, corresponding with saturated,

monoenoic, dienoic and polyenoic FAME. The FAME had higher Rf the more unsaturated they were, so that saturated FAME were found just behind the solvent front and polyenoic FAME near the origin.

Bands of separated FAME were scraped off the plates, eluted with diethyl ether into scintillation vials, dried under a continuous stream of nitrogen at 60-70^{o} on a sand bath and were counted with 10 ml liquid scintillation fluid (see section 2.8.).

2.6. Measurement of rates of de novo synthesis of lipids by incorporation of radioisotopes

Measurement of the total rates of fatty acid synthesis by following the incorporation of hydrogen isotopes from their oxides into long-chain fatty acids was first introduced by Schoenheimer and Rittenberg (1937). The method relies on the stability of the carbon-hydrogen bonds of fatty acids to proton exchange, such that, incorporation of hydrogen only occurs during fatty acid synthesis (Van Heynigen et al, 1938).

Total rates of lipogenesis have been measured in the present work by the incorporation of ^{3}H from $^{3}H_2O$ into fatty acids and cholesterol. The quantity of fatty acid or cholesterol synthesised was calculated from the relative total activity (RTA) with a correction for the isotope discrimination effect of ^{3}H compared to ^{1}H. The relative total activity was calculated as follows:-

$$RTA = \frac{DPM\ ^{3}H \text{ in tissue fatty acids or cholesterol/g wet tissue}}{DPM\ ^{3}H/\mu g \text{ H in serum or perfusate water}}$$

The isotope discrimination effect for incorporation of ^{2}H into fatty acids was measured by Bernhardt and Schoenheimer (1940), who maintained the body water of mice on a fat free diet at a constant deuterium content of 1.5% by giving them $^{2}H_2O$ in their drinking water for 3 months. By the end of that period the deuterium content of the fatty acids had equilibrated at 43% of that in the body water. In the

present work a factor of 0.5 has been used since Jungas (1968) suggested that isotopic equilibrium might not have been reached in the above experiments. In experiments with both $^{3}H_2O$ and $^{2}H_2O$ present, a preferential incorporation into long-chain fatty acids of ^{2}H over that of ^{3}H by a factor of 1.19 was found (Eidinoff et al, 1953 and Jungas, 1968). By combining these two factors a discrimination factor for the incorporation of ^{3}H from $^{3}H_2O$ into long-chain fatty acids compared to ^{1}H from $^{1}H_2O$ was calculated thus 1.19/0.5 = 2.38 (Windmuller and Spaeth, 1966). The validity of these calculations has been discussed by Brunengraber et al (1972), Wadke et al (1973) and Salmon et al (1974). The use of $^{3}H_2O$ for measuring total rates of lipogenesis appears to be valid unless an excess of reducing substrates such as lactate is present (Clark et al, 1974).

The total fatty acid synthesised was calculated, as acetyl units incorporated, by dividing the RTA by 4/2.38 = 1.68 since there are 4 hydrogen atoms in each C_2-unit and expressed as μmoles C_2-units/g wet tissue.

The amount of cholesterol synthesised was calculated using similar considerations since Brunengraber et al (1972) demonstrated a common precursor pool of acetyl-CoA for both cholesterol and long chain fatty acid synthesis. These workers found similar $^{14}C/^{3}H$ ratios for sterols and fatty acids when [^{14}C]-glucose and $^{3}H_2O$ were provided simultaneously as precursors. The RTA was divided by 1.4 to calculate C_2-units of cholesterol synthesised since there are less than 4 hydrogen atoms per C_2-unit in cholesterol.

The amount of fatty acid or cholesterol synthesised from ^{14}C labelled glucose or lactate was calculated by dividing the quotient:-

$$\frac{\text{DPM } ^{14}C \text{ incorporated into fatty acid or cholesterol/g wet tissue}}{\text{DPM } ^{14}C/\mu\text{g atom glucose or lactate carbon}}$$

by 2 to give the number of acetyl units incorporated (since there are 2 carbon atoms per acetyl unit).

(a) Measurement of the lipogenic rate in perfused livers

The perfused livers were allowed to stabilize for one hour before 10 mCi of $^{3}H_2O$ were added as a single dose (usually 50 microlitres of 200 mCi/ml $^{3}H_2O$). In some experiments the incorporation of ^{14}C into fatty acids from [U-^{14}C]labelled D-glucose or L-lactate was used to measure the contribution of glucose, or lactate, to fatty acid synthesis simultaneously with the measurement of the total rate of fatty acid synthesis by the incorporation of ^{3}H from $^{3}H_2O$. In these experiments 10-12 μCi of [U-^{14}C]labelled D-glucose or L-lactate were added together with the $^{3}H_2O$. After 2 minutes had elapsed to allow for complete mixing of the isotopes in the medium pool a sample was removed for determination of perfusate specific radioactivity. One or two hours after the addition of isotopes a liver sample was taken and frozen in liquid nitrogen for extraction of fatty acids and glycogen. The incorporation of ^{3}H from $^{3}H_2O$ and ^{14}C from [U-^{14}C]-glucose or [^{14}C]-L-lactate into fatty acids

is approximately linear for 3 hours (Salmon et al, 1974).

(b) Measurement of the lipogenic rate in vivo

Rats were injected intraperitoneally or intravenously, through the tail vein, with 10 mCi of $^{3}H_2O$ alone or 10 mCi of $^{3}H_2O$ and 12μCi of [U-^{14}C]-D-glucose. Doses were made up in normal saline to a total volume of 0.2 ml for I.P. injections or 0.1 ml for I.V. injections. After one hour the rats were stunned by a blow on the head and a sample of blood was removed for determination of SRA. Liver and adipose tissue samples were taken for lipid extraction.

2.7. Analytical techniques

2.7.1. Determination of glycogen, glucose and lactate

(a) Glycogen

Glycogen was determined as glucose following hydrolysis by amyloglucosidase. About 0.5 g of crushed frozen liver was boiled in 10 volumes of aqueous 30% (w/v) potassium hydroxide for about two minutes until completely dispersed. The sample could then be stored and boiled later or boiled immediately for a further 30 minutes, after which the glycogen was precipitated by the addition of 3 volumes of cold ethanol (Good et al, 1933). After standing overnight at 4°, the glycogen was sedimented by centrifugation at 4° and 20,000 xg for 15 minutes. The pellet was resuspended in 10 ml distilled water, using a motor driven glass pestle. A sample of the glycogen suspension containing between 0.05 and 0.3 μ moles of glycogen-glucose (usually 10 or 50 microlitres) was made up to 1 ml with 25 mM sodium acetate pH 4.8 and hydrolysed with 30 microlitres of amyloglucosidase at 37° for 1 hour (Lee and Whelan, 1966). The complete 1 ml sample was then used for glucose estimation (see below). The recovery of added glycogen by this procedure was 98%.

(b) Glucose

Glucose was determined by the glucose oxidase method of Krebs et al (1964), modified after Trinder (1969). A sample of perchloric acid extracted perfusion medium, containing 0.05-0.3 μ moles glucose, made up to 1 ml with

water or the 1 ml of glycogen digest (see above) was mixed with 3 ml of colour reagent (10 ml phenol reagent; 3 g NaCl and 0.334 g phenol made up to 100 ml with distilled water, 1 ml 3.33% (w/v) aqueous 4-aminophenazone solution, 13 mg glucose oxidase, 5 mg peroxidase, made up to 100 ml with 0.5 M sodium phosphate pH 7.3 containing 0.1 M Tris) and incubated at 37° for 30 minutes. The resulting purple coloration was read within 30 minutes in a spectrophotometer at 515 nm against a reagent blank. Glucose standards were run with each batch of samples.

(c) Lactate

L-lactate concentration was determined enzymatically in PCA extracts of perfusate by the method of Hohorst (1963). A 10 microlitre sample of neutralized PCA extract of perfusate (6% PCA:perfusate, 1:1 v/v) containing less than 25 μmol L-lactate was added to a spectrophotometer cuvette of 1 cm light path containing 0.6 mmol hydrazine sulphate, 1 mmol glycine, 15 μmol EDTA, and 3 μmol NAD^+ at pH 9.6 to produce a final assay volume of 3.11 ml. After mixing, the cuvette was allowed to stand until a constant OD 340 nm was obtained, 10 microlitres lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase from pig heart, 1 mg/ml, 360 U/mg) was then added and the cuvette again allowed to stand (following mixing) until a constant OD 340 nm was obtained. Since the molar extinction coefficient of NADH was known to be 6.22 in a 1 cm light path and assuming that each 1.0 μmol of L-lactate caused the reduction of 1.0 μmol of NAD^+ the

amount of L-lactate in the cuvette was calculated from the ΔOD 340 nm. With the total assay volume in the cuvette of 3.11 ml, 0.1 μmol L-lactate gave ΔOD 340 nm of 0.2. Blank cuvettes with L-lactate omitted were subjected to the same procedure and any ΔOD 340 nm was subtracted from the ΔOD 340 nm for the samples (and standards) before calculating the amount of L-lactate. Linearity of reaction with increasing concentration of L-lactate was checked with standard L-lactate.

2.7.2. Determination of serum lipid concentrations

(a) Unesterified long-chain fatty acids

Spectrophotometric determination of serum unesterified fatty acid concentration was carried out following reaction of the copper soaps with diethyldithiocarbamate to produce a yellow colour, the method used being essentially as described by Lauwerys (1969). Serum lipids were extracted in a glass-stoppered tube with 20 volumes modified Doles' extraction reagent (isopropanol:n-heptane:2 N H_2SO_4, 40:10:1), usually 0.5 ml serum with 10 ml Doles' reagent. Two phases were then produced by the addition of 6 ml n-heptane and 4 ml distilled water to every 10 ml of modified Doles' extraction reagent. After shaking, 5 ml of the upper phase was mixed with an equal volume of 0.1 N H_2SO_4 in a clean glass-stoppered tube and shaken automatically for at least 2 minutes. 3 ml of the upper phase was transfered to a clean glass-stoppered tube

containing 3 ml chloroform and 3 ml copper reagent (3.25 g $Cu(NO_3)_2.3H_2O$, 6.25 g K_2SO_4, 17.0 g Na_2SO_4, 7.0 ml triethanolamine and 0.3 ml glacial acetic acid made up to 100 ml with distilled water). After a further 2 minutes shaking, the tube was centrifuged at 10,000 xg for 10 minutes. 3 ml of the upper phase (containing the copper soaps) was mixed in a clean tube with 0.5 ml freshly prepared diethyldithiocarbamate (DDC) reagent (0.1% w/v sodium DDC in n-butanol). The yellow colour produced was read against a reference blank of chloroform:n-heptane:DDC reagent (3:3:1) at 440 nm. Standards diluted from a stock solution of palmitic acid (2 μmol/ml; 12.8 mg palmitic acid in 25 ml, 100 mM Tris buffer pH 8.5 containing 7% fatty acid free bovine serum albumin, see 2.2) were taken through the above procedure together with a blank of buffered 7% fatty acid free albumin solution to give a calibration curve. The increase in OD 440 nm with increasing concentrations of palmitic acid was linear from 0.001 μmol/ml to 2 μmol/ml.

(b) Neutral glycerides

Total serum neutral glycerides were determined enzymatically as glycerol, essentially as described by Pinter et al (1967). Lipids were extracted from serum with 25 volumes of Dole's extraction reagent (see previous section). An aliquot of the upper phase (containing less than 1.0 μmol glyceride-glycerol) was dried in a clean glass-stoppered tube on a sand tray at 70-80° under a

stream of nitrogen, prior to saponification at 70° for 30 minutes with 0.5 ml of freshly made alcoholic KOH (absolute alcohol:33% KOH, 47:3; Albrink, 1959). Free fatty acids were precipitated as the magnesium salts by the addition of 1 ml, 0.15 M $MgSO_4$. An aliquot of the supernatant containing less than 0.4 μmol glycerol was made up to 1.1 ml and added to a cuvette containing 2 ml of assay mixture (0.1 ml, 2 mg/ml pyruvate kinase; 0.1 ml, 0.5 mg/ml lactate dehydrogenase; 0.25 ml, 75 mM ATP; 1 ml,2 mM phosphoenol pyruvate and 1 ml, 2.5 mM NADH made up to 20 ml with 0.1 M triethanolamine-HCl pH 7.6 containing 6 mM $MgSO_4$ and 2 mM KCl). After a steady rate of drift was attained reactions were initiated by the addition of 10 λ, 0.5 mg/ml glycerokinase. Since it was known that each 1.0 μmol glycerol in the cuvette caused the oxidation of 1.0 μmol NADH according to the reaction sequence:-

i) Glycerol + ATP $\xrightarrow{\text{glycerokinase}}$ ∝glycerol phosphate + ADP

ii) ADP + phosphoenol pyruvate $\xrightarrow{\text{pyruvate kinase}}$ pyruvate + ATP

iii) Pyruvate + NADH + H^+ $\xrightarrow{\text{lactate dehydrogenase}}$ lactate + NAD^+

and the molar extinction coefficient of NADH at 340 nm with a 1.0 cm light path was 6.22, the number of μmol glycerol could be calculated from the ΔOD 340 nm. With the total volume in the cuvette of 3.11 ml, 0.1 μmol glycerol gave ΔOD 340 nm of 0.2. Assay mixtures were checked with standard glycerol solution and blanks were run with each

batch. Recovery of triolein added to the serum was between 95 and 105%.

Endogenous serum glycerol was excluded at the Dole's extraction step, and extra glycerol added to the serum had no effect on the assay result.

Phospholipids are hydrolysed to 1- and 2-glycerophosphates under the saponification procedure used, and glycerokinase shows no activity on these products (Eggstein, 1966). No change in OD 340 nm occured when phospholipid was treated as above.

2.7.3. Separation and analysis of fatty acid methyl esters (FAME) by gas-liquid chromatography (GLC)

FAME were separated by GLC using a Pye 104 gas chromatograph (Salmon and Hems, 1976). 2-5 µl samples of FAME in diethyl ether were injected into a 2.44 m x 5.0 mm glass column packed with 10% (w/w) PEGA (polyethylene glycol adipate) liquid phase on Diatomite 'C' solid phase. Separation was carried out isothermally at 190° with argon carrier gas flowing at 60 ml/minute. Emerging FAME were detected using flame ionization detection (FID) and identified by comparing their retention times with those of authentic FAME standards. The detector was supplied with hydrogen at 60 ml/minute and air at >200 ml/minute to maintain the temperature at 250°.

The linearity of response of the FID was established by injecting known quantities of FAME standards. Constant peak area/weight responses ($\pm$ 1%) were found for 16:0-18:3 and correction factors were determined for FAME of greater chain length.

Results were quantified by the addition of internal standards of heptadecanoic acid and triheptadecanoyl glycerol (6 mg/g wet liver and 1.2 mg/g wet liver respectively) to liver samples before homogenising.

2.8. Determination of radioactivity in lipid and aqueous samples by liquid scintillation counting

(a) ^{14}C or ^{3}H alone

Dried lipid samples were dissolved directly in 10 ml of liquid scintillation fluid (8 g Butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4 diazole] in 1 l toluene) in a glass scintillation vial ready for counting. Aqueous samples (never more than 0.1 ml of water) were mixed with 10 ml liquid scintillation fluid and sufficient 2-methoxyethanol to produce a single phase system.

The vials were counted at 7^{o} in a Packard Tri-Carb liquid scintillation spectrometer. The DPM present in each vial were calculated from the recorded CPM by a channels ratio method (Baillie, 1960). The method used two channels, one recording the counts over the whole energy spectrum of the radioisotope, and the other only recording counts at the lower energy end (see Fig. 5).

A series of standard vials containing liquid scintillation fluid, 2-methoxyethanol and ^{14}C or ^{3}H labelled hexadecane, quenched with various amounts of water, were used to produce a graph of counting efficiency vs channels ratio. The counting efficiencies of experimental vials were determined from this standard curve using the channels ratio of the counts obtained for each sample. The efficiency of ^{14}C counting was usually 70-75% for lipid samples and 50-65% for aqueous samples. The efficiency of ^{3}H counting was approximately 30-35% for

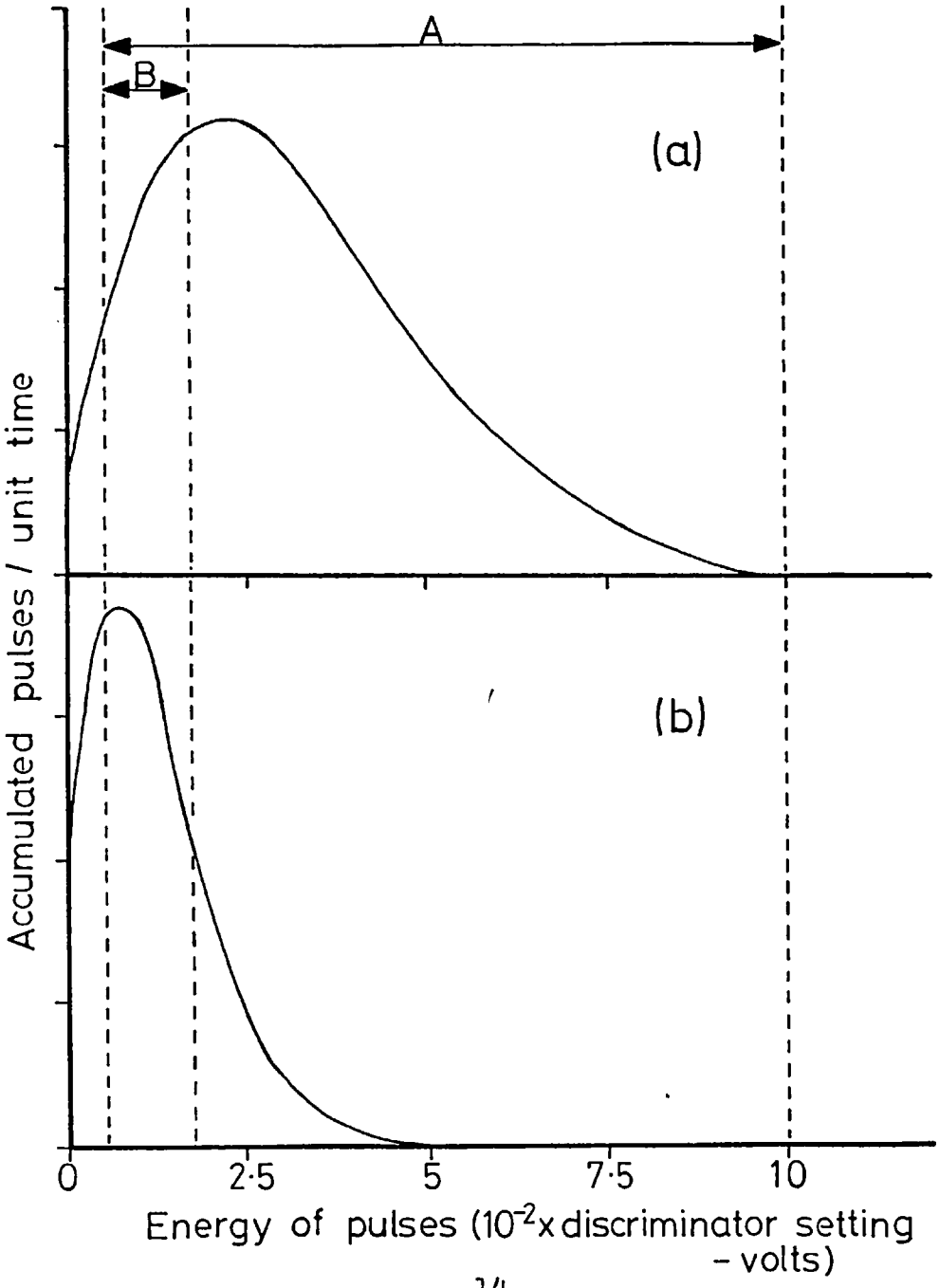

Fig. 5 Energy spectrum of ^{14}C and discriminator settings used to calculate counting efficiency.

(a) Shows the unquenched energy spectrum of ^{14}C and the discriminator settings used to determine the efficiency of counting (see 2.8.). (b) Quenching of the sample with water caused the spectrum to be compressed into the lower energy range. This led to an increase in the proportion of counts in channel 'B' and a decrease in the total number of counts recorded (channel 'A').

lipid samples and 20-25% for aqueous samples.

(b) ^{14}C and ^{3}H together (double label)

Samples containing ^{14}C and ^{3}H together were prepared for counting as above. The method used for counting ^{14}C and ^{3}H simultaneously was essentially as described by Hendler (1964). The discriminators of the Packard Tri-Carb liquid scintillation spectrometer were set to count the scintillations due to β-particles in three different energy ranges or channels (see Fig. 6).

Two of these channels ('A' & 'B') were selected to exclude 99.9% of all tritium counts, and to record the higher energy emissions from ^{14}C. The settings for these two channels were also selected so that the ratio of counts from an unquenched ^{14}C sample was 0.3

i.e. $\frac{\text{CPM in channel 'B'}}{\text{CPM in channel 'A'}} = 0.3 = R_1$.

Using the channels ratio method the counts recorded in these two channels were used to calculate the efficiency of ^{14}C counting and the proportion of counts recorded in the third channel (channel 'C') which were due to ^{14}C. By subtracting the number of counts in channel 'C' calculated to be due to ^{14}C from the total count and then correcting for the efficiency of counting ^{3}H, the DPM due to ^{3}H present in the samples could be calculated.

A set of standard vials were set up containing equal quantities of ^{14}C and/or ^{3}H labelled hexadecane and variably quenched with chloroform. Using the counts from

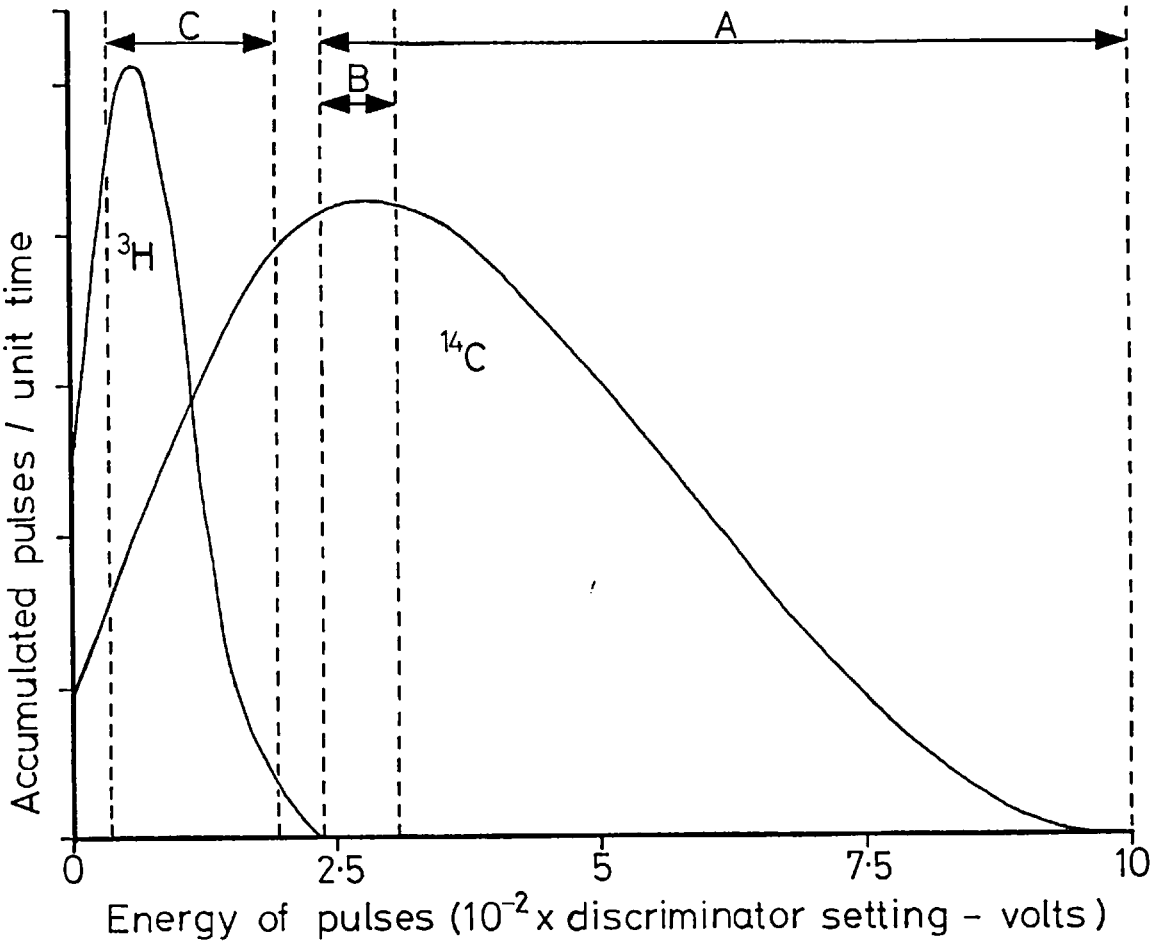

Fig. 6 Discriminator settings for simultaneous determination of ^{3}H and ^{14}C in samples.

The energy spectra of ^{14}C and ^{3}H are shown together with the channels used for counting the two isotopes simultaneously (see section 2.8.). Channels A & B were selected to exclude 99.9% of all counts due to tritium. The settings for these two channels were also selected so that the ratio of counts recorded from an unquenched ^{14}C sample was 0.3 (i.e. CPM in channel B/CPM in channel A = 0.3). Channel C was set to record low energy ^{14}C pulses and pulses due to ^{3}H.

these standard vials three graphs were plotted to show the relationships between

1. R_1 and the ^{14}C counting efficiency.
2. R_2 $(= \frac{\text{CPM in channel C}}{\text{CPM in channel A}})$ and the ^{14}C counting efficiency
3. R_1 and the efficiency of counting ^{3}H

(see Fig. 7)

Equations were fitted to these curves and a computer programmed to calculate the DPM and counting efficiencies of unknown samples. The standard vials were counted with each set of samples and the values obtained were fitted into the equations by the computer prior to calculating the DPM of the samples. The efficiency of counting ^{14}C and ^{3}H by this method was 35-40% and 25-30% respectively for lipid samples and 20-25% for both ^{14}C and ^{3}H for aqueous samples. The validity of the method was checked by recounting some samples after the addition of internal standards of ^{14}C or ^{3}H hexadecane. The method was not found to be accurate for highly quenched samples, that is when R_1 exceeded 0.6. Counts where R_1 exceeded 0.6 were therefore discarded.

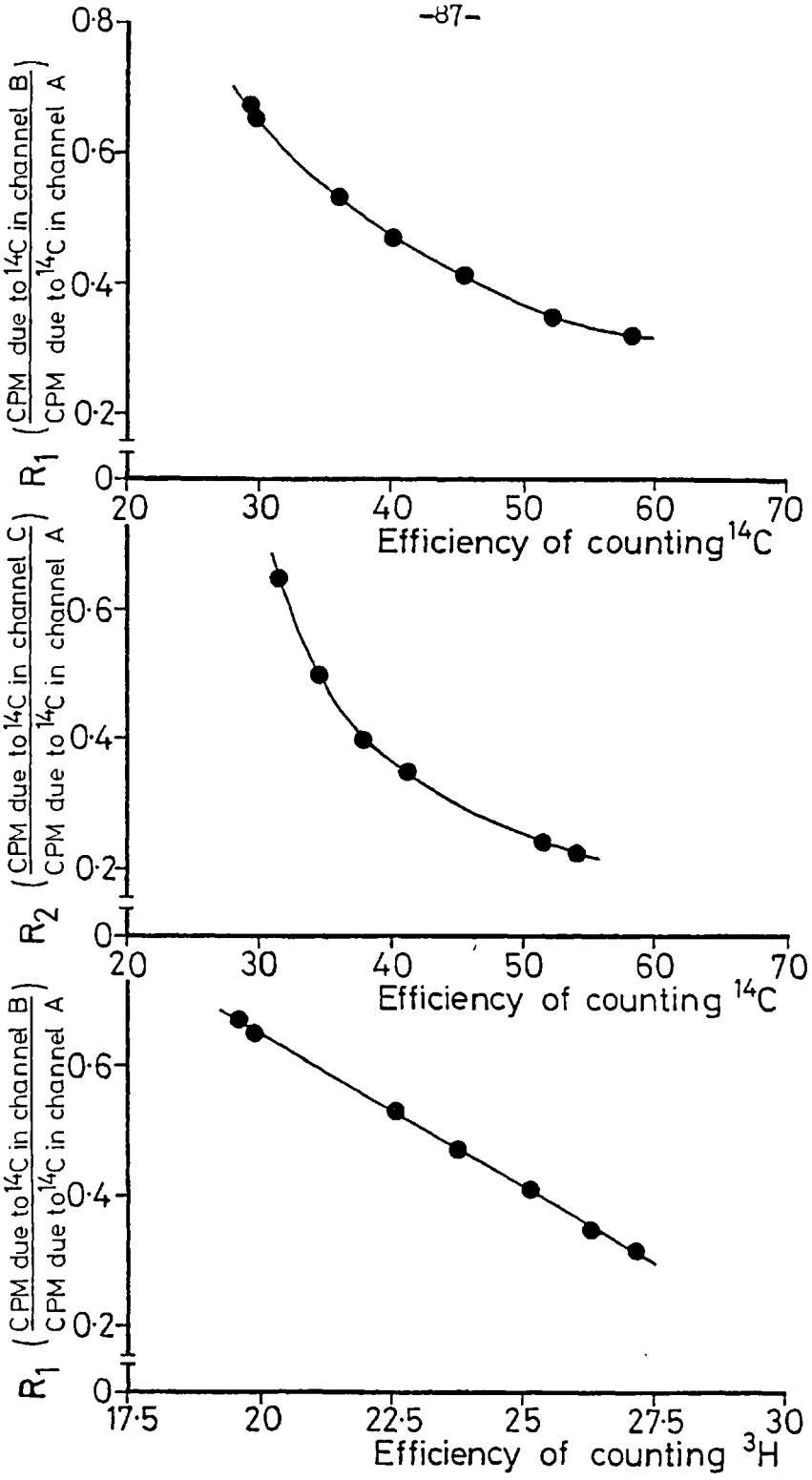

Fig. 7 Calibration curves for the simultaneous determination of ^{14}C & ^{3}H by liquid scintillation spectrometry.

The counts from a set of standard scintillation vials containing equal quantities of ^{14}C and/or ^{3}H were used to determine the relationship between (a) R1 (CPM due to ^{14}C in channel B/CPM due to ^{14}C in channel A) and the efficiency of counting ^{14}C, (b) R_2 (CPM due to ^{14}C in channel C/CPM due to ^{14}C in channel A) and the efficiency of counting ^{14}C and (c) R_1 and the efficiency of counting ^{3}H. Equations were fitted to these curves and a computer programmed to calculate the efficiencies of counting and the DPM due to ^{14}C and ^{3}H in unknown samples (section 2.8.).

2.9. Assay of acetyl-CoA carboxylase [E.C. 6.4.1.2., acetyl-CoA:CO_2 ligase (ADP)]

2.9.1. Preparation of samples

Tissue required for the assay of acetyl-CoA carboxylase activity was freeze-clamped between aluminium tongs, chilled in liquid nitrogen, and stored below -20°.

Rats or mice were killed by stunning and cervical dislocation, the abdomen was opened by a transverse incision and a piece of liver cut off and freeze clamped within 10 seconds. Liver samples which took longer than 10 seconds to freeze-clamp were discarded. Ischaemic liver samples were prepared by rapidly severing the blood supply to the liver after the first (zero-time) sample had been freeze-clamped. The severed liver was left in situ to remain at 37°, pieces being cut off and freeze-clamped at 0.5, 1 and 2 minute intervals after stunning. In rats it was possible to obtain four samples from each liver (i.e. one zero-time and three ischaemic). The mouse liver however was too small to provide more than two samples, so a zero-time sample and one ischaemic sample was obtained from each liver.

2.9.2. Assay procedure

Quick frozen liver samples were homogenised in 100 mM potassium phosphate pH 7.4, containing EDTA 0.5 mM, bovine serum albumin (fatty acid-free) 5 mg/ml, and dithioerythritol

(DTE) 2 mM, with twenty passes of a motor driven pestle in a Potter-Elvehjem homogeniser at 0-4°.

For most assays homogenates were centrifuged at 38,000 xg and 4° for 60 minutes. Following removal of the fat-cake the supernatant was transferred to a clean plastic tube. The supernatants were then divided into two parts, one assayed without further treatment (initial activity) and the other incubated at 37° for 30 minutes in the presence of 10 mM potassium citrate and 15 mM magnesium chloride (10 microlitres each of 1 M and 1.5 M stock solutions respectively added to 1 ml supernatant) to give the total activity. Following incubation the acetyl-CoA carboxylase activity was assayed immediately.

For some assays (where indicated) homogenates were centrifuged at 41,000 xg for 1 minute at 4°, the fat-cake was rapidly removed and an aliquot of the supernatant used immediately for assay of the initial activity. By this procedure the initial activity was assayed within 6 minutes of starting to homogenise the sample. The total enzyme activity was assayed after treatment with potassium citrate and magnesium chloride as described above.

Acetyl-CoA carboxylase was assayed by measuring the incorporation of $^{14}CO_2$ from $KH^{14}CO_3$ into malonyl-CoA by a modification of the method of Martin and Vagelos (1962) after Halestrap and Denton (1973) and Carlson and Kim (1974 a & b). To 0.45 ml of reaction mixture in a stoppered plastic tube was added 50 λ of liver homogenate high speed

supernatant to initiate the reaction. The reaction mixture contained 10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM potassium citrate, 5 mg/ml fatty acid free bovine serum albumin, 0.5 mM EDTA, 2 mM DTE, 5 mM ATP, 0.15 mM acetyl-CoA and 15 mM $KH^{14}CO_3$ (approximate specific activity 0.75 μCi/1 μmole). Following incubation at 37° for 3 minutes the reaction was terminated by the addition of 0.2 ml of 5 M HCl. Precipitated proteins were pelleted by centrifuging at 10,000 xg for 10 minutes, 0.5 ml of the supernatant was a dried under vacuum below 80° in a scintillation vial, taken up in 0.1 ml water and mixed with 14 ml of scintillation fluid (composition:- 10 volumes toluene containing 8 g butyl-PBD per litre : 3 volumes 2-methoxy ethanol) to produce a single phase system for counting. Tissue blanks were assayed in the absence of acetyl-CoA and the small number of counts incorporated were subtracted from the sample counts. Each sample had its initial, total and tissue blank activity measured, and counts were corrected for quenching before calculation of activities.

2.9.3. Factors affecting the assay of acetyl-CoA carboxylase

The assay of acetyl-CoA carboxylase is limited by several factors including the apparent reduction in the rate of incorporation of ^{14}C which occurs a few minutes after initiation of the reaction. This non-linearity of incorporation is probably in part due to the action of

malonyl-CoA decarboxylase (Nakada et al, 1957), which uses the end product of the acetyl-CoA carboxylase reaction as its substrate and releases the $^{14}CO_2$ incorporated during the assay. In this study the finding of Halestrap and Denton (1973) for rat adipose tissue acetyl-CoA carboxylase, that the incorporation of $^{14}CO_2$ was linear for the first 3 minutes, was confirmed for both the initial and total activities of the rat liver enzyme (Fig. 8).

Inhibition of acetyl-CoA carboxylase by high concentrations of one of the substrates, ATP, has been demonstrated previously (Carlson and Kim, 1974 b). Under the assay conditions used in this study the activity of the enzyme was maximal at ATP concentrations of between 2.5 and 5 mM. 4.5 mM was chosen for the substrate concentration in most assays since utilization of nearly 70% of the available ATP would only effect the rate of reaction by a maximum of 10% (see Fig. 9).

Since citrate is an important modifier of acetyl-CoA carboxylase activity (Carlson and Kim, 1974 b), the effect of potassium citrate in the assay was investigated. 20 samples from perfused rat livers (8 treated with AVP and 12 control samples) were assayed with and without 10 mM citrate present. No difference in the recorded total activity was found (i.e. after activation with 10 mM potassium citrate and 15 mM magnesium chloride) for any of the samples. The initial activity was increased by about 30%, in the presence of citrate, in both the control and AVP treated livers. Citrate was included in the assay to

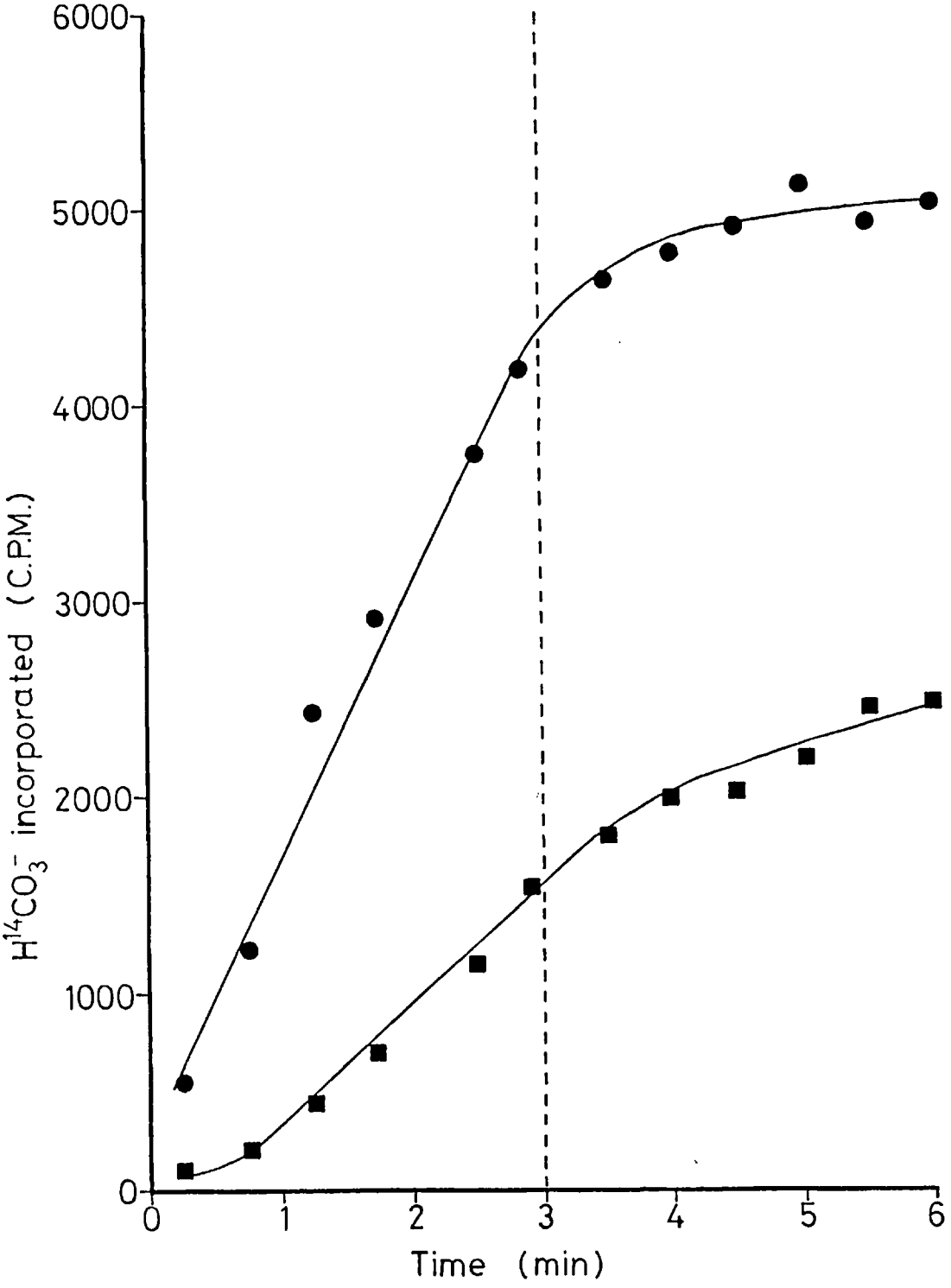

Fig. 8 Incorporation of $^{14}CO_2$ into malonyl-CoA during the assay of acetyl-CoA carboxylase as a function of time.

At various times, as shown, during the assay of acetyl-CoA carboxylase (section 2.9.2.) samples were removed for determination of non-volatile ^{14}C. The counts shown are less those incorporated in the absence of acetyl-CoA, and are the means of two determinations. The reaction was more or less linear for the first 3 min of the reaction, for both the initial (■) and total (●) activities.

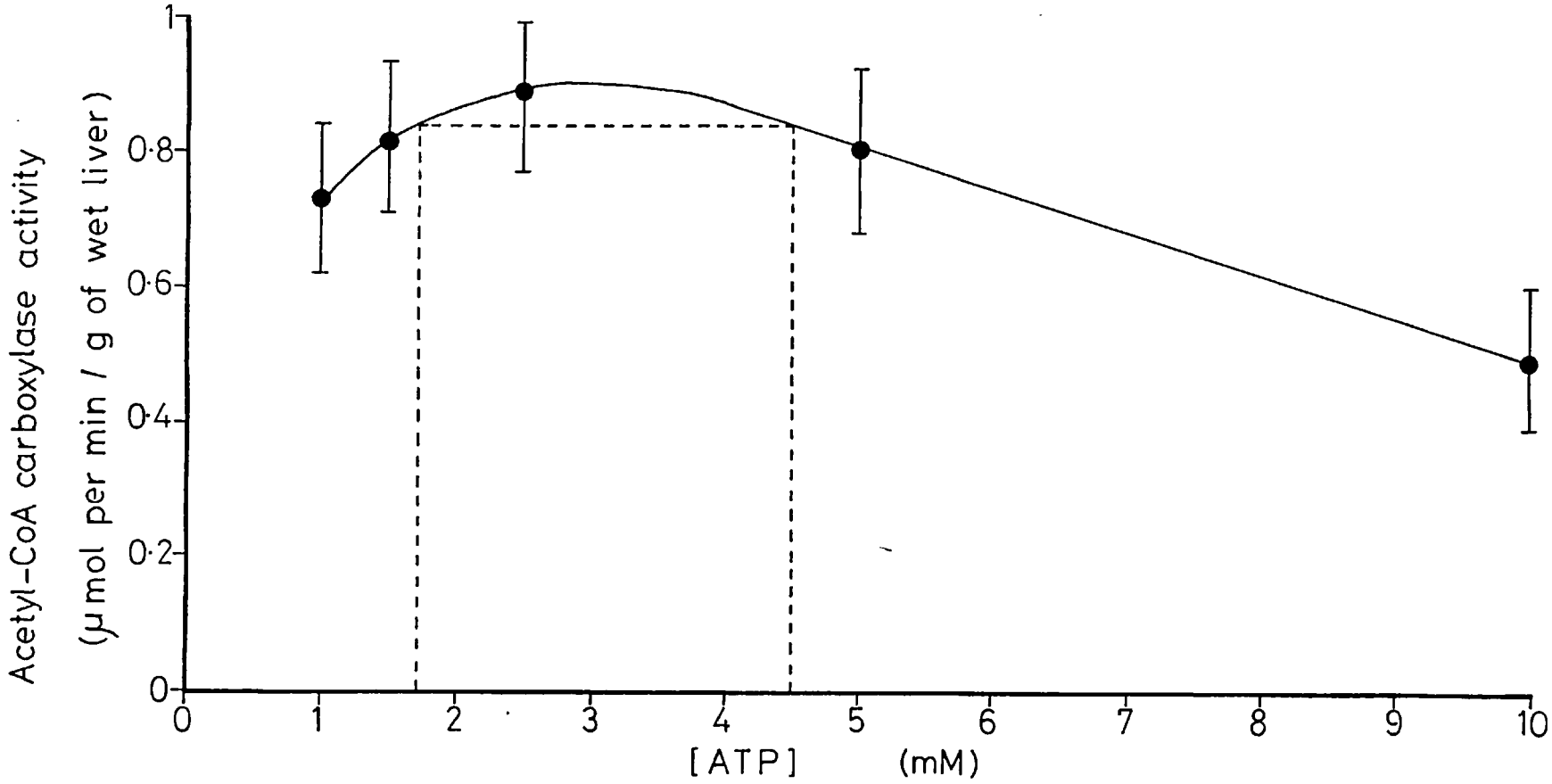

Fig. 9 Influence of ATP concentration on the activity of hepatic acetyl-CoA carboxylase.

The total activity of acetyl-CoA carboxylase was measured in the high speed supernatant of fed rat livers in the presence of various concentrations of ATP. Results are the means ± S.E.M. of 3 observations.

systematise the concentration of this important modifier.

With increasing amounts of liver high speed supernatant the increase in incorporation of $^{14}CO_2$ was proportional to the tissue present at least up to the equivalent of 12 mg fresh liver (see Fig. 10). This is similar to the findings of Halestrap and Denton (1973),in rat adipose tissue. The amount of supernatant added to each tube in the assay was therefore kept to the equivalent of between 4 and 10 mg fresh liver.

To test the effect of delay during the assay of liver acetyl-CoA carboxylase on the activity recorded, a series of liver homogenates were centrifuged at 41,000 xg and 4^{o} for 1 minute and assayed immediately and after 30 and 120 minutes standing in ice. The samples were also assayed by the long assay method (i.e. centrifuged at 38,000 xg and 4^{o} for 60 minutes). Delay during the assay of up to 120 minutes was found to have no significant effect on either the initial or total activity recorded (see Table 1). There was a significant ($P<0.05$) change in the % initial/total activity when the samples were stored in ice for 120 minutes. Samples assayed by the long method were found to have slightly higher initial and total activities than when assayed by the short method but the % initial/total activity remained the same. This may be a reflection of the slightly higher proportion of counts incorporated in the absence of acetyl-CoA in the short assay. Other factors affected by the longer spin may include the removal of mitochondria, with which a large part of the malonyl-CoA

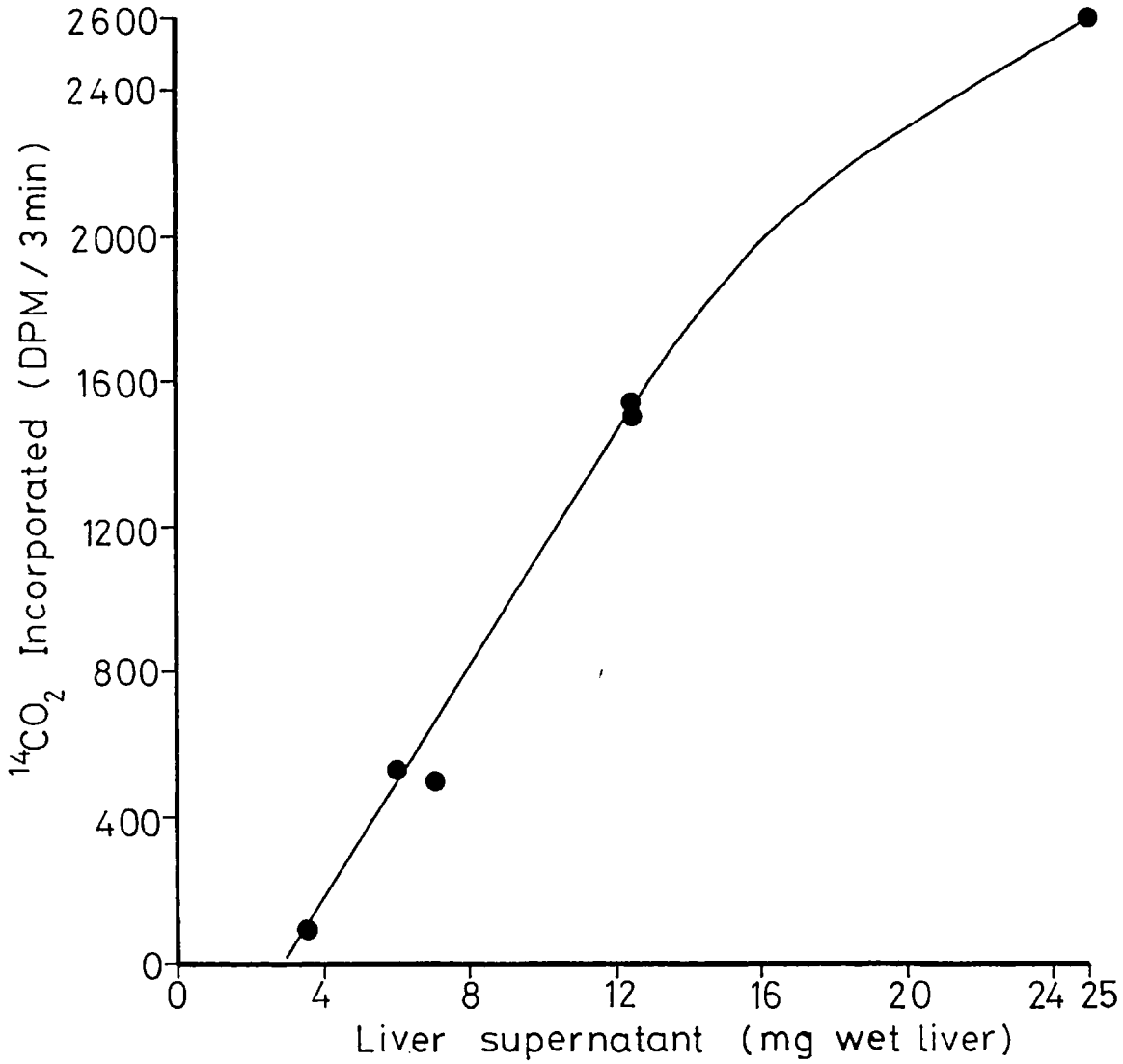

Fig. 10 Relationship between the incorporation of $^{14}CO_2$ by hepatic acetyl-CoA carboxylase and the amount of tissue present.

The incorporation of $^{14}CO_2$ by acetyl-CoA carboxylase was followed as a function of the concentration of rat liver high-speed supernatant. At the end of 3 min the reaction was terminated and the number of DPM incorporated was determined (see 2.9.2. for details). Each point is the mean of two determinations.

Table 1.

Effect of delay during the assay of acetyl-CoA carboxylase

Livers were rapidly homogenised in 9 vols. phosphate buffer pH 7.3 (see 2.9.2.). The homogenate was divided, one part was centrifuged for 1 min and 41,000 xg at 4°, followed by assay of the initial acetyl-CoA carboxylase activity immediately and after standing on ice. The second part of the homogenate was centrifuged for 60 min at 41,000 xg and 4° before the initial enzyme activity was measured. Total activities were measured after 30 min incubation with 10 mM citrate and 15 mM magnesium at 37°. Results are means ± S.E.M. for 3 determinations. Other details are in the text.
* $P<0.01$ for initial activity at 8 and 90 min.

Time delay (min)	Duration of centrifugation at 41,000 xg & 4° (min)	Acetyl-CoA carboxylase activity (μmoles CO_2 incorporated/min/g wet tissue)		
		Initial	Total	% initial/total
8	1	0.36±0.01*	-	-
30	1	0.35±0.01	0.7 ±0.09	51±4.6
120	1	0.31±0.02	0.87±0.09	36±2.1
90	60	0.55±0.04*	0.98±0.08	57±1.2

decarboxylase activity seems to the associated (Nakada et al, 1957). In view of these findings samples were generally assayed by the long method with the minimum of delay. Frozen liver samples were stored at -20° prior to assay for periods up to 7 days without detectable loss of activity.

In early experiments involving hormone treatment of perfused mouse livers serial samples were taken. The samples were cut from the perfused mouse livers immediately before and at various times after hormone treatment, loss of perfusate was prevented by ligaturing the cut lobe. It soon became apparent that the serial biopsy affected the acetyl-CoA carboxylase activity of later samples in the absence of hormone treatment (see Table 2). The practice of only taking single samples from perfused liver was therefore adopted for all hormone studies.

Table 2.

Effect of serial biopsy on the acetyl-CoA carboxylase activity of perfused mouse livers

Livers from lean mice were perfused under standard conditions (see 2.4.2.) with recirculating medium containing 15 mM glucose and 10 mM lactate for 30 min. Recirculation of the medium was then discontinued and a liver biopsy sample was taken, further samples were taken 10 and 20 min later. Liver samples were immediately frozen and stored at -20° until acetyl-CoA carboxylase activity was measured. Results are the means ± S.E.M. of the numbers of observations in parentheses.

* $P<0.05$ cf. 0 min sample.

Time after first sample (min)	Acetyl-CoA carboxylase activity (μmoles CO_2 incorporated/min/g fresh liver)		
	Initial	Total	% initial/total
0	0.15±0.01 (4)	0.49±0.06 (4)	31
10	*0.11±0.01 (3)	0.38±0.08 (3)	29
20	*0.11±0.01 (3)	0.34±0.06 (3)	32

CHAPTER 3

3. Results - Effects of hormones on the rate of lipogenesis in the mouse liver

3.1. Introduction

There are a number of hormones which have been shown to exert short-term catabolic effects on hepatic glycogen metabolism, e.g. glucagon, adrenaline, vasopressin and angiotensin II. Two of these hormones, glucagon and vasopressin have been shown to inhibit long-chain fatty acid synthesis in perfused mouse livers (section 1.3.2.). The effect of these two hormones on acetyl-CoA carboxylase, the first committed step of fatty acid synthesis, was therefore investigated.

Since adrenaline and angiotensin II show elevated plasma concentrations in certain stress and adaptive conditions, it appeared interesting to investigate the effects of these two hormones on long-chain fatty acid synthesis and the activity of acetyl-CoA carboxylase, using the perfused mouse liver.

The genetically obese (ob/ob) mouse shows a remarkable increase in the rate of hepatic lipogenesis when compared with lean littermates (Jansen et al, 1967; Salmon and Hems, 1973). This is associated with increases in the activities of several enzymes involved in fatty acid synthesis including acetyl-CoA carboxylase. It has

recently been reported that the livers of obese (ob/ob) mice show a resistance to inhibition of fatty acid synthesis by vasopressin (Hems and Ma, 1976). This raises the question as to whether lipogenesis, in the obese (ob/ob) mouse liver, is resistant to other hormones which inhibit lipogenesis in the lean mouse liver. The rate of lipogenesis and activity of acetyl-CoA carboxylase was therefore measured in perfused livers from obese (ob/ob) mice after treatment with adrenaline and angiotensin II.

3.2. Effects of glucagon on the rate of lipogenesis and the activity of acetyl-CoA carboxylase in the perfused lean mouse liver

In perfusions of fed, lean, mouse livers with recirculating medium, containing glucose (15 mM) and lactate (10 mM)and lasting 2 hours, glucagon at concentrations of 10^{-10} and 10^{-9}M inhibited both fatty acid and cholesterol synthesis (Table 3). The % inhibition of lipogenesis was similar for the total rate (calculated from the incorporation of ^{3}H from $^{3}H_2O$) and the rate from [^{14}C]-L-lactate, and was greater with the higher concentration of glucagon.

Glucose output, from the liver, was stimulated by glucagon at the concentrations used and resulted in the depletion of liver glycogen stores by the end of the perfusions (Table 3). Stimulation of gluconeogenesis by glucagon (10^{-9}M) was evident, from the significant increase in the rate of conversion of lactate to glucose, from the control value of 216 $\pm$ 8 to 295 $\pm$ 20 μg atoms per 2 hours/g of wet liver after hormone ($P<0.01$).

Since glucagon caused depletion of liver glycogen stores and increased the rate of conversion of lactate into glucose, in these experiments, the inhibition of lipogenesis could have been secondary to alterations in the supply of substrates. Experiments of shorter duration were therefore carried out,to attempt to clarify the mechanism by avoiding depletion of glycogen stores. In these experiments, livers

Table 3.

Effect of glucagon on the rate of lipogenesis, glucose output, and glycogen content of perfused lean mouse livers

Lean mouse livers were perfused with recirculating medium containing 15 mM glucose and 10 mM lactate for 3 hours (see section 2.4.2.). Hormone was added after 45 min followed by 8 further doses at 15 min intervals, each dose being sufficient to produce the concentration indicated. $^{3}H_2O$ and ^{14}C-lactate were added at 60 min (i.e. 15 min after the first glucagon dose). At 180 min a liver sample was taken for analysis of glycogen content and extraction of fatty acids and cholesterol. Glucose output was determined from input and output medium samples taken 10 min after the addition of hormone. Lactate conversion to glucose was calculated from the final medium content of ^{14}C-glucose. Results are the means ± S.E.M. for 3 or 5 perfusions as indicated.

* $P<0.01$ cf. control (no glucagon)

Treatment	No. of perfusions	Lipid synthesis (μmol C_2-units/g fresh liver/2 h)				Final glycogen content (μmol glucose/g fresh liver)	Glucose output (μmol/g fresh liver)	Lactate converted to glucose (μg atoms/2h/g fresh liver)
		Total rate		Rate from lactate				
		Fatty acid	Cholesterol	Fatty acid	Cholesterol			
Control	5	44 ±3.7	2.3±0.4	21 ±2	1.1±0.2	170±31	0.4±0.1	216± 8
Glucagon 10^{-10} M	3	17 ±1.1*	1.8±0.4	6.8±0.8*	0.7±0.2	16± 2*	3.7±0.3*	—
Glucagon 10^{-9} M	5	9.2±2*	0.6±0.1*	3.8±0.8*	0.3±0.1*	21± 4*	5 ±0.7*	295±20*

from fed lean mice were perfused with recirculating medium, containing glucose (15 mM) and lactate (10 mM), for 30 minutes. Recirculation of the medium was then stopped. $^{3}H_2O$, [^{14}C]-L-lactate and glucagon were added to the main reservoir. 40 minutes later a liver sample was taken for lipid extraction. Glucose output was determined from input and output medium samples, taken simultaneously 10 minutes after the addition of hormone.

Under these conditions, lipogenesis was not significantly inhibited, by glucagon, at concentrations below 10^{-8}M (Fig. 11 'a'). The incorporation of [^{14}C]-L-lactate carbon into fatty acids and cholesterol showed parallel inhibition to that of the total rate of fatty acid synthesis, measured with $^{3}H_2O$. Glucose output was stimulated by glucagon at concentrations as low as 10^{-10}M (Fig. 11 'b').

Acetyl-CoA carboxylase catalyses the first committed step of fatty acid synthesis and is widely held to be rate limiting for the process. If glucagon has a direct, short-term effect on the rate of fatty acid synthesis it would be most likely to be exerted on this enzyme. The activity of acetyl-CoA carboxylase was therefore determined after treatment of livers with glucagon in short, flow-through perfusions carried out exactly as above. Samples were freeze-clamped for enzyme assay at the intervals shown (Table 4). Glucagon (10^{-9} & 10^{-8} M) had no effect on the initial, total or % initial/total activities of acetyl-CoA carboxylase within

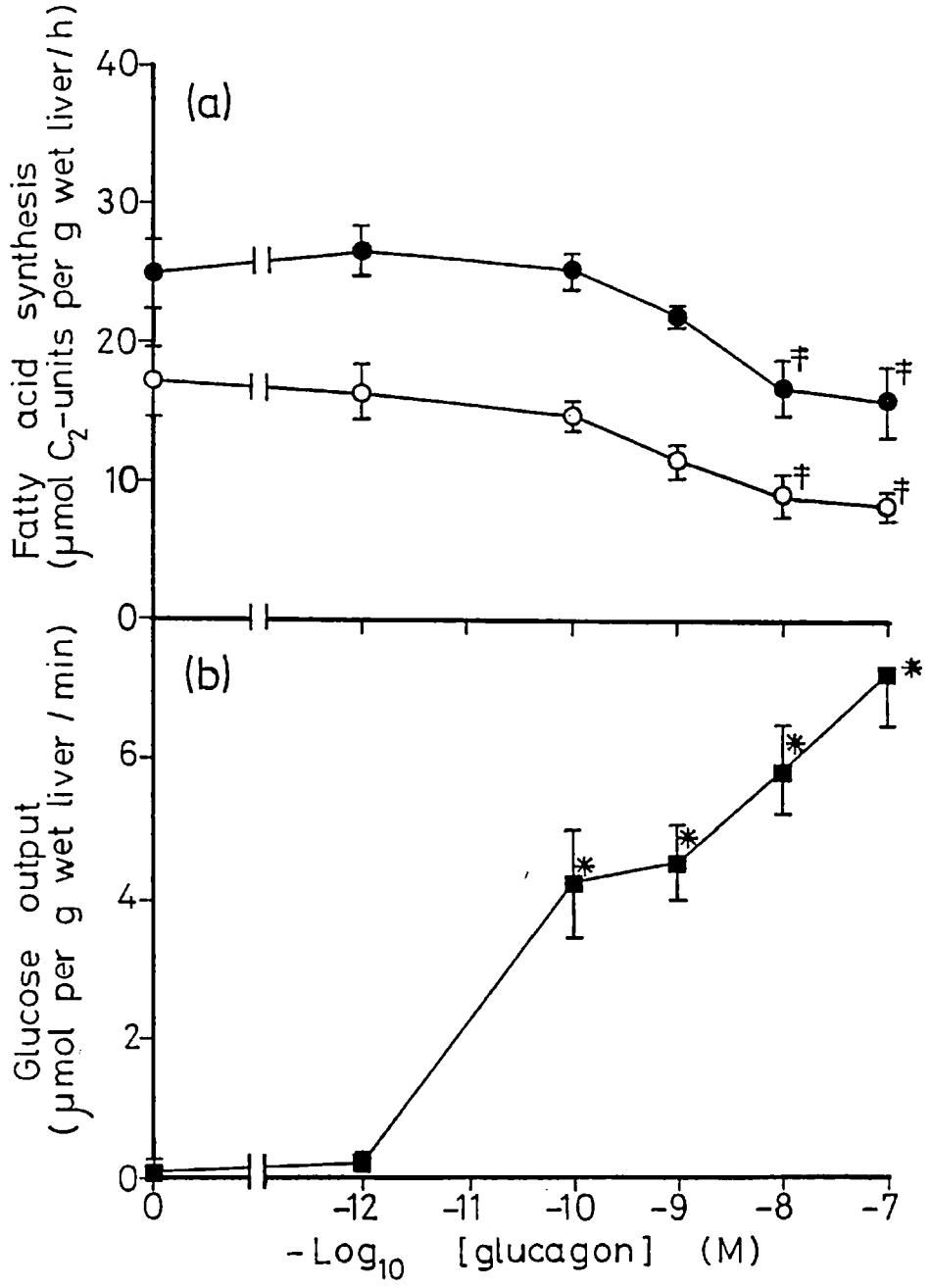

Fig. 11 Effect of glucagon on the rate of fatty acid synthesis and glucose output of perfused livers from lean mice.

Livers from lean mice were perfused under standard conditions with recirculating medium containing 15 mM glucose and 10 mM lactate for 30 min. Recirculation of the medium was discontinued and ^3H_2O, ^{14}C-lactate and glucagon were added. (a) 40 min after the start of non-recirculating perfusion a liver sample was taken for determination of both the total rate of fatty acid synthesis (●) and the rate of synthesis from lactate (○). (b) 10 min after the start of flow-through perfusion input and effluent medium samples were taken for determination of glucose output (■). Results are means ± S.E.M. for 4-5 observations. (After Ma, 1976).
* $P<0.01$ cf. control (no glucagon).
≠ $P<0.05$ cf. control (no glucagon).

Table 4.

The effect of glucagon on the acetyl-CoA carboxylase activity of perfused livers from lean mice

Livers from lean mice were perfused as described in Fig. 11, no isotopes were added to the perfusate and liver samples for the assay of acetyl-CoA carboxylase activity were frozen after 10, 20 or 40 min (only one sample from each perfusion) of non-recirculating perfusion. Other details are given in the text. Results are means ± S.E.M. for the number of perfusions indicated.

Treatment	Time after start of flow-through perfusion (min)	No. of perfusions	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
			Initial	Total	% initial/total
Control	10	3	0.12±0.02	0.52±0.06	23±1.7
Control	20	3	0.12±0.02	0.48±0.04	25±0.2
Glucagon (10^{-9} M)	10	4	0.13±0.01	0.51±0.03	24±1.8
Glucagon (10^{-9} M)	20	4	0.15±0.01	0.59±0.05	25±1.2
Glucagon (10^{-9} M)	40	4	0.19±0.03	0.73±0.1	27±2
Glucagon (10^{-8} M)	10	4	0.11±0.02	0.45±0.03	24±3.4
Glucagon (10^{-8} M)	20	4	0.12±0.04	0.46±0.14	27±2.9

40 minutes, even though the higher concentration of hormone was capable of inhibiting hepatic fatty acid synthesis within 40 minutes (Table 4; Fig. 11 'a').

3.3. Effects of vasopressin on the rate of lipogenesis and the activity of acetyl-CoA carboxylase in the perfused, lean mouse liver

The sensitivity of fatty acid synthesis, in the lean mouse liver, to inhibition by vasopressin has been reported previously (Ma and Hems, 1975).

Fig. 12, included here by permission of Drs. G.Y. Ma and D.A. Hems shows the concentration dependence of the inhibition of fatty acid synthesis and stimulation of glucose release in perfused lean mouse liver in response to vasopressin.

Vasopressin inhibited fatty acid synthesis over the concentration range 500 μU/ml - 2 mU/ml in short non-recycling perfusions of lean mouse livers, carried out as in the glucagon experiments (Fig. 12 'a'). The stimulation of glucose output from the liver, in response to vasopressin, showed parallel concentration dependence to the effect on fatty acid synthesis (cf. Fig. 12 'a' & 12 'b'). Glucose output from the liver in response to vasopressin was sustained mainly by degradation of hepatic glycogen stores. This was demonstrated in perfusions lasting 2 hours in which the medium (containing glucose, 15 mM; lactate, 10 mM; and vasopressin, 4 mU/ml) was recirculated (Ma and Hems, 1975). No difference in the rate of conversion of medium lactate into glucose was found between control and vasopressin treated livers (241 ± 20 and 243 ± 19 μg atoms per g of wet liver/2 hours respectively).

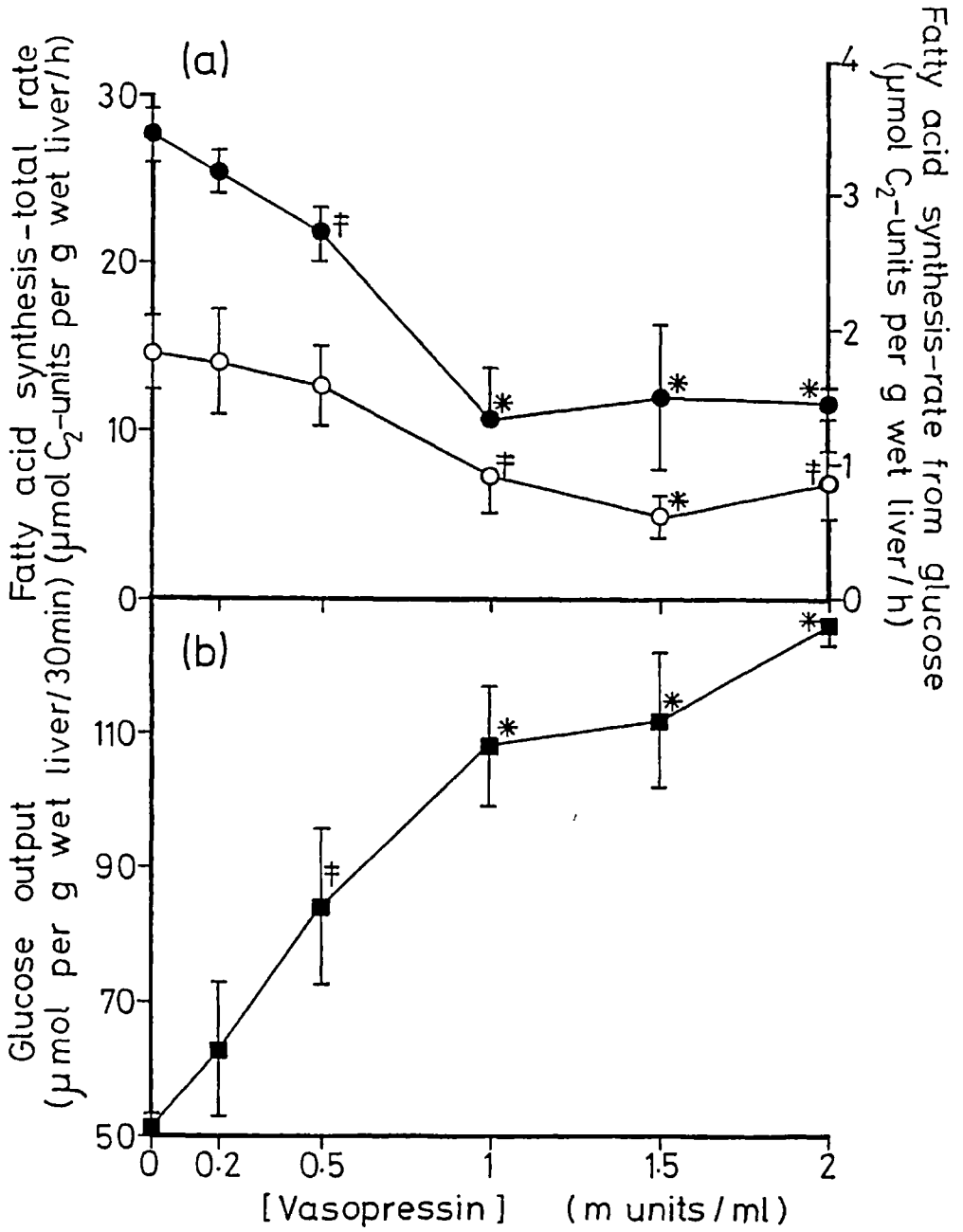

Fig. 12 Effect of vasopressin on the rate of fatty acid synthesis and glucose output of perfused livers from lean mice.

Livers from lean mice were perfused under standard conditions with 120 ml medium containing 15 mM glucose and 10 mM lactate. The medium was recirculated for 30 min when $^{3}H_2O$, [U-^{14}C]-glucose and hormone were added, recirculation of the medium was then discontinued. Perfusion continued until the medium ran out (40-70 min), when a final liver sample was taken for determination of the total rate of fatty acid synthesis (●) and the rate from glucose (○). Glucose output (■) was determined from effluent and input blood samples taken 5 min after hormone addition by which time glucose output had reached a steady value. Results are means of 3-4 perfusions and bars indicate S.E.M. (After Ma & Hems, 1975).

Although vasopressin caused simultaneous stimulation of glycogenolysis and inhibition of fatty acid synthesis, it seems unlikely that depletion of hepatic glycogen stores was the cause of this effect on fatty acid synthesis, as it was apparent in short perfusions in which glycogen depletion was avoided. A more likely explanation seems to be a direct, primary action of vasopressin on fatty acid synthesis, since the rate of cholesterol synthesis was unaffected even in longer perfusions. A likely site for inhibition of fatty acid synthesis would be the conversion of acetyl-CoA to malonyl-CoA catalysed by the enzyme acetyl-CoA carboxylase. Inhibition of this enzyme would not affect cholesterol synthesis since incorporation of carbon from acetyl units into cholesterol proceeds via β-hydroxy β-methylglutaryl-CoA and mevalonic acid and not by carboxylation of acetyl-CoA (see Bloch, 1965).

The activity of acetyl-CoA carboxylase was therefore determined after 20, 30 or 40 minutes treatment of perfused livers with vasopressin carried out exactly as described for glucagon (section 3.2.). Vasopressin inhibited both the initial and total activities of the enzyme at 30 minutes after hormone addition. Little change in the % initial/total activity was observed, since both activities declined to a similar extent (Table 5).

Table 5.

Effect of vasopressin on the acetyl-CoA carboxylase activity of perfused livers from lean mice

Livers from lean mice were perfused as described in Table 4, vasopressin was used in place of glucagon and samples of liver for determination of acetyl-CoA carboxylase activity were taken after 20, 30 and 40 min of non-recirculating perfusion. Other details are in the text. Results are the means ± S.E.M. of 3 or 4 observations as indicated.

* $P<0.01$ compared with control at 30 min after start of non-recirculating perfusion.

Treatment	Time after start of flow-through perfusion (min)	No. of perfusions	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
			Initial	Total	% initial/total
Control	20	3	0.21±0.05	0.65±0.15	32±1.5
Control	30	3	0.3 ±0.02*	0.89±0.06*	32±1.8
Control	40	3	0.28±0.07	0.86±0.19	32±4.9
AVP (4 mU/ml)	20	3	0.18±0.03	0.65±0.14	29±2.8
AVP (4 mU/ml)	30	4	0.16±0.02*	0.55±0.05*	28±1.6
AVP (4 mU/ml)	40	3	0.17±0.02	0.58±0.09	30±4.3

3.4. Effect of adrenaline and angiotensin II on the rate of long-chain fatty acid synthesis and the activity of acetyl-CoA carboxylase in the perfused livers of lean mice

The effects of adrenaline and angiotensin II on hepatic fatty acid synthesis, glucose output and acetyl-CoA carboxylase activity were investigated in short, flow-through perfusions carried out as described for glucagon (section 3.2.) except that medium was recirculated for 60 minutes prior to hormone addition. Hormone doses were selected to produce roughly equal glucose output for the two hormones, using dose curves obtained in perfused rat liver (Hems et al, 1976).

Adrenaline (5 ng/ml) caused a small increase in hepatic glucose output. At this concentration no effect on the rate of fatty acid synthesis occurred. At 50 ng/ml adrenaline caused a considerably larger stimulation of glucose output and fatty acid synthesis from both $^{3}H_2O$ and [^{14}C]-L-lactate was inhibited by about 45%. Hepatic glycogen stores were unaffected by the lower dose of adrenaline but were considerably depleted at the higher dose (Table 6).

Angiotensin II (1 ng/ml) had no effect on the rate of either glucose output or fatty acid synthesis in the perfused livers. When the concentration of hormone was raised to 10 ng/ml there was a significant stimulation of glucose output with a concomitant fall in the final

Table 6.

Effects of adrenaline and angiotensin II on the rate of lipogenesis and glucose output of perfused livers from fed lean mice

Livers from lean mice were perfused with recirculating medium containing 15 mM glucose and 10 mM lactate for 60 min followed by 40 min non-recirculating perfusion (see 2.4.1.). $^{3}H_2O$, ^{14}C-lactate and hormone were added at 60 min and a liver sample for extraction of lipids and glucogen was taken at 100 min. Glucose output was determined from input and effluent medium samples taken simultaneously 10 min after the start of flow-through perfusion. Results are the means ± S.E.M. for the number of perfusions indicated. Other details are given in the text.

* $P<0.01$ Compared with relevant control

≠ $P<0.05$ Compared with relevant control

Treatment	No. of perfusions	Fatty acid synthesis (µmol of C_2-Units/hr/g fresh liver)		Glucose output (µmol/g/min)	Final glycogen content (µmol glucose/g fresh liver)
		Total rate	Rate from lactate		
Control	10	22±1.2	9±0.7	0.9±0.2	151±14
Adrenaline (5 ng/ml)	3	22±1.2	8±0.9	2 ±0.3	130±32
Adrenaline (50 ng/ml)	5	12±1.2*	4±0.6*	9 ±0.7*	86±20≠
Angiotensin II (1 ng/ml)	3	20±6	10±3.4	1.2±0.1	151±14
Angiotensin II (10 ng/ml)	4	8±0.9*	3±0.5*	3 ±0.2*	118±16

glycogen content of the liver. Fatty acid synthesis measured both with $^{3}H_2O$ and [^{14}C]-L-lactate showed over 60% inhibition in response to the higher dose of angiotensin II (Table 6).

Since the inhibition of fatty acid synthesis in these experiments was accompanied by depletion of hepatic glycogen stores (one of the favoured precursors for fatty acid synthesis in the liver) the effects of adrenaline and angiotensin II on the activity of acetyl-CoA carboxylase was investigated in order to distinguish between direct effects on fatty acid synthesis and effects due to altered substrate supply.

In perfusions carried out exactly as above, the initial activity of acetyl-CoA carboxylase was inhibited following treatment of the livers with adrenaline (50 ng/ml) and angiotensin II (10 ng/ml). The total activity of the enzyme was unaffected by adrenaline (50 ng/ml) but decreased in response to angiotensin II (10 ng/ml). The ratio % initial/total activity of the enzyme was significantly reduced by both hormones at these concentrations (Table 7).

Table 7.

Effects of adrenaline and angiotensin II on the acetyl-CoA carboxylase activity of perfused livers from fed lean mice

Livers from lean mice were perfused with non-recirculating medium as described in Table 6, no isotopes were added. Hormone, was added at the start of non-recirculating perfusion and a liver sample was frozen 40 min later. Results are the means ± S.E.M. of the number of perfusions indicated. Other details are given in the text.

* $P<0.01$ Compared with control

≠ $P<0.05$ Compared with control

Treatment	No. of perfusions	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
		Initial	Total	% initial/total
Control	8	0.36±0.04	0.70±0.14	56±5
Adrenaline (50 ng/ml)	6	0.27±0.05	0.72±0.29	37±2*
Angiotensin II (10 ng/ml)	5	0.22±0.05	0.53±0.14	42±3≠

3.5. Effects of adrenaline and angiotensin II on the rate of fatty acid synthesis and the activity of acetyl-CoA carboxylase in perfused livers of freely fed and diet-restricted genetically obese (ob/ob) mice

The actions of adrenaline and angiotensin II in livers from freely fed and diet-restricted mice (section 2.1.), were investigated in short, flow-through perfusion experiments carried out as described for lean mouse livers (section 3.4.).

In livers from freely fed obese (ob/ob) mice, adrenaline (50 ng/ml) and angiotensin II (10 ng/ml) stimulated glucose output and did not caused a significant fall in final glycogen concentration. There was also a small, but not significant, decline in the rate of fatty acid synthesis both from $^{3}H_2O$ and [^{14}C]-L-lactate under these conditions (Table 8).

Adrenaline (50 ng/ml) slightly reduced the initial activity of acetyl-CoA carboxylase, causing a decrease in the ratio of % initial/total activities (Table 9). This effect persisted in 2 hour recycling perfusions.

Angiotensin II (10 ng/ml) had no effect on acetyl-CoA carboxylase activity (Table 9).

No differences in the responses of glucose output, fatty acid synthesis or acetyl-CoA carboxylase to adrenaline or angiotensin II were found between livers from freely fed and diet restricted obese (ob/ob) mice (Tables 8 & 9).

Table 8.

Effects of adrenaline and angiotensin II on the rate of fatty acid synthesis and glucose output of perfused livers from freely fed obese (ob/ob) and diet restricted (D.R. ob/ob) mice

Livers from ob/ob and D.R. ob/ob mice were perfused as described in Table 6. Results are the means ± S.E.M. of the number of observations indicated. Other details are in the text.

* $P<0.01$ Compared with relevant control

≠ $P<0.05$ Compared with relevant control

Mouse	Treatment	No. of perfusions	Fatty acid synthesis (μmol of C_2-Units/hr/g fresh liver)		Glucose output (μmol/g fresh liver/min	Final glycogen content (μmol glucose/g fresh liver
			Total rate	Rate from lactate		
ob/ob	Control	4	29±2.6	13±1	0.8±0.14	151±22
ob/ob	Adrenaline (50 ng/ml)	3	21±4.5	9±1.4	4 ±1.1≠	94±15
ob/ob	Angiotensin II (10 ng/ml)	3	22±4.4	8±2	2 ±0.5	138± 4
D.R. ob/ob	Control	4	25±4.3	8±0.6	0.6±0.3	145±20
D.R. ob/ob	Adrenaline (50 ng/ml)	4	21±2.8	8±1	5 ±0.4*	107+11
D.R. ob/ob	Angiotensin II (10 ng/ml)	4	24±3.4	7±1	3 ±0.5*	121±28

Table 9.

Effects of adrenaline and angiotensin II on the acetyl-CoA carboxylase activity of perfused livers from freely fed obese (ob/ob) and diet restricted obese (D.R. ob/ob) mice

Livers from ob/ob and D.R. ob/ob mice were perfused as described in Table 6, no isotopes were added. Hormone was added at the start of non-recirculating perfusion and a liver sample was frozen 40 min later. In one group of experiments livers from ob/ob mice were perfused with recirculating medium throughout, hormone was added at 60 min and a liver sample was frozen at 180 min. Results are the means ± S.E.M. for the numbers of observations indicated. Other details are in the text.

* $P<0.01$ Compared with relevant control

≠ $P<0.05$ Compared with relevant control

Mouse	Treatment	Time after start of experiment (min)	No. of perfusions	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
				Initial	Total	% initial/total
ob/ob	Control	40	4	0.47±0.02	1.2 ±0.1	41±2·6
ob/ob	Adrenaline (50 ng/ml)	40	3	0.32±0.02	1.2 ±0.06	27±1·7
ob/ob	Angiotensin II (10 ng/ml)	40	3	0.43±0.09	1.2 ±0.06	37±5.3
ob/ob	Control	120	3	0.40±0.05	1 ±0.06	29±2.4
ob/ob	Adrenaline (50 ng/ml)	120	4	0.27±0.03	1.3 ±0.07	21±1.8≠
D.R. ob/ob	Control	40	4	0.25±0.03	0.47±0.04	53±4.4
D.R. ob/ob	Adrenaline (50 ng/ml)	40	4	0.19±0.02	0.5 ±0.03	38±2.6≠
D.R. ob/ob	Angiotensin II (10 ng/ml)	40	4	0.23±0.02	0.47±0.01	49±2

CHAPTER 4

4. Results - Influence of hypoxia and adenine nucleotides on hepatic acetyl-CoA carboxylase activity

4.1. Introduction

A rapid reduction in the initial activity of acetyl-CoA carboxylase was found in response to short periods of hepatic anoxia *in vivo*. Since the rats used to provide these samples were killed by stunning and cervical dislocation there was a possibility that the effects were triggered by nerve action. Similar experiments were therefore carried out using deeply anaesthetised rats and perfused livers. A similar decrease in the initial activity of acetyl-CoA carboxylase was found in these experiments. Rats and mice show differences in the sensitivity of fatty acid synthesis to inhibition by hormones. Thus vasopressin at physiological concentrations, will inhibit fatty acid synthesis in perfused liver from lean mice but not from obese (*ob/ob*) mice or rats. The effects of ischaemia on the hepatic acetyl-CoA carboxylase activity of lean and obese (*ob/ob*) mice was investigated. The time course of the reduction in the initial activity of the enzyme in response to ischaemia was similar in rats and lean and obese (*ob/ob*) mice.

The rapid reduction in the initial activity of hepatic acetyl-CoA carboxylase in response to a decline in tissue oxygenation poses the question as to the

mechanism involved. A possible answer is that one or more of the metabolites or nucleotides which increase in concentration in the liver in response to hypoxia inhibit(s) the enzyme.

Adenine nucleotides are extremely sensitive to tissue oxygenation and have been shown to exert regulatory influence on several key enzymes including some of those of carbohydrate metabolism. The effects of a variety of purine nucleotides, on the activity of acetyl-CoA carboxylase, were therefore investigated. The most potent inhibition was caused by AMP and ADP.

In the extra mitochondrial phase of the liver parenchymal cell, AMP is a particularly sensitive indicator of the adenine nucleotide status or 'charge' as it undergoes proportionally larger changes in concentration than does ATP or ADP. The kinetics of the inhibitory effect of AMP on acetyl-CoA carboxylase was therefore investigated.

4.2. Effects of hypoxia on acetyl-CoA carboxylase activity in livers of rats and mice

4.2.1. Effects of ischaemia in rat liver in vivo

The initial activity of acetyl-CoA carboxylase, in livers from consciousfed rats, was reduced by over 65% in response to 30 sec of ischaemia, and fell further to 20% of the original activity within 1 min (Table 10, Fig. 13). This reduction in the initial activity of acetyl-CoA carboxylase could largely be overcome by incubation with citrate and magnesium for 30 min at 37°. Thus the total activity of the enzyme was reduced by only 20% after 2 min of ischaemia (Table 10, Fig. 13). This alteration in the % initial/total activity, induced by the ischaemia, would therefore seem to reflect a change in the state of polymerisation and/or phosphorylation of the enzyme.

Deep anaesthesia with 'nembutal' caused a 60% reduction in both the initial and total activities of hepatic acetyl-CoA carboxylase, when compared to the activities in the livers of fed conscious rats (Table 10). No change in the % initial/total activities or in the response to ischaemia was found in these rats when compared to conscious fed rats (Table 10, Fig. 13). A similar reduction in initial and total enzyme activities was found in the livers of fed rats anaesthetised with diethyl ether (data not shown).

Table 10.

Inhibition of acetyl-CoA carboxylase activity in the rat liver in response to ischaemia in vivo

The initial and total activities of acetyl-CoA carboxylase were measured in liver samples freeze-clamped after various periods of ischaemia. In one set of experiments the rats were stunned and a liver sample was immediately freeze-clamped (0 min sample), the blood supply to the remainder of the liver was severed and further samples freeze-clamped at 0.5, 1 and 2 min intervals. In the second set of experiments, rats were deeply anaesthetised with nembutal before a zero time liver sample was freeze-clamped. The blood supply to the liver was severed and further samples were taken 0.5 and 1 min later. Results are the means ± S.E.M. for the number of observations indicated. Other details are in the text.

* $P<0.01$ cf. 0 min sample

≠ $P<0.05$ cf. 0 min sample

Treatment	Duration of ischaemia (min)	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
		Initial	Total	% initial/total
Normal fed rats	0	0.32±0.04 (6)	0.90±0.19 (5)	38±2.7 (5)
	0.5	0.11±0.02*(5)	0.58±0.08 (4)	16±1.7*(4)
	1	0.06±0.01*(6)	0.62±0.12 (5)	10±1.6*(5)
	2	0.04±0.01*(5)	0.61±0.13 (4)	8.8±2.8*(4)
Deeply anaesthetised rats (nembutal for 30 min)	0	0.11±0.01 (3)	0.44±0.04 (3)	25±1.6 (3)
	0.5	0.05±0.01*(3)	0.38±0.03 (3)	14±1.9≠(3)
	1	0.03±0.01*(3)	0.31±0.04 (3)	9.4±1.2*(3)

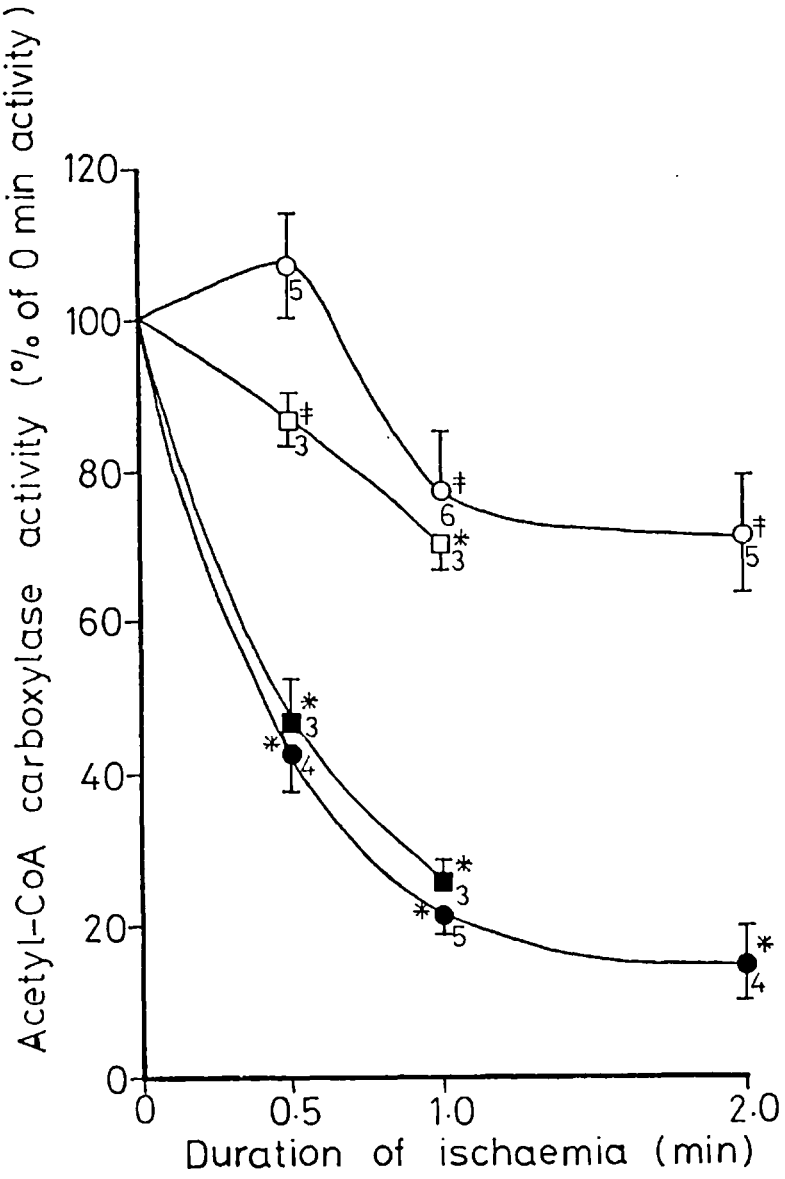

Fig. 13 Decrease in the activity of acetyl-CoA carboxylase in the rat liver in response to ischaemia in vivo

The percentage change in the initial (●, ■) and total (○, □) activities of acetyl-CoA carboxylase was determined in the liver samples from the stunned (●, ○) and deeply anaesthetised (■ , □) rats described in table 10. Results are means ± S.E.M. for the numbers of observations indicated. Other details are in the text.
* $P<0.01$ cf. 0 min sample.
‡ $P<0.05$ cf. 0 min sample.

4.2.2. Effects of anoxia in perfused rat livers

The effects of hypoxia on hepatic acetyl-CoA carboxylase were examined in two types of perfusion experiment. In the first (ischaemic), the livers were perfused with recirculating medium containing glucose (10 mM) for 1 hr. The flow of medium to the liver was then stopped and samples taken at 0, 0.5, 1 and 2 min intervals. In the second (anoxic), the livers were perfused as above for 1 hr, but then the medium was changed to one lacking red cells and gassed with nitrogen:CO_2 (95%:5%). A liver sample was taken immediately before and 3 min after the medium was changed.

Initial and total activities of acetyl-CoA carboxylase were similar in the perfused fully oxygenated liver to those observed in the livers of deeply anaesthetised rats (Tables 10 & 11). Ischaemia caused a similar decline in the initial activity of the enzyme in perfused livers to that observed *in vivo* except that little change had occurred within the first 30 sec (Tables 10 & 11 and Figs. 13 & 14). This may reflect a higher level of tissue oxygenation in the perfused liver system, since the medium was gassed with O_2:CO_2 (95%:5%). Perfusion of the liver for 3 min with oxygen free medium caused a 90% reduction in the initial activity of acetyl-CoA carboxylase (Table 11, Fig. 14). Reduction of the total activity of acetyl-CoA carboxylase in response to hypoxia was similar in perfusion and *in vivo* (Tables 10 & 11, Figs. 13 & 14).

Table 11.

Inhibition of acetyl-CoA carboxylase activity in response to ischaemia and anoxia in perfused livers from fed rats

Livers from fed rats were perfused with medium containing 10 mM glucose for 60 min (see 2.4.2.). In one series of experiments (ischaemia) the flow of medium to the liver was stopped and liver samples were taken immediately and 0.5, 1 and 2 min later. In a second series of experiments (anoxia) the medium perfusing the liver was changed to one lacking red cells and gassed with N_2:CO_2 (95:5% v/v). Liver samples were taken immediately before and 3 min after the change of medium. Results are means ± S.E.M. for the number of experiments shown. Other details are given in the text.

* $P<0.01$ cf. 0 min sample

≠ $P<0.05$ cf. 0 min sample

Treatment	Duration of ischaemia/anoxia (min)	No. of perfusions	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
			Initial	Total	% initial/total
Ischaemia	0	5	0.15±0.04	0.46±0.06	30±5.6
	0.5	3	0.13±0.01	0.51±0.05	25±0.5
	1	5	0.03±0.01≠	0.41±0.04	7.8±2*
	2	4	0.02±0.01≠	0.34±0.03	4.7±1*
Anoxia	0	3	0.14±0.02	0.49±0.03	28±3.2
	3	3	0.02±0.01*	0.46±0.03	3.5±1.8*

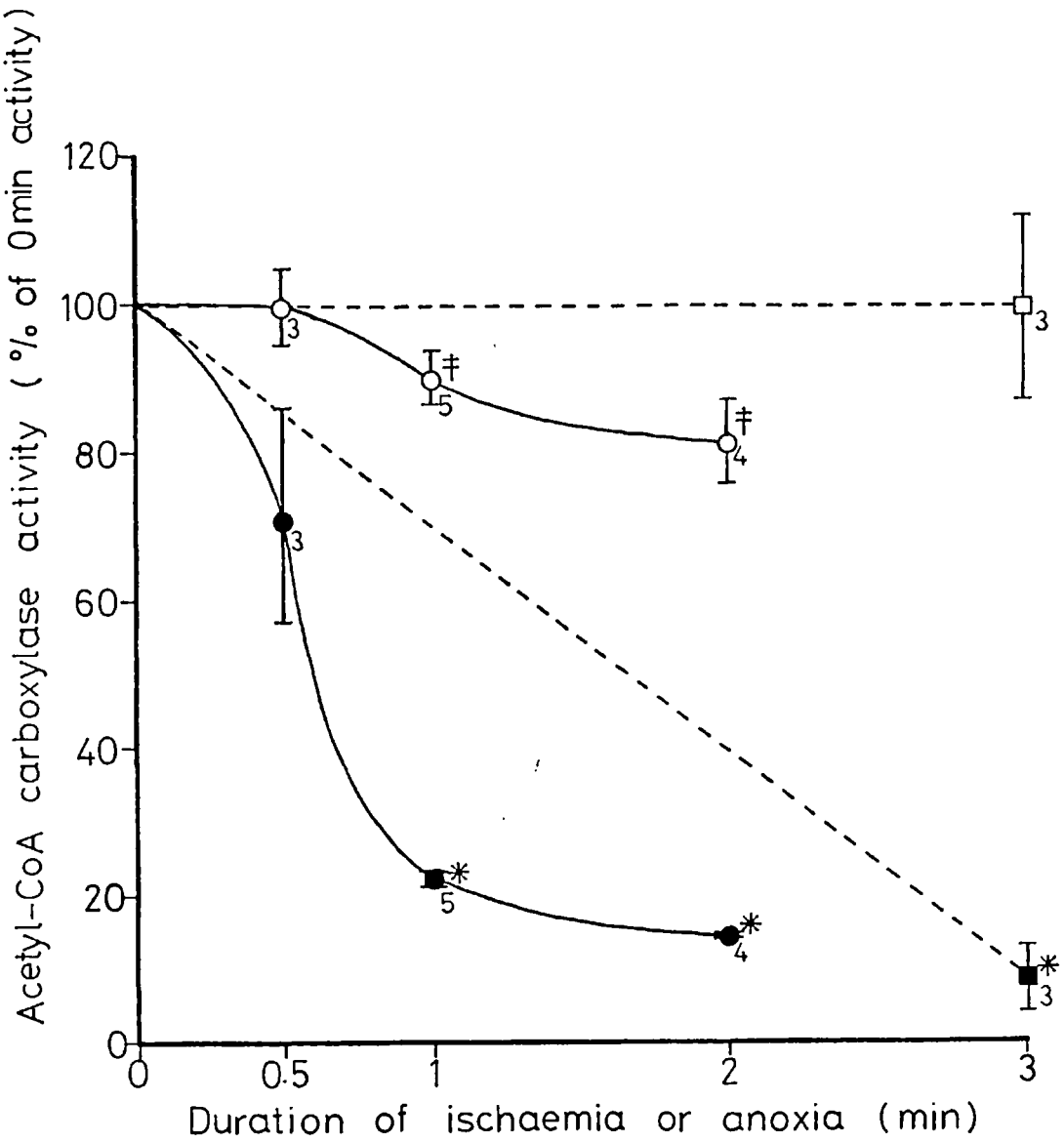

Fig. 14 Decrease in the acetyl-CoA carboxylase activity of perfused livers from fed rats in response to ischaemia and anoxia.

The percentage change in the initial (●, ■) and total (○, □) activities of acetyl-CoA carboxylase was determined in the liver samples from the ischaemic (●, ○) and anoxic (■ , □) liver perfusions described in table 11. Results are means ± S.E.M. for the numbers of observations indicated. Other details are in the text.

* $P<0.01$ cf. 0 min sample.

‡ $P<0.05$ cf. 0 min sample.

4.2.3. Effects of ischaemia in lean and genetically obese (ob/ob) mouse livers *in vivo*

The initial activity of acetyl-CoA carboxylase in lean mouse liver, *in vivo*, decreased by 70% within 1 min in response to ischaemia (Fig. 15). The time course of inactivation of the lean mouse liver enzyme was similar to that of the rat liver enzyme (cf. Figs. 13 & 15). The initial activity of acetyl-CoA carboxylase was similarly reduced in the obese (ob/ob) mouse liver, in response to ischaemia, although the reduction in activity was slower in onset (Table 12). This would seem to indicate that the intracellular mechanisms controlling the activity of acetyl-CoA carboxylase are still functional in the obese (ob/ob) mouse liver.

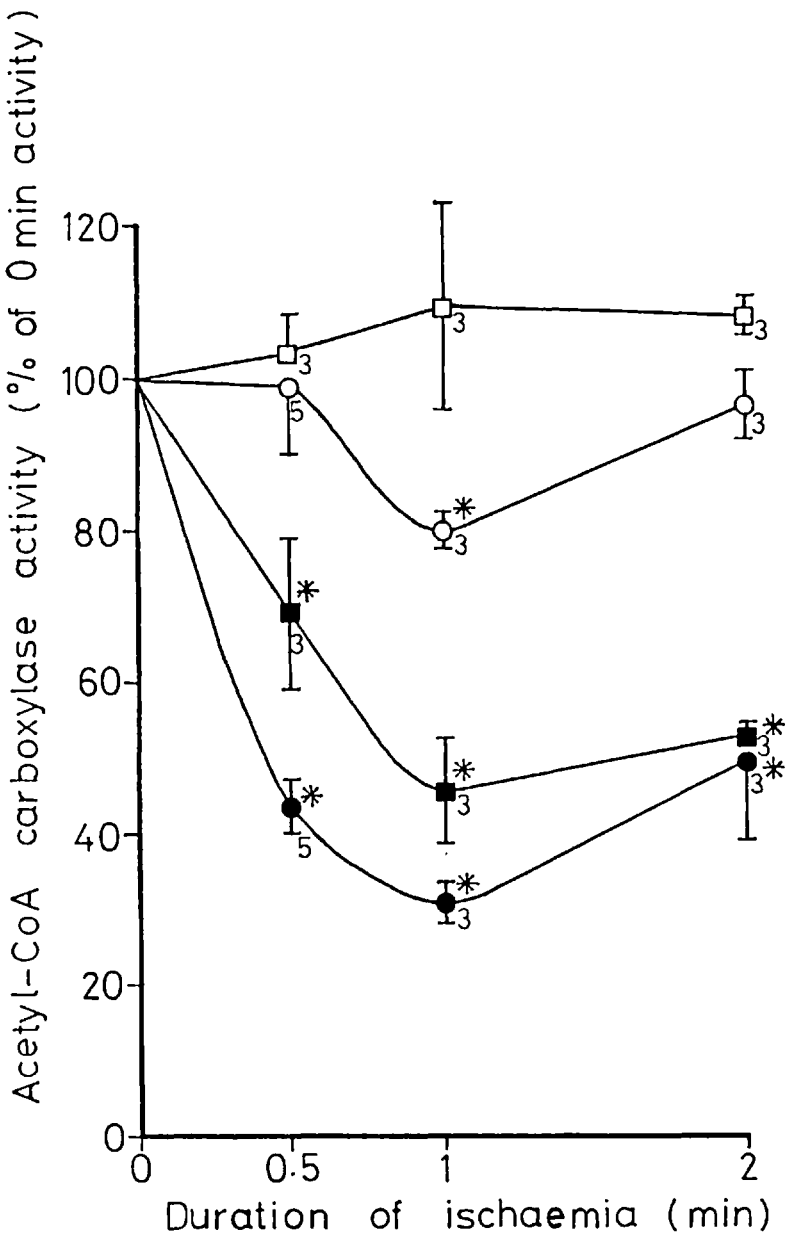

Fig. 15 Decrease in the activity of hepatic acetyl-CoA carboxylase in vivo in lean and obese (ob/ob) mice in response to ischaemia.

The percentage decrease in the initial (●, ■) and total (○, □) activities of acetyl-CoA carboxylase was determined in the liver samples from the lean (●, ○) and obese (■ , □) mice described in table 12. Results are the means ± S.E.M. for the numbers of observations indicated. Other details are given in the text.
* $P<0.01$ cf. control (i.e. 0 min sample).

Table 12.

Effect of ischaemia on the hepatic acetyl-CoA carboxylase activity of fed lean and obese (ob/ob) mice *in vivo*

The initial and total activities of acetyl-CoA carboxylase were measured in liver samples taken from lean and obese mice after various periods of ischaemia. The mice were quickly stunned and a sample of liver freeze-clamped. The blood supply to the rest of the liver was quickly severed and a second liver sample was freeze-clamped 0.5, 1 or 2 min later. Results are the means ± S.E.M. of the numbers of observations shown. Other details are in the text.

* $P<0.01$ cf. 0 min sample
≠ $P<0.05$ cf. 0 min sample
ϖ $P<0.01$ cf. lean and ob/ob after 2 min of ischaemia

Mouse	Duration of ischaemia	No. of animals	Acetyl-CoA carboxylase activity (μmol CO_2 incorporated/min/g fresh liver)		
			Initial	Total	% initial/total
Lean mouse	0	11	0.41±0.04	0.74±0.06	56±3.4
	0.5	5	0.18±0.03*	0.67±0.1	27±1.2*
	1	3	0.12±0.02*	0.56±0.09	21±0.3*
	2	3	0.20±0.03*	0.84±0.08	ϖ23±0.9*
ob/ob mouse	0	12	0.75±0.06	1.3 ±0.1	60±2.7
	0.5	3	0.51±0.08≠	1.3 ±0.2	40±8.8≠
	1	3	0.43±0.05*	1.6 ±0.2	29±5.7*
	2	3	0.44±0.04*	1.5 ±0.2	ϖ30±1*

4.3. Effects of nucleotides on hepatic acetyl-CoA carboxylase

4.3.1. Effects of naturally occurring purine compounds on hepatic acetyl-CoA carboxylase

Both AMP and ADP, at a concentration of 0.5 mM, inhibited the total activity of acetyl-CoA carboxylase activity by about 20% (Table 13). None of the other nucleotides tested inhibited the enzyme at this concentration. Other purine nucleotide monophosphates added with the AMP did not effect the extent of inhibition.

When the concentration was increased to 5 mM all the nucleotides except adenosine, hypoxanthine and IMP caused a significant inhibition of the enzyme. By far the greatest inhibition was caused by AMP and ADP, at this concentration (Table 13). Since the assay procedure was only 3 min in duration these data clearly demonstrate a short-term inhibitory effect of AMP and ADP on acetyl-CoA carboxylase.

4.3.2. Inhibition of hepatic acetyl-CoA carboxylase by AMP

The concentration dependence of the inhibition of the initial activity of acetyl-CoA carboxylase by AMP is shown in Fig. 16 'a'. The most steeply rising part of the curve occurred at those concentrations known to occur *in vivo* viz 0-1 mM. The plot of % inhibition vs log [AMP]

Table 13.

The effects of various nucleotides and metabolites on the acetyl-CoA carboxylase activity of rat liver high speed supernatant

The total activity of acetyl-CoA carboxylase in the high speed supernatant of liver from fed rats was measured in the usual way (see 2.9.2.). Aliquots of concentrated stock solutions of the nucleotides and metabolites shown (adjusted to pH 7.1-7.4) were added to the assay mixture immediately before the liver high speed supernatant. Results are the means of 3 determinations ± S.E.M. and other details are in the text

Nucleotide/metabolite		Adenine	Adenosine	Inosine	Xanthosine	Hypoxanthine	IMP	XMP	AMP	ADP	IMP XMP	IMP AMP	XMP AMP	IMP XPM AMP
Concentration														
0.5 mM	% Inhib.	0.34	-1.2	-1.2	-0.22	1.5	1.2	0	20.3	18.8	-0.22	16.8	17.4	18.1
	S.E.M.	±2.2	±2.3	±1.1	±1.8	±1	±2.3	±2.7	±5	±3.3	±1.8	±1.2	±0.7	±1.4
5.0 mM	% Inhib.	27.8	-1.8	12.2	19.9	6.1	9.7	16	62	76.8	18.5	38.7	49.1	52.4
	S.E.M.	±3.6	±1.6	±1.3	±2.7	±1.8	±2.5	±0.3	±5	±2.1	±0.3	±1.4	±0.7	±1.1

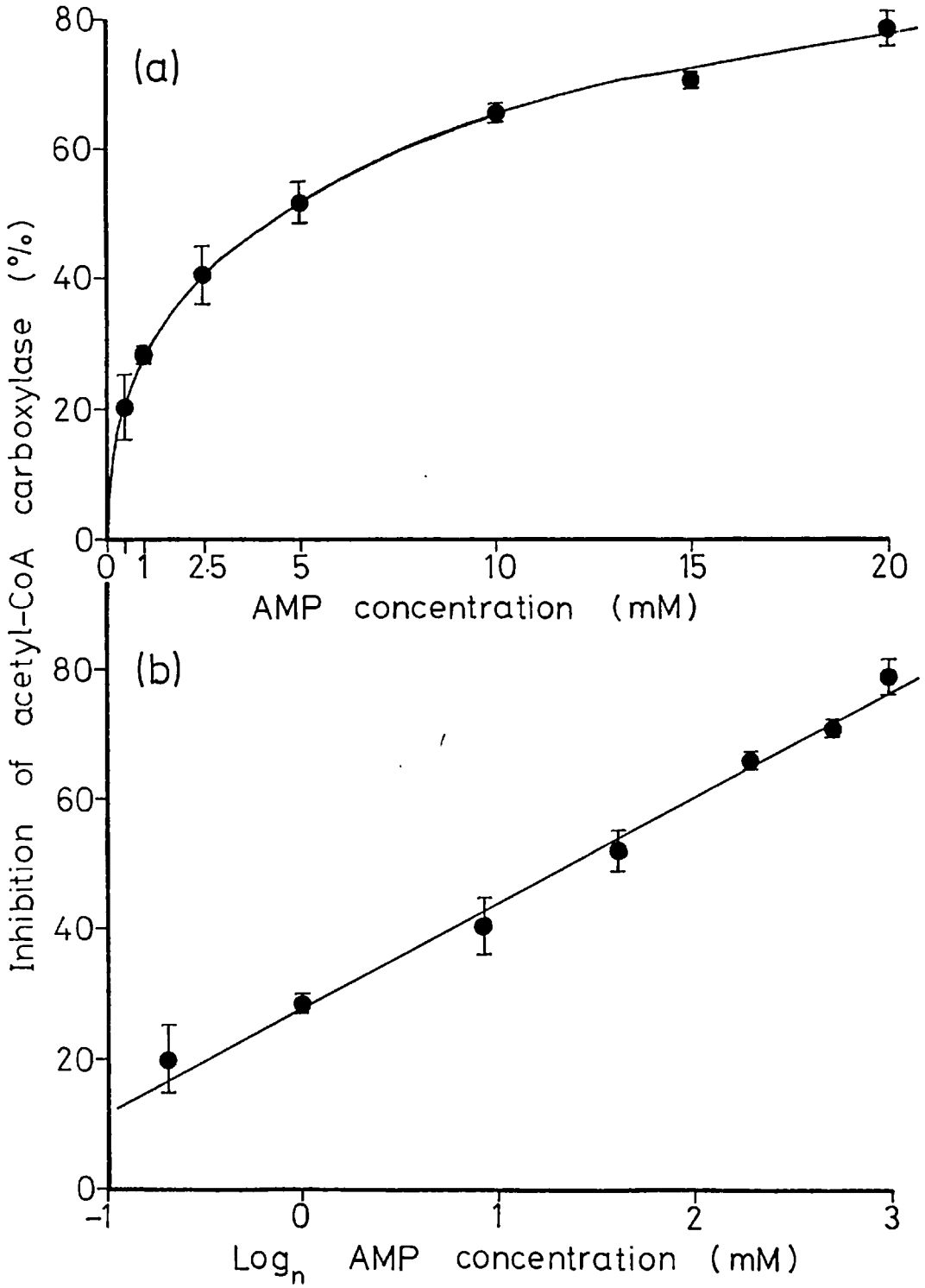

Fig. 16 The concentration dependence of the inhibition of hepatic acetyl-CoA carboxylase by AMP.

The initial activity of acetyl-CoA carboxylase in the high speed supernatant of fed rat livers was measured in the presence of AMP over the concentration range 0.5-20 mM. Results are the means ± S.E.M. of three observations and are expressed as % inhibition of the relevant control activity, and are plotted (a) vs. AMP concentration and (b) vs. $\log_n$ AMP concentration. All points are significantly ($P<0.01$) different from the control activity.

is linear over the concentration range 0.5-20 mM (Fig. 16'b'). The regression line fitted to these data gave the AMP concentration which exerted 50% inhibition as 4.6 mM.

Since ATP is a substrate for acetyl-CoA carboxylase, competition of AMP and ADP with ATP for the ATP binding site may be one possible mechanism for the inhibition of the enzyme.

The inhibition of acetyl-CoA carboxylase by AMP was investigated under conditions of increasing substrate (ATP) concentration in the presence of fixed concentrations of AMP. Plots of these data in the form 1/v vs 1/[S], in the absence and presence of 0.5 and 5 mM AMP, showed the inhibition to be of the partially competitive type (Fig. 17). Extrapolation of the curve obtained in the absence of AMP gave a value of 0.39 mM for the Km of the enzyme for ATP. Calculation of the Ki for AMP gave a value of 4.1 mM under the assay conditions used. The non-linearity of the curves near the 'Y' axis in Fig. 17 is due to inhibition of the enzyme by high concentrations of the substrate, ATP (see section 2.9.3., Fig. 9).

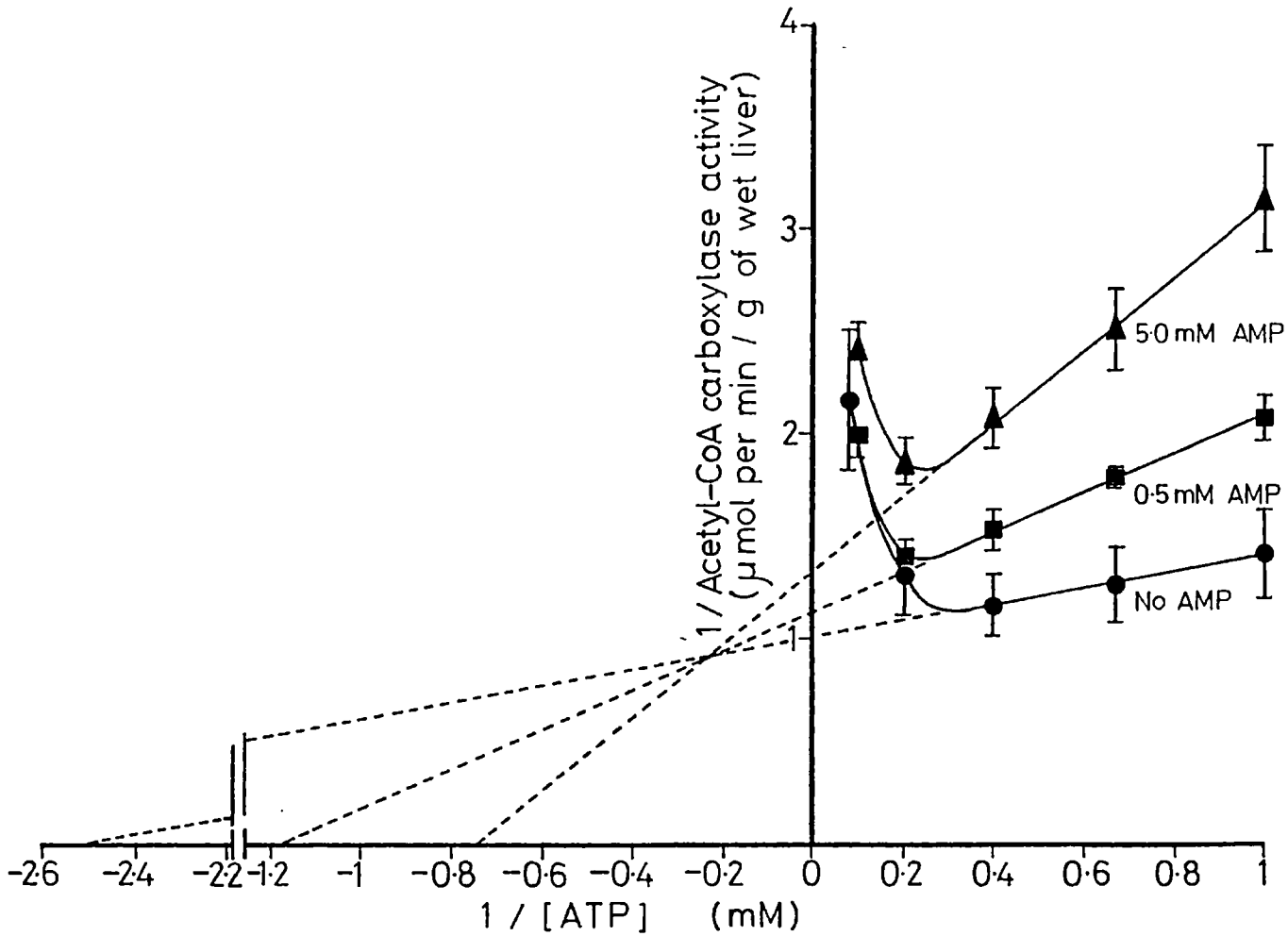

Fig. 17 Inhibition of hepatic acetyl-CoA carboxylase by AMP in the presence of increasing concentrations of ATP.

The total activity of acetyl-CoA carboxylase in the high speed supernatant of liver from fed rats, was assayed in the absence (●) and in the presence of AMP (0.5 mM ■ and 5 mM, ▲), at various concentrations of ATP over the range 1-10 mM. The results are plotted as the reciprocal of the enzyme activity vs. the reciprocal of the ATP concentration and are the means ± S.E.M. of three observations.

CHAPTER 5

RESULTS

5. Fatty acid synthesis in the regenerating liver of the rat

5.1. Introduction

In common with the genetically obese (ob/ob) mouse, the partially hepatectomised rat has a fatty hypertrophic liver. In this study it was found that the regenerating liver, like that of the obese (ob/ob) mouse, had an increased rate of de novo fatty acid synthesis. This increased rate of fatty acid synthesis in the regenerating liver was paralleled by an increase in the rate of fatty acid desaturation.

No intrinsic alteration in the capacity of the regenerating liver for fatty acid, could be found, when compared with livers from sham operated rats. Thus no difference in the activity of hepatic acetyl-CoA carboxylase, in vivo, or the rate of de novo fatty acid synthesis in perfused livers, could be detected between sham operated and partially hepatectomised rats.

5.2. General characteristics of rats following partial hepatectomy and sham operation

One of the problems associated with studies of the regenerating liver is the effects of non-specific changes in the size and function of the liver caused by the stresses of the operative procedure. To overcome this difficulty sham-operations were performed in parallel with the partial hepatectomies.

Following the operation there was a decline in the food intake and body weight of both the sham-operated and partially hepatectomised rats (Figs. 18 & 19). These effects were slightly more pronounced in the partially hepatectomised rats, but by four days post operation they were eating and growing normally once more. The sham-operated rats showed a slight drop in liver weight immediately after operation, as would be expected during a period of partial food deprivation (Fig. 20 'a'). The regenerating liver showed an increase in weight even by the first post operation day and had reached approximately 75% of the normal weight by the fourth day (Fig. 20 'b'). The % regrowth of the liver remnant was calculated as a function of body weight since the partially hepatectomised rats showed a set back to their growth and were approx. 16 g lighter than non-operated rats by the sixth day (Fig. 19).

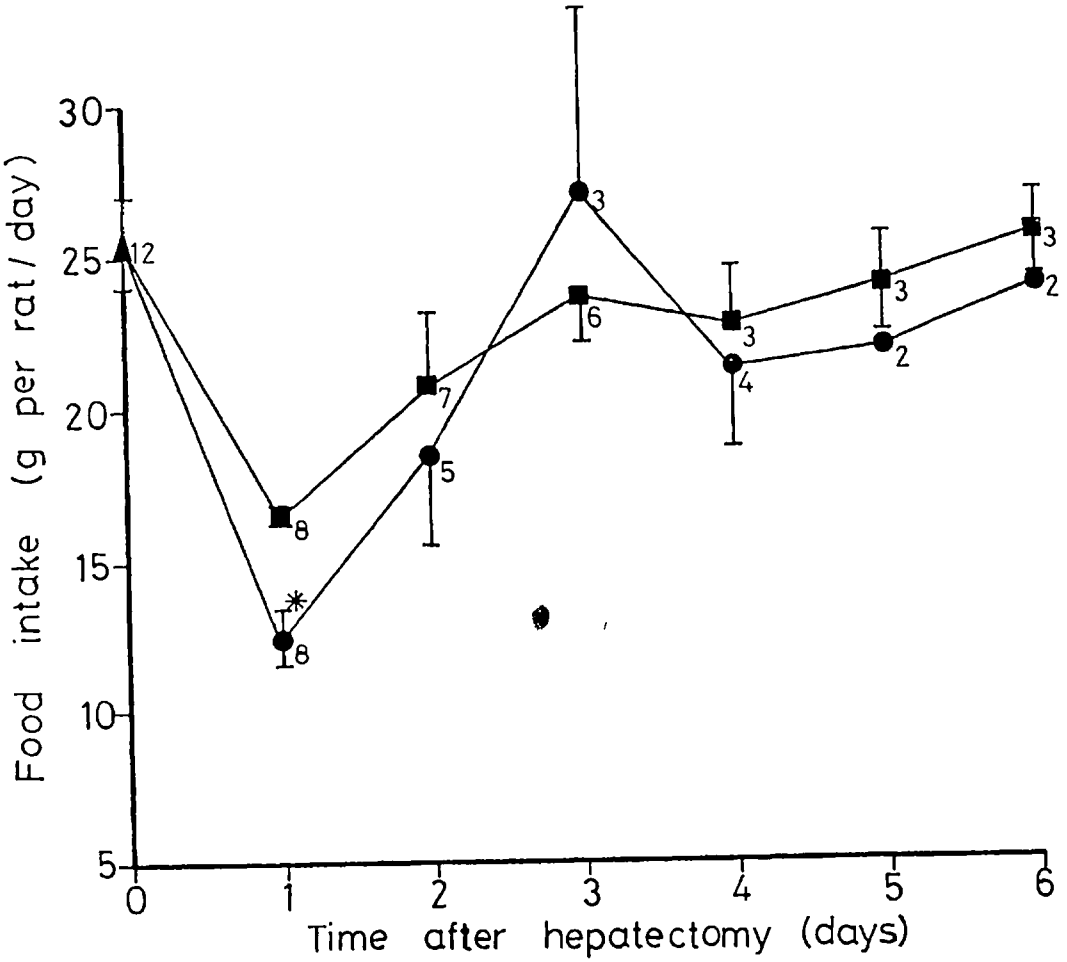

Fig. 18 Food intake of rats after partial hepatectomy and sham-operation.

The food intake of rats was recorded before (▲) and at 24 hour intervals after partial hepatectomy (●) or sham-operation (■). Exact details are given in the text (section (2.3.1.). Results are means ± S.E.M. of the numbers of observations indicated.
* $P<0.01$ cf. sham-operated values.

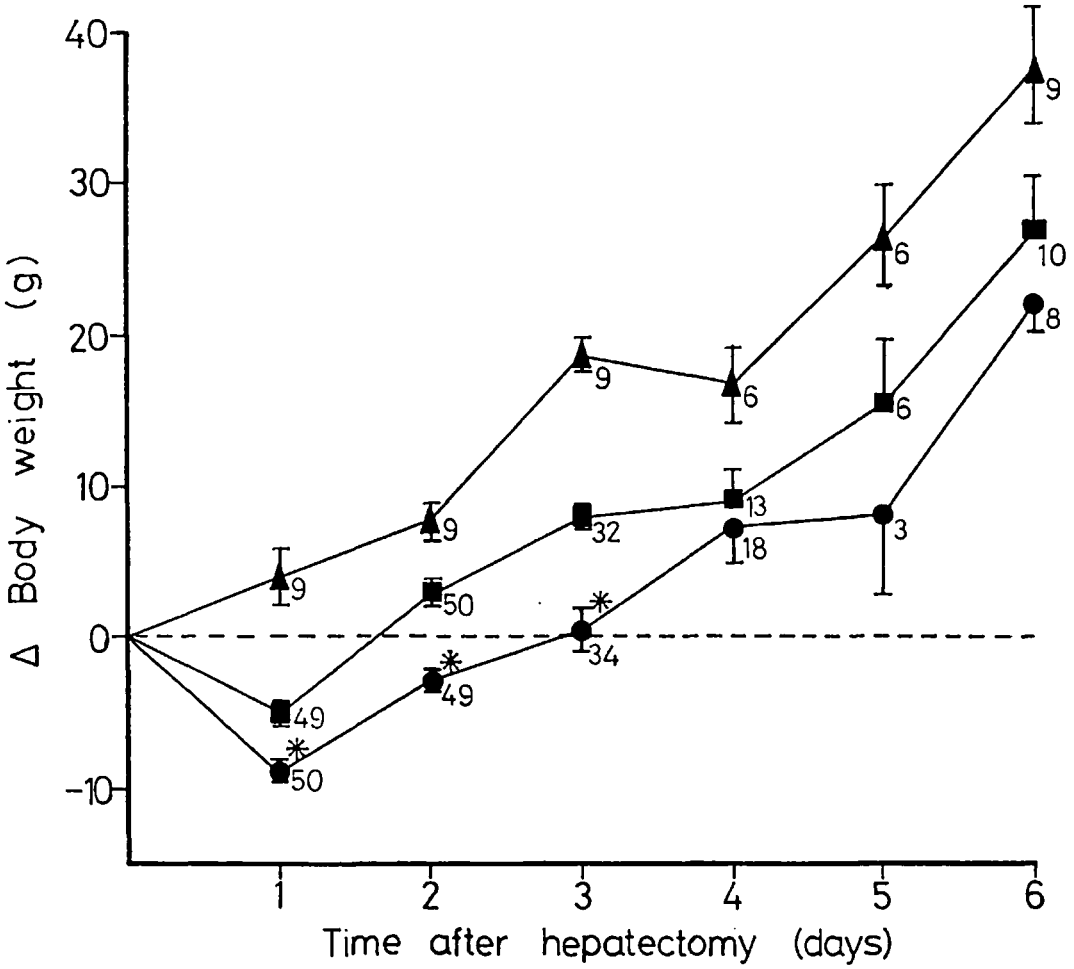

Fig. 19 Body weight changes of rats after partial hepatectomy or sham-operation.

Male Sprague-Dawley rats weighing 200 ± 1 g (99) underwent partial hepatectomy (●) or sham-operation (■), and the subsequent changes in body weight, compared with the immediate post-operative weight, were recorded over the period 1-6 days post-op. Other details are in the text. The body weight changes of unoperated rats (initial weight 202 ± 4 g, 12) (▲) were also recorded over a six day period for comparison. Results are means ± S.E.M. of the numbers of observations indicated.
* $P<0.001$ cf. sham-operated values.

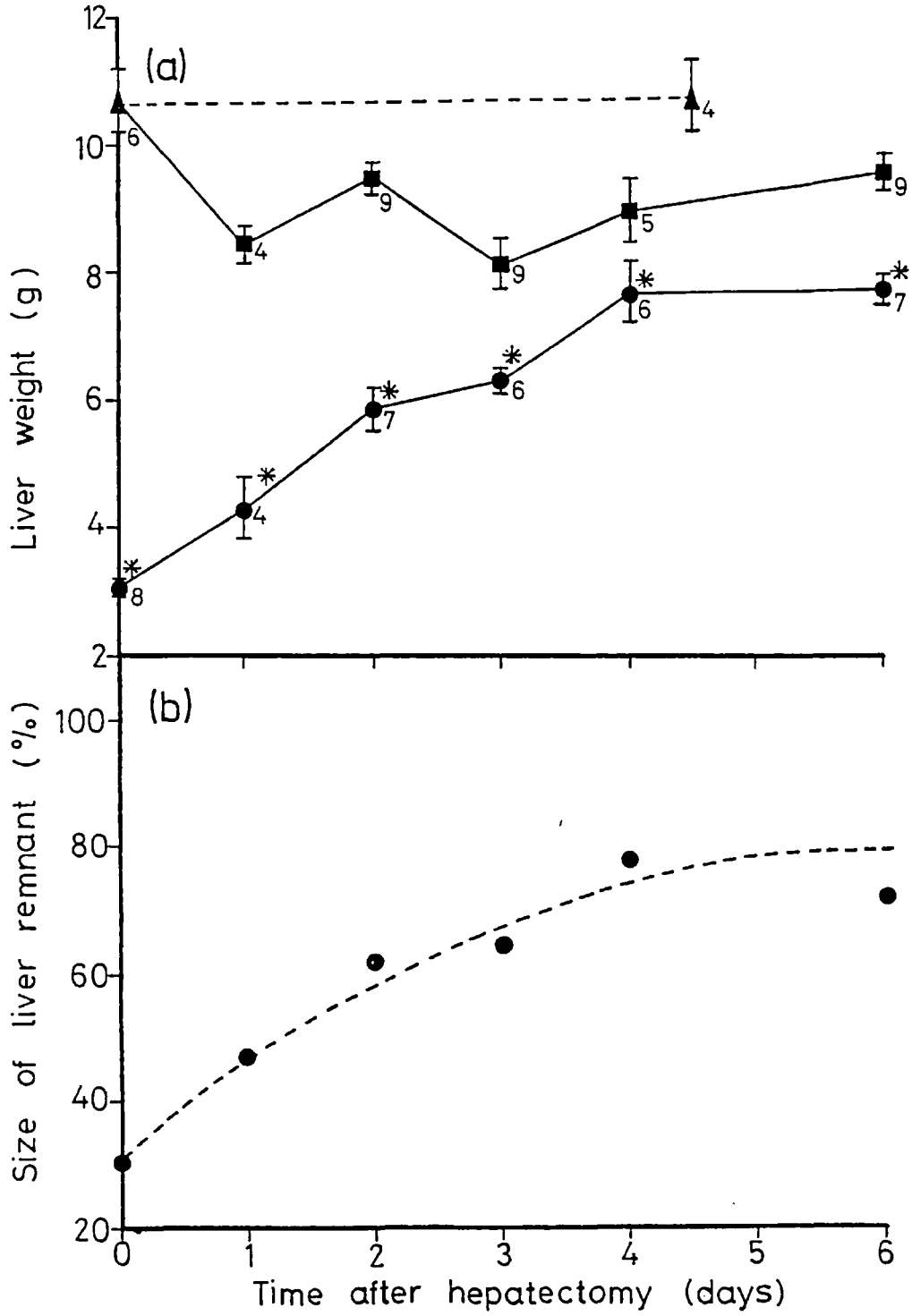

Fig. 20 Liver weight of rats after partial hepatectomy and sham operation.

(a) The livers of rats were removed and weighed at various times after partial hepatectomy (●) or sham-operation (■). The liver weights of two groups of non-operated rats fed ad libitum of comparable body weights to the experimental animals are shown for comparison (▲). Results are the means ± S.E.M. of the numbers of observations indicated.
(b) The size of the liver remnant in rats over the period 1-6 days after partial hepatectomy was expressed as % of the total liver present in weight-paired non-operated rats. Each point represents the means of at least 4 observations.
* $P<0.01$ cf. sham-operated values.

5.3. De novo synthesis of fatty acids in the regerating liver

The rates of de novo fatty acid synthesis in the regenerating liver at various times over the period 1-6 days post partial hepatectomy were measured using the incorporation of ^{3}H from $^{3}H_2O$. The rate of fatty acid synthesis, expressed per g of fresh liver, was increased in the regenerating liver over the period 1-3 days post-op., compared with either sham-operated or non-operated rats (Fig. 21 'a'). This increase in the rate of fatty acid synthesis coincided with the period when liver regrowth was most rapid (Fig. 20). The rate of fatty acid synthesis per whole liver was also higher in partially hepatectomised rats compared with sham-operated rats (Fig. 21 'b').

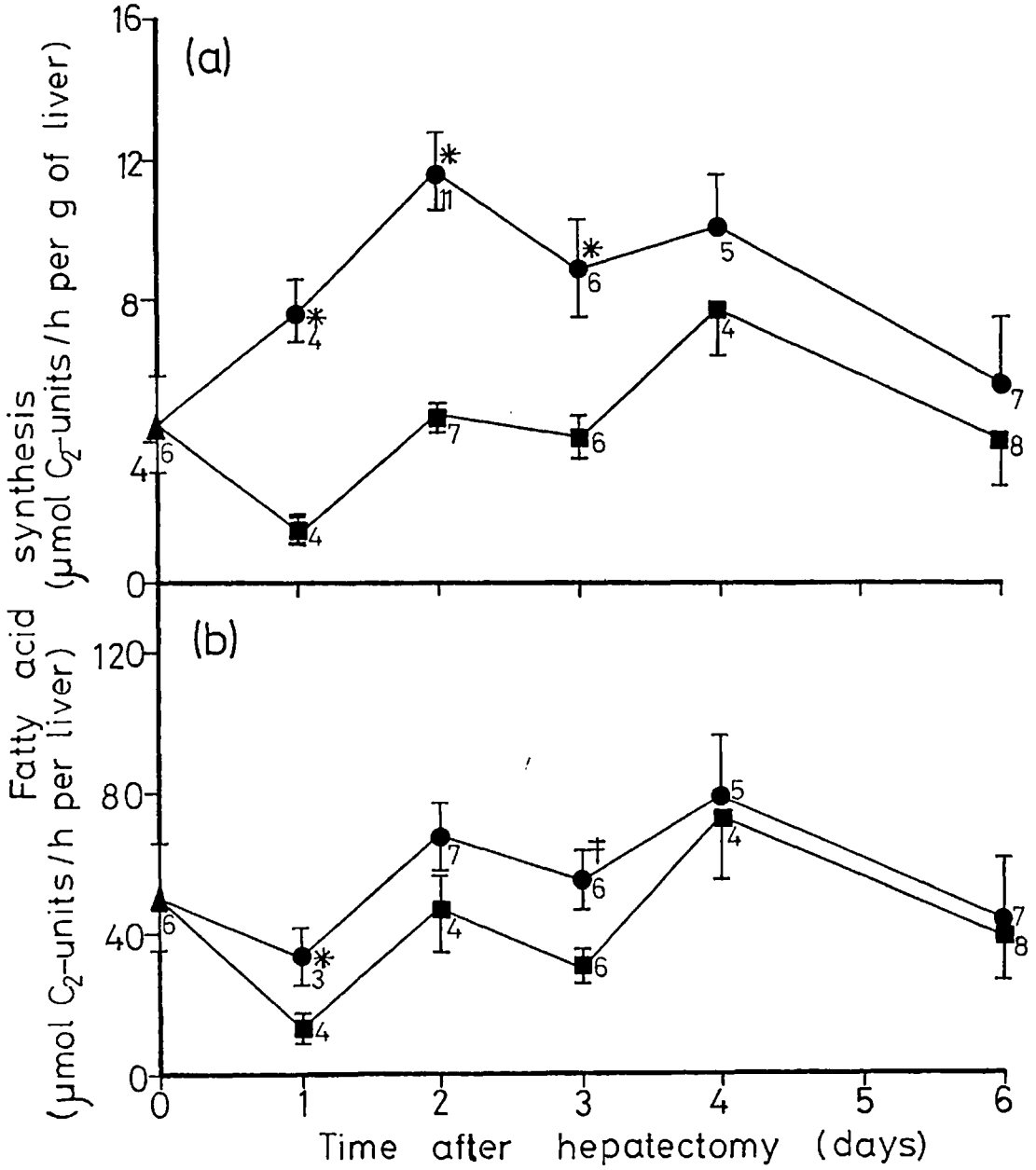

Fig. 21 Hepatic fatty acid synthesis in rats after partial hepatectomy or sham-operation.

At various times over the period 1-6 days after partial hepatectomy (●) or sham-operation (■) rats were injected with ^3H_2O. Liver fatty acids were analysed for incorporation of ^3H after 1 hour. The values for non-operated rats (▲) are also given. Rates of fatty acid synthesis are expressed as μmoles of C_2-units/hour, either (a) per g of fresh liver, or (b) per whole liver. Other details are given in the text. Results are means ± S.E.M. of the numbers of observations indicated.

* $P<0.01$ cf. sham-operated values.

ǂ $P<0.05$ cf. sham-operated values.

5.4. General aspects of lipid metabolism *in vivo* following partial hepatectomy

5.4.1. Fatty liver after partial hepatectomy

The phenomenon of fat accumulation in the liver following partial hepatectomy is well established (see section 1.5.). In this study the lipid content of the regenerating liver was increased only on the first day post-op. when compared with non-operated rats (Table 15). The sham-operated rats showed a relative decline in hepatic fat content probably due to mobilization of fat stores in response to the decrease in food intake following the operation. Thus in comparison with the sham-operated rats, the partially hepatectomised rats had a higher hepatic fat content for 2 days post-op.

The increased *de novo* synthesis of fatty acids (section 5.3.) could account for the acretion of lipids in the liver; although this seems unlikely to be the only cause since the total lipid content / g of fresh liver had decreased to normal by the second day post-op. when the rate of hepatic fatty acid synthesis/g was at a maximum. If *de novo* synthesis of fatty acids significantly contributed to the fatty liver a relative increase in the major products of fatty acid synthesis (i.e. palmitic, stearic and oleaic acids) would be expected. The hepatic triacylglycerol fatty acid and total fatty acid contents of the liver were therefore analysed two days after partial hepatectomy by quantitative GLC (Salmon and Hems, 1976).

Table 15.

Hepatic total lipid content and cholesterol synthesis in rats following partial hepatectomy and sham operation

Rates of cholesterol synthesis and total lipid contents were determined in some of the livers from the rats described in Fig. 21. The rate of cholesterol synthesis was assessed from the incorporation of ^3H from ^3H_2O (see Fig. 21 for details). Results are the means ± S.E.M. for the number of observations in parentheses. Other details are in the text.

	Time after operation (days)	Partial hepatectomy	Sham operation
Hepatic cholesterol	0	3.1±0.4(6)	
synthesis	2	5.02±1.5(3)	4.2±0.3(3)
(μmol C_2-Units/h/g)	3	3.5 ±0.4(3)	2.4±0.8(3)
	4	3.4 ±0.7(5)	3.1±0.4(4)
	6	2.2 ±0.1(6)	2.3±0.1(6)
Liver total lipid	0	55 ±3(6)	
content	1	71±16(5)	46±2(4)
(mg/g wet wt.)	2	55±7.5(8)	39±2(6)
	3	58±11(6)	56±7(6)
	4	59± 4(6)	55±3(5)
	6	58± 6(7)	44±1(8)

Most of the extra lipid present in the regenerating liver could be accounted for by the increased content of triacylglycerols (Table 16). The proportional composition of the hepatic triacylglycerol fatty acids was virtually unaltered in partially hepatectomised rats compared with sham-operated control rats. In the total hepatic fatty acid fraction, the increases in the content of individual fatty acids in the regenerating liver remained fairly proportional except for the octadecenoates, the proportion of which nearly doubled following partial hepatectomy (Table 16). This increase in the regenerating liver compared to sham-operated control livers is in general agreement with the finding of Glende and Morgan (1968) and is explicable by the influx of plasma fatty acids (produced by adipose tissue lipolysis) to the liver, as well as by the increased rate of de novo fatty acid synthesis.

5.4.2. Synthesis of unsaturated fatty acids in the regenerating liver

The process of fatty acid desaturation was investigated in liver following partial hepatectomy (using the incorporation of ^{3}H from $^{3}H_2O$), since a parallelism has been demonstrated between the rate of lipogenesis and the rate of fatty acid desaturation (Gellhorn and Benjamin, 1966). Fatty acids were separated according to their degree of unsaturation by argentation-TLC following methylation (section 2.5.4.). The rate of incorporation of ^{3}H into all fatty acid fractions was increased in

Table 16.

Fatty acid composition of hepatic total triacylglycerols and total fatty acids of rats 2 days after partial hepatectomy or sham-operation

2 days after partial hepatectomy or sham-operation the component fatty acids of the triacylglycerol and total fatty acid fractions of rat liver were separated by quantitative G.L.C. (Salmon and Hems, 1976). Results are the means ± S.E.M. of 3 observations. Other details are in the text

* $P<0.01$ cf. sham-operated value

≠ $P<0.05$ cf. sham-operated value

		Triacylglycerol fatty acids						
		16:0	16:1	18:0	18:1	18:2		Total
Partial hepatectomy	μmol/g fresh liver	10.9±1.4≠	2 ±0.6	0.8±0.08≠	12.4±1.7≠	8.4±1.4≠		34.6±3.3*
	% of total	31.5	5.9	2.3	35.9	24.2		—
Sham-operation	μmol/g fresh liver	4.5±0.3	0.7±0.1	0.4±0.03	5.5±0.6	3.7±0.3		14.7±0.9
	% of total	30.3	4.7	2.6	37.2	25.1		—
		Total fatty acids						
		16:0	16:1	18:0	18:1	18:2	20:4	Total
Partial hepatectomy	μmol/g fresh liver	31.4±2.4	3.3±1.1	14.9±1.8	24.9±2.5*	17.9±0.3	10.6±1.5	103±0.6≠
	% of total	30.5	3.2	14.4	24.2	17.4	10.3	—
Sham-operation	μmol/g fresh liver	22.3±2.5	2.3±0.7	12.1±1.4	10.6±1.5	14.7±2	13.6±2.1	76±10
	% of total	29.5	3	16	14	19.5	18	—

regenerating liver at 2 days post-op. when compared with the rate in the livers of sham-operated rats (Table 14). The incorporation of ^{3}H into hepatic di and poly-enoic fatty acids was also increased following partial hepatectomy, which suggests that elongation of 'essential' fatty acids was increased in parallel with the increase in the rate of synthesis of other fatty acids.

5.4.3. Cholesterol synthesis in the regenerating liver

Several processes associated with the provision of new tissue components show increased activity in the regenerating liver (eg. protein and DNA synthesis). It is therefore possible that an increase in the rate of synthesis of cholesterol (for use in membrane production) would occur following partial hepatectomy. The rate of cholesterol synthesis was followed with $^{3}H_2O$ over the period 1-6 days post partial hepatectomy and sham-operation. The regenerating liver showed slightly higher rates of cholesterol synthesis over the period 1-4 days post-op. compared with the livers of sham-operated rats, although no significant difference was found (Table 15).

5.4.4. Synthesis of fatty acids in adipose tissue, in vivo, after partial hepatectomy and sham-operation

Rats were injected with $^{3}H_2O$ and [U-^{14}C]-D-glucose, intraperitonealy, at various times over the period

Table 14.

Synthesis of unsaturated fatty acids in the liver of rats 2 days after partial hepatectomy or sham-operation

2 days after partial hepatectomy or sham-operation rats were hightly anaesthetised with diethyl-ether and injected I.V. with ^3H_2O. After 100 min a sample of liver was taken for extraction of total fatty acids. Fatty acids were separated according to degree of saturation by argentation-T L C and rates of synthesis were determined from the ^3H content. Other details are in the text. Results are means ± S.E.M. of 5 determinations.

* $P<0.01$ cf. sham operated value

		Total	Saturated	Mono-enoic	Di-enoic	Poly-enoic
Partial hepatectomy	μmoles C_2-units/h/g fresh liver	10±3	6±2	1.6±0.8	0.3±0.08	1.5±0.1*
	% of total	—	65.3	16.3	3.1	15.4
Sham operation	μmoles C_2-units/h/g fresh liver	3.9±0.4	2.5±0.3	0.4±0.05	0.2±0.4	0.9±0.08
	% of total	—	63.6	10.1	4.4	22

1-6 days after partial hepatectomy or sham-operation. There were no significant differences in the rates of fatty acid synthesis in the adipose tissue of partially hepatectomised and sham-operated rats over this period (Table 17). Both groups of rats showed a decline in the rate of adipose tissue fatty acid synthesis when compared with non operated rats. Accurate rates of fatty acid synthesis could not be calculated from the incorporation of ^{14}C from [U-^{14}C]-glucose, into fatty acids, since the specific radioactivity of the plasma glucose was continuously changing. An estimate of the contribution of glucose carbon to fatty acid synthesis in the liver compared with that in adipose tissue can however be obtained from the quotient:-

$$\frac{[(\text{DMP due to } ^{14}C/\text{DMP due to } ^{3}H) \text{ in adipose tissue fatty acids}]}{[(\text{DMP due to } ^{14}C/\text{DMP due to } ^{3}H) \text{ in liver fatty acids }]}$$

(Hems et al, 1975). This quotient varied between 4-9 in the present study showing that blood borne glucose contributed at most 25% of the carbon required for fatty acid synthesis in the liver (Table 17). No differences in the quotient were found between partially hepatectomised and sham-operated rats, although the ratio was decreased after operation compared with non-operated rats.

5.4.5. The concentration of liver glycogen and serum lipids after partial hepatectomy and sham-operation

There are established correlations between the rate of fatty acid synthesis in liver and both hepatic

Table 17.

Synthesis of fatty acids in adipose tissue and the relative contribution of glucose carbon to fatty acid synthesis in liver and adipose tissue in rats after partial hepatectomy

In some of the rats described in Fig. 21 and Table 15 the rate of fatty acid synthesis in the epididymal fat pad was measured using ^3H_2O. In some of these rats a trace dose of [U-^{14}C]-glucose (12 μCi; S.R.A. of stock 3 mCi/mmol was given with ^3H_2O, and the ratio [^{14}C/^{3}H in adipose tissue fatty acids]/[^{14}C/^{3}H in liver fatty acids] was measured one hour later. Other details are in the text. Results are means ± S.E.M. for the number of observations in parentheses.

* $P<0.05$ Compared with all other values

	Time after operation (days)	Partial hepatectomy	Sham operation
Adipose tissue fatty acid synthesis (μmol C_2-Units/h/g)	0	7.7±1.6(6)*	
	1	—	2.6±0.3(4)
	2	1.7±0.5(10)	2.1±0.5(8)
	3	2.5±0.8(6)	1.1±0.3(5)
	4	2.9±0.9(4)	2.9±1.3(4)
	6	2.6±0.4(7)	3.2±1.2(7)
Ratio $\left[\frac{^{14}C \text{ in D.P.M.}}{^3H \text{ in D.P.M.}} \text{ adipose tissue}\right] / \left[\frac{^{14}C \text{ in D.P.M.}}{^3H \text{ in D.P.M. in fatty acids}} \text{ liver}\right]$	0	10±3(5)	
	2	5±2 (10)	4±0.6(9)
	3	5±0.3(4)	6±3 (3)
	6	9±2 (3)	7±2 (4)

glycogen content (Salmon et al, 1974) and the concentration of serum unesterified fatty acids (Mayes and Topping, 1974). To assess the importance of these factors in regenerating liver the concentrations of liver glycogen, serum total unesterified fatty acids (TUFA) and serum triacylglycerols were measured in rats over the period 1-6 days post partial hepatectomy or sham-operation.

The liver glycogen content declined rapidly following partial hepatectomy and remained low over the period of maximum lipogenesis i.e. 1-3 days post-op. In contrast, the liver glycogen concentration of the sham-operated rats fell immediately following the operation but had returned to normal levels by the second day post-op. (Fig. 22).

The serum unesterified fatty acid concentration of the rats increased after partial hepatectomy and sham-operation (Fig. 23 'a'). Both groups showed a transient fall in serum triacylglycerol concentration immediately after operation but levels had returned to normal by the second day post-op. (Fig. 23 'b').

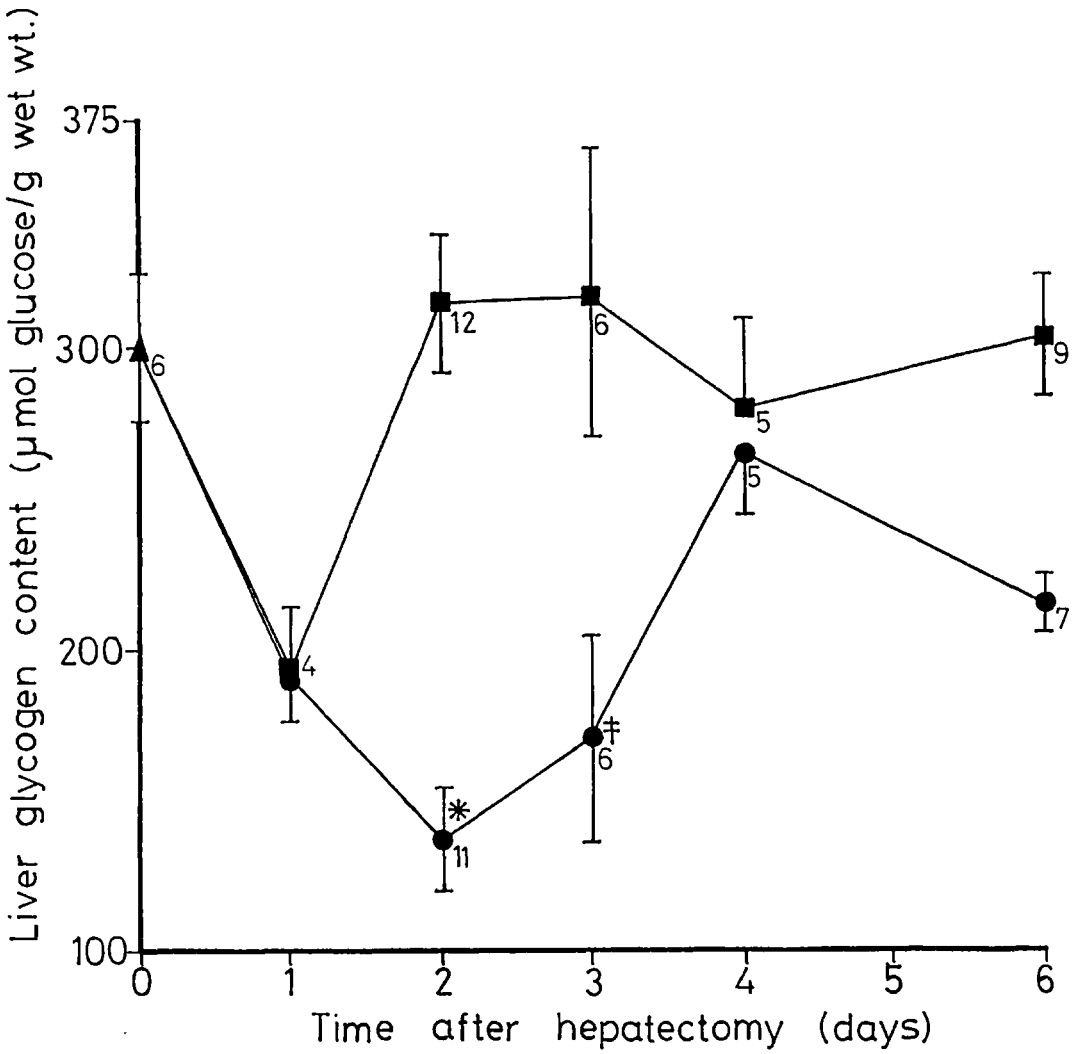

Fig. 22 Hepatic glycogen content of rats after partial hepatectomy or sham-operation.

The liver glycogen concentration of rats was measured at various times over the period 1-6 days post partial hepatectomy (●) or sham-operation (■). The value obtained from non-operated rats (▲) (i.e. zero time) is also given. Other details are in the text. Results are means ± S.E.M. of the numbers of observations indicated.
* $P<0.001$ cf. sham-operated value.
‡ $P<0.05$ cf. sham-operated value.

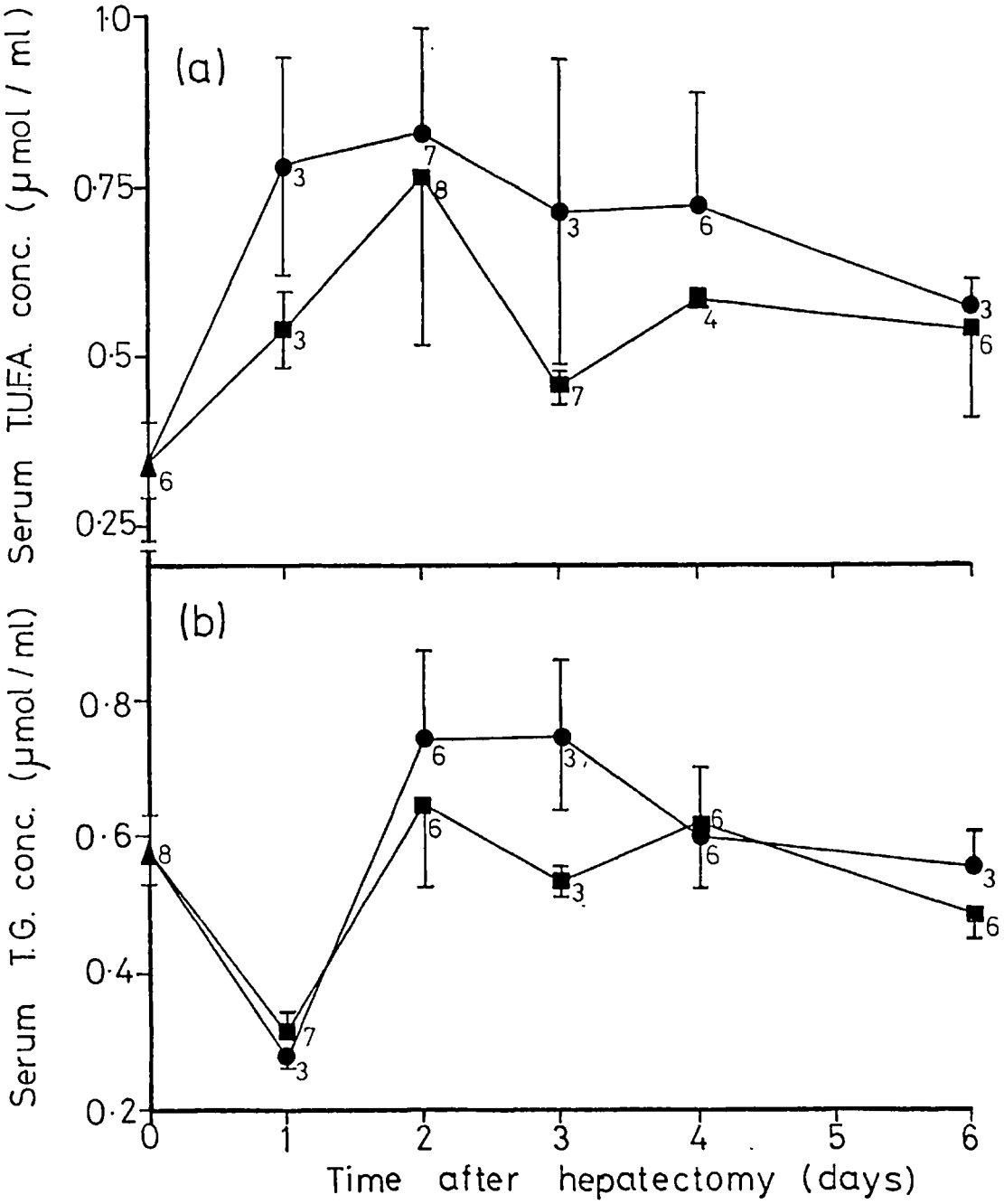

Fig. 23 Concentrations of unesterified fatty acids and triacylglycerols in the serum of rats after partial hepatectomy or sham-operation.

At various times after partial hepatectomy (●) or sham-operation (■) rats were quickly stunned and serum was prepared for determination of (a) total unesterified fatty acids (T.U.F.A.) and (b) total triacylglycerols. The values for non-operated rats (▲) are given for comparison. Results are means ± S.E.M. of the numbers of observations indicated.

5.5. Control of fatty acid synthesis in the regenerating liver

5.5.1. The activity of acetyl-CoA carboxylase in the regenerating rat liver

Acetyl-CoA carboxylase is widely held to be the site for controlling the rate of fatty acid synthesis (section 1.3.2.). The activity of this enzyme was therefore measured in regenerating liver on the second post-op. day when fatty acid synthesis/g fresh liver was maximal. No differences in either the initial or total activities of acetyl-CoA carboxylase were found, although the ratio % initial activity/total activity was slightly (but not significantly) higher (Table 18). Comparison of Fig. 21 and Table 18 shows that the initial activities of the enzyme were of the same order as the rates of fatty acid synthesis.

5.5.2. Fatty acid synthesis in perfused livers from partially hepatectomised and sham-operated rats

To decide whether the increased rate of fatty acid synthesis observed in the regenerating liver *in vivo* reflected an intrinsic alteration in the capacity of the liver for fatty acid synthesis, livers from partially hepatectomised and sham-operated rats were perfused under controlled conditions (Table 19). The three sets of conditions used were chosen because of the significance of

Table 18.

The activity of acetyl-CoA carboxylase in the liver of rats 2 days after partial hepatectomy and sham-operation

2 days after partial hepatectomy or sham-operation the initial and total (incubated with 10 mM citrate and 15 mM magnesium at 37° for 30 min) activities of acetyl-CoA carboxylase were assayed in the liver. Other details are in the text. Results are means ± S.E.M. for 7 observations

	Acetyl-CoA carboxylase activities (μmoles CO_2 incorporated/g wet liver/min)		
	Initial	Total (plus citrate & magnesium)	% initial/total
Partial hepatectomy	0.26±0.02(7)	0.57±0.05(7)	47±4(7)
Sham operation	0.22±0.02(7)	0.6 ±0.04(7)	31±2(7)

Table 19.

Fatty acid synthesis in perfused livers from rats 2 days after partial hepatectomy or sham-operation

Livers were perfused with perfusion medium (see 2.4.2.) containing the additives shown below. Glucose was present from the begining of all perfusions at the concentrations shown. In those perfusions with C_3-substrates, lactate was present at an initial concentration of 10 mM and a mixture of lactate, glycerol and pyruvate (3:2:1 molar ratio, total concentration 0.33 M) was infused at the rate of 3 ml/hour. Sodium oleate was added slowly as a warm solution (20 mM pH 8.5) to the perfusion medium containing albumin. 10 mCi of ^3H_2O and 12 μCi of [U-^{14}C]-glucose (S.R.A. 3 mCi/mmol) were added 30 min after the start of perfusion and a liver sample was taken 60 min later for analysis of liver fatty acids. Other details are in the text. Results are means ± S.E.M. for the numbers of perfusions indicated

Substrates in perfusate	No. of perfusions	Fatty acid synthesis (μmol C_2-units/h/g fresh liver)			
		Partial hepatectomy		Sham operation	
		Total rate	Rate from glucose	Total rate	Rate from glucose
Glucose (30 mM)	3	13.9±2.9	2.8±0.6	17.2±5.1	4.6±1.5
Glucose (15 mM) Lactate (10 mM) C_3 substrates	3	12.8±5.2	0.11±0.05	19.0±1.5	0.16±0.02
Glucose (5 mM) Oleate (0.5 mM)	4	3.2±0.5	—	3.8±0.5	—

the constituents added to the perfusate in the regulation of hepatic fatty acid metabolism. No difference could be found in the rate of fatty acid synthesis, in the regenerating liver compared with that in livers from sham-operated animals, under any of the three conditions used (Table 19).

CHAPTER 6

6. DISCUSSION

6.1. Hormonal control of fatty acid synthesis in the normal mouse liver

Comparison of the concentration dependence of the effects of a hormone on different processes within a tissue can shed light on the mechanisms involved.

Thus there was a difference in sensitivity between the inhibition of fatty acid synthesis and the stimulation of glucose output in the perfused lean mouse liver in response to glucagon. Glucagon did not inhibit fatty acid synthesis in short-term perfusions at concentrations below 10^{-9} M. Glucose output was stimulated at glucagon concentrations of 10^{-11} - 10^{-10} M, i.e. the same concentration range as has been shown to occur in plasma (Unger, 1976).

There was no inhibition of lipogenesis in short-term flow-through perfusions under optimal substrate conditions in which fatty acid synthesis was measured using tritium oxide and [U-^{14}C]-L-lactate, two of the most suitable substrates for measuring the process (Salmon et al, 1974).

In longer, recycling perfusions, glucagon at a concentration of 10^{-9} M, was found to inhibit fatty acid synthesis. This agrees with the observations of Regen and Terrell (1968) and Exton et al (1972) using the perfused

rat liver, Haugaard and Stadie (1953); Akhtar and Bloxham (1970); Bloxham and Akhtar (1972); Allred and Roehrig (1972, 1973); Bricker and Levey (1972 a & b); Bricker and Marraccini (1975); Meikle et al (1974) and Raskin et al (1974) using liver slices and Capuzzi et al (1971, 1974); Harris (1975); Edwards (1975); and Muller et al (1976) using cell suspensions. During these experiments it is likely that glycogen, which is one of the prefered substrates for hepatic fatty acid synthesis (Salmon et al, 1974, Clark et al, 1974), was depleted from the liver cells. Glucagon at concentrations above 10^{-9} M caused rapid loss of glycogen from perfused livers and in experiments lasting longer than 25 min there was almost total depletion of hepatic glycogen stores.

Glucagon also caused a stimulation in the rate of conversion of lactate into glucose. This apparent stimulation of gluconeogenesis may operate through a reduction of the activity of pyruvate kinase (Rognstad and Katz, 1977) caused by glucagon stimulated phosphorylation of the enzyme (Feliu et al, 1976) leading to inhibition of glycolysis.

From the above it can be seen that livers treated with glucagon would convert lactate and glycogen, the favoured precursors for fatty acid synthesis, into the less favoured precursor glucose (Salmon et al, 1974). Inhibition of fatty acid synthesis would be more rapid in preparations which already have a reduced glycogen content, such as, liver slices and cell suspensions, and it is in these preparations that glucagon produces the greatest inhibitory

effects. In cell suspensions cyclic-AMP has an increasing inhibitory effect on lipogenesis with time (Harris, 1975), as would be expected from the above.

In the experiments conducted in this study glucagon had no effect on either the total assayable activity of acetyl-CoA carboxylase or on the ratio of initial/total activities. The alteration in the proportion of hepatic acetyl-CoA carboxylase in the active form reported in liver slices (Allred and Roehrig, 1972, 1973), cell suspensions (Muller et al, 1976) and in intact rats (Klain and Weiser, 1973) in response to glucagon may not have been due to a direct primary short-term action of the hormone. The effect, particularly in cells, may have been secondary to alterations in substrate supply. Acetyl-CoA carboxylase is sensitive to alteration in the concentration of a variety of intracellular modifiers (Numa et al, 1965; Carlson and Kim, 1974 b) although cyclic-AMP does not appear to be one of them (Carlson and Kim, 1974 a; Cook et al, 1977).

McGarry et al (1975) have reported an increase in the hepatic capacity for ketogenesis in response to glucagon which they suggested was secondary to glycogen depletion. Since ketogenesis is the result of β-oxidation of fatty acids, the first step of which is the activation of fatty acids to long-chain acyl-CoA, a stimulation of the process would also tend to inhibit fatty acid synthesis since long-chain acyl-CoAs are potent inhibitors of acetyl-CoA carboxylase (Numa et al, 1965; Carlson and Kim, 1974 b).

If the inhibition of fatty acid synthesis by glucagon is secondary to alterations in carbohydrate metabolism it may still have a role in the control of hepatic lipid metabolism in the intact animal, but would be slower in onset than some of the more rapid effects of the hormone. Glucagon can also prevent the induction of acetyl-CoA carboxylase and fatty acid synthetase in the intact animal in response to refeeding (Volpe and Marasa, 1975a).

Vasopressin (Ma and Hems, 1975) adrenaline and angiotensin II (this study) all showed potent inhibition of fatty acid synthesis (whether measured with $^{3}H_2O$ or [U-^{14}C]-labelled glucose or lactate) and stimulation of glucose output in the perfused mouse liver. This inhibition of hepatic fatty acid synthesis by adrenaline shows agreement with the findings of Haugaard and Stadie (1953) in rat liver slices.

Adrenaline and angiotensin II at the lower doses tested (5 ng/ml and 1 ng/ml respectively) did not stimulate glucose release or cause an inhibition of fatty acid synthesis. A ten-fold higher dose, of the two hormones, stimulated glucose release and inhibited fatty acid synthesis. These results indicate equivalence in the sensitivity of the effects on glycogen metabolism and fatty acid synthesis in the perfused mouse liver in response to vasopressin, adrenaline and angiotensin II. This equivalence indicates that they are probably both of a primary nature, and may share a common mechanism of action. At least in the rat, neither vasopressin (Kirk and Hems, 1974) nor

angiotensin II (Hems et al, 1978) act via cyclic-AMP, since no rise in cyclic-AMP levels is seen in response to these two hormones. Adrenaline also may not act via cyclic-AMP since at least one major action has been shown to be independent of cyclic-AMP concentration (Tolbert et al, 1973; Tolbert and Fain, 1974; Saitoh and Ui, 1976).

Treatment of the perfused mouse liver with concentrations of adrenaline, angiotensin II or vasopressin, sufficient to inhibit fatty acid synthesis, caused a reduction in the initial activity of acetyl-CoA carboxylase. Adrenaline and angiotensin II had little effect on the total activity of the enzyme and so altered the ratio of initial/total activities to a significant extent. Vasopressin on the other hand had an effect on both the total and initial activities of the enzyme so that the ratio of initial/total activities remained virtually unaltered. The magnitude of these effects on acetyl-CoA carboxylase was of a similar order to those on fatty acid synthesis and may indicate the site of a direct hormonal effect in controlling hepatic lipogenesis in the mouse. Since the carboxylation of acetyl-CoA is the first committed step of fatty acid synthesis alterations in the activity of acetyl-CoA carboxylase in response to hormones would provide a very specific mechanism for controlling the rate of the complete pathway.

Vasopressin, adrenaline and angiotensin II are all potent vasoconstrictor agents, in vivo, which are released into the blood under conditions of acute stress (e.g.

haemorrhagic shock), in order to maintain blood pressure. They also show vasoconstrictor activity in the perfused rat liver (Hems et al, 1976). At the concentrations used in this study none of the hormones caused vasoconstriction in the perfused mouse liver (assessed from the total flow of perfusate through the liver). Although no overall change in the rate of flow of perfusate through the liver occurred, alterations in the flow within the liver cannot be ruled out, as has previously been suggested for adrenaline (Boobis and Powis, 1974).

The inhibition of fatty acid synthesis by vasopressin (Ma and Hems, 1975) occurs at concentrations which fall within the upper range of those shown to occur in plasma in stress states such as haemorrhagic shock (Ginsburg, 1968). The effects of adrenaline and angiotensin II on hepatic fatty acid synthesis and acetyl-CoA carboxylase activity occur at concentrations about ten-fold greater than the upper range reported to occur in plasma in various stress and pathological conditions such as diabetes, haemorrhagic shock and hypertension. Thus plasma adrenaline concentrations have been shown to be increased in diabetes melitus (Christensen, 1974), haemorrhagic shock (Jakschik et al, 1974), during exercise and hypertension (Chadakowska et al, 1975; Christensen et al, 1975) and in response to insulin (Goldfien et al, 1958; Christensen, 1974). In diabetes mellitus plasma renin activity has been reported to be raised (Christlieb et al, 1975) which would probably lead to increased angiotensin II concentrations. Plasma angiotensin II levels have also been shown to be raised in other states

such as haemorrhagic shock (Tuck et al, 1975) Vallotton et al, 1967; Scornik and Paladini, 1964), hypertension (Boyd and Peart, 1974) and during starvation (Nocenti et al, 1975).

It is therefore possible that these hormones play a part in the decline in the rate of fatty acid synthesis which occurs in diabetes, or in reducing the rate of the process in periods of acute food deprivation. They may also have a role in supressing fatty acid synthesis in 'catabolic' states such as haemorrhagic shock or during exercise when hepatic glycogen stores are broken down to increase plasma glucose concentrations.

6.2. Resistance of hepatic fatty acid synthesis to inhibition by adrenaline and angiotensin II in genetically obese (ob/ob) mice

Resistance of fatty acid synthesis in perfused livers from obese (ob/ob) mice to inhibition by vasopressin has been reported previously (Hems and Ma, 1976). This resistance, which was not reduced after relatively severe restriction of food intake, does not reflect a generalised insensitivity of the obese (ob/ob) hepatocyte to vasopressin. Thus glycogen breakdown was stimulated normally in perfused livers from diet restricted obese (ob/ob) mice (Hems and Ma, 1976).

In the present study resistance to inhibition of fatty acid synthesis in response to angiotensin II and adrenaline was found in the obese (ob/ob) mouse liver. This defect in response to adrenaline and angiotensin II, like that of vasopressin, was still apparent even in animals which had had their food intake severely limited. Such limitation of food intake is capable of abolishing the hyperglycaemia and reducing the hyperinsulinaemia which occurs in freely fed obese (ob/ob) mice (Abraham et al, 1971). Such diet-restricted obese mice have a similar body weight range to their lean littermates although their percentage body fat content is still higher (Alonso and Maren, 1955). While the alterations in carbohydrate metabolism, such as the excessive synthesis and turn over of glycogen and hyperglycaemia, can largely be reversed in the intact obese mouse by food deprivation (Abraham et al, 1971; Elliott et al, 1971) the

alterations in lipid metabolism do not show such remission. Thus there is no reduction in the increased rate of fatty acid synthesis (Jansen et al, 1967; Volpe and Marasa, 1975 b; Hems et al, 1975) or the increased assimilation and turnover of triacylglycerols (Salmon and Hems, 1973; Rath et al, 1974) and of glycerol and free fatty acids (Elliott et al, 1974). The obese (ob/ob) mouse liver also shows resistance to insulin action, which is associated with a reduction in the number of insulin receptors when compared with matched lean mouse livers (Kahn et al, 1973; Soll et al, 1975 a & b; Chang et al, 1975; Baxter and Lazarus, 1975). This resistance is however reversed by food deprivation (Chang et al, 1975; Soll et al, 1975 a) showing it to be secondary to the obesity. The defect in the response of the process of fatty acid synthesis to inhibition by vasopressin, adrenaline and angiotensin II is of a more fundamental nature since it cannot be reversed by food deprivation (Hems and Ma, 1976 and this study). This defect does not reside in the cyclic-AMP system since adenylate cyclase and hepatic glycogenolysis are stimulated by glucagon even in free fed obese (ob/ob) mice (Chang et al, 1975; Ma et al, 1978), and vasopressin (Kirk and Hems, 1974; Keppens and De Wulf, 1975) and angiotensin II (Hems et al, 1978) do not act via cyclic-AMP in the liver. This resistance of hepatic fatty acid synthesis to catabolic control by hormones does not seem to be total, since high concentrations of vasopressin were found to reduce the rate of fatty acid synthesis in perfused livers from freely fed obese mice (Hems and Ma, 1976).

The reistance of hepatic fatty acid synthesis in ob/ob mice to inhibition by vasopressin, adrenaline and angiotensin II discussed above may contribute to the pathogenesis of obesity, particularly since the rate of lipogenesis in the obese (ob/ob) mouse liver is increased over that occurring in the livers of lean mice (Hems et al, 1975). While the liver is not the major site of fatty acid synthesis in the obese (ob/ob) mouse (Hems et al, 1975), a lack of catabolic control over the process in this organ may still have serious consequences. The resistance of hepatic fatty acid synthesis to inhibition by adrenaline is paralleled by the resistance of adipose tissue lipolysis to stimulation by adrenaline which has been demonstrated in vitro (Enser, 1970; Yen and Steinmetz, 1972).

The resistance of the processes of lipid metabolism to catabolic control by hormones in the obese mouse may be the direct manifestation of the genetic lesion while the alterations in carbohydrate metabolism and insulin sensitivity are secondary to obesity. This is particularly likely since diet restriction had no effect on the resistance of hepatic fatty acid synthesis to inhibition by vasopressin (Hems and Ma, 1976) adrenaline or angiotensin II (this study) in the obese (ob/ob) mouse, but did reverse the alterations in carbohydrate metabolism (Abrahams et al, 1971; Elliott et al, 1971).

Thus the genetic lesion in the obese (ob/ob) mouse probably operates through an alteration in the mechanism which mediates the responses of lipid metabolism to extracellular effectors.

6.3. Lipid metabolism in the regenerating liver of the rat

The partially hepatectomised rat shows certain similarities to the genetically obese (ob/ob) mouse. Both have a hypertrophic, fatty liver, and show increased rates of hepatic fatty acid synthesis. They also show increased rates of synthesis and export of triacylglycerols from the liver.

During the period of rapid growth i.e. 1-4 days after operation) the regenerating liver requires new tissue components. The processes of DNA and protein synthesis and the regenerating liver have been studied extensively (see reviews by Bucher, 1963; Leduc, 1964 and Bucher and Malt, 1971). The growing liver also needs extra lipids for the production of new membranes and fat stores. The need for extra lipid could be met by uptake of circulating non-esterified fatty acids and cholesterol or by increased de novo synthesis. From the results obtained in this study it would appear that the need for extra fatty acids is met, at least in part, by increased synthesis de novo.

The synthesis of both saturated and monoenoic long-chain fatty acids was accelerated in the regenerating liver. The increases in the rates of fatty acid synthesis and desaturation were almost exactly proportional. A parallelism between the processes in other adaptive situations was observed by Gellhorn and Benjamin (1966).

The extra cholesterol which must be required by the growing liver, for membrane synthesis, was probably obtained

mainly from dietary intake. Using liver slices, from partially hepatectomised and sham-operated rats, Takeuchi et al (1976) found that the incorporation of ^{14}C-acetate into cholesterol and fatty acids was increased nearly three-fold in the regenerating liver, two days after operation. This increase, in the rate of cholesterol synthesis, could be prevented by feeding the animals a diet enriched in cholesterol. In the present study only a slight increase in the rate of cholesterol synthesis was observed, in the regenerating liver, when measured in vivo using $^{3}H_2O$. The discrepancy in the rate of hepatic cholesterol synthesis observed in this study and that of Takeuchi et al (1976) could be due to a difference in the dietary intake of cholesterol between the two groups of animals. Another explanation is that the total rate of cholesterol synthesis alters little following partial hepatectomy, but that the rate of incorporation of acetate into cholesterol is increased.

An explanation of the increased rate of fatty acid synthesis in the regenerating liver is necessary. Causes associated with post-operative stress can be excluded since the increase was not observed in the sham-operated rats. The transient phase of diminished food intake, decline in hepatic glycogen stores or increase in serum non-esterified fatty acid concentration would be unlikely to increase fatty acid synthesis. If these non-specific influences were responsible then adipose tissue fatty acid synthesis would also be likely to be stimulated. Adipose tissue fatty

acid synthesis declined however after the operation, which is the response which would generally be expected during post operative stress. The increased rate of hepatic fatty acid synthesis, following partial hepatectomy, must therefore reflect a regulatory response which is specific to the regenerating liver. This response does not seen to be due to an intrinsic increase in the capacity of the regenerating liver for fatty acid synthesis, since the rates of fatty acid synthesis in perfused livers from partially hepatectomised and sham-operated rats were similar. The total activity of acetyl-CoA carboxylase also showed no difference between the two groups of animals. The conditions under which the livers were perfused were carefully chosen for the significance in the regulation of hepatic fatty acid synthesis of the constituents added to the perfusate. No difference could however be found in the responses of the regenerating liver when compared to livers from sham-operated rats. It would therefore seen likely that the increase in the rate of fatty acid synthesis observed in the regenerating liver *in vivo* is brought about by the continuous action of unidentified intracellular or bloodborne modifiers such as hormones or substrates.

The rise in glucagon concentration, and fall in insulin concentration, in the plasma of the rats following partial hepatectomy (Leffert *et al*, 1975; Morley *et al*, 1976) would seem unlikely to lead to an increase in the rate of hepatic fatty acid synthesis. Other changes in hormone balance or substrate supply brought about either by alteration in the rate of release or through diminished

clearance may however be responsible for the observed increase in the rate of hepatic fatty acid synthesis. One interesting feature of liver regrowth following experimental injury is that the hypertrophy is more rapid the greater the proportion of the liver which is removed (Harkness, 1957; Islami and Pack, 1959; Rabinovici and Wiener, 1961; Hays et al, 1968). This would seem to imply that reduced clearance or release of a humoral factor or factors, by the liver, may have some role in controlling liver regeneration, although experiments designed to demonstrate this possibility have given equivocal results (see Bucher and Malt, 1971 and Leduc, 1964). Whether such a factor has a role in stimulating the de novo synthesis of fatty acids during liver regeneration remains to be clarified.

Following partial hepatectomy the rats showed a transient increase in the hepatic total lipid content ('fatty liver') as has been observed previously (Ludewig et al, 1939; Harkness, 1952; Bartsch and Gerber, 1966; Infante et al, 1969; Delahunty and Rubinstein, 1970). Analysis of the liver lipids by quantitative GLC showed that the increase in fatty acid content could almost entirely be accounted for by the increase in triacylglycerol concentration, in agreement with the findings of Glende and Morgan (1968) and Fex (1970 a). In contrast, the phospholipid concentration in the regenerating liver has been shown to remain constant throughout the growth period (Glende and Morgan, 1968; Tata, 1970; Fex, 1970 b; Bergelson et al 1974; and Fex and Thorzell, 1975). The regenerating liver shows an increase in the rate of triacylglycerol

synthesis per unit mass, as has been demonstrated with the use of labelled fatty acids (Fex and Olivecrona, 1968 a; Olivecrona and Fex, 1970 and Fex 1970 a) and glycerol (Olivecrona and Fex, 1970). It seems probable that the increase in synthesis and activity of phosphatidate phosphohydrolase which has been demonstrated in the liver remnant (Mangiapane et al, 1973) has a role in increasing the rate of triacylglycerol synthesis while the rate of phospholipid synthesis remains unaltered (Fex 1970 b and Girard et al, 1971).

The rate of triacylglycerol export is rapidly increased per gram tissue in the regenerating liver, mainly by increasing the amount of lipid coupled to the protein of the β-lipoprotein to compensate for the loss of liver mass, the rate of export per whole liver is thus maintained (Fex and Olivecrona, 1968 a & b; Delahunty and Rubinstein, 1970). The cause of the fatty liver following resection seems to be the inability of the liver remnant to synthesise lipoproteins at a sufficient rate initially to cope with the increase in the rate of synthesis of triacylglycerols (Infante et al, 1969; Delahunty and Rubinstein, 1970; Girard et al, 1971). The increase in the capacity of the liver remnant for triacylglycerol synthesis occurs in response to the increased flux of plasma nonesterified fatty acids through the liver. The increased flux of nonesterified fatty acids seems to be brought about by the decreased size of the liver following resection and the increase in plasma non-esterified fatty acid concentrations

due to mobilization of adipose tissue fat stores in response to post-operative stress (Delahunty and Rubinstein, 1970). This seems particularly likely since the accretion of lipid by the liver remnant can be prevented by continuous infusion of glucose into rats for 21 hrs following partial hepatectomy (see Bucher and Malt, 1970). This effect of glucose presumably operates by preventing mobilization of adipose tissue fat stores. Mobilization of adipose tissue fat stores can also be prevented by adrenalectomy or cordotomy, and the liver remnants of animals treated in this way do not show accumulation of lipid (see Bucher, 1963).

6.4. Effects of hypoxia on hepatic acetyl-CoA carboxylase activity

The activity of hepatic acetyl-CoA carboxylase can be regulated by alterations in the state of polymerisation of the enzyme and by phosphorylation and dephosphorylation of the enzyme protein. One or both of these mechanisms seems to be involved in the rapid loss of activity observed in response to ischaemia.

The initial activity of rat liver acetyl-CoA carboxylase was found to diminish by up to 80% in response to one min. of ischaemia. The degree of reduction in activity in response to ischaemia was similar in liver samples from normal fed rats and lean mice, deeply anaesthetised fed rats and perfused rat livers made ischaemic by cessation of medium flow. This implies that the reduction in the initial activity of acetyl-CoA carboxylase, occurs in response to ischaemia and is not caused by nerve stimulation *in vivo*, since the response also occurred in perfused liver preparations. A similar response to hypoxia was found in those samples taken from livers after 3 min of perfusion with anoxic medium.

The reduction in the initial activity of acetyl-CoA carboxylase within the liver, in response to hypoxia, could be brought about in one of the following ways. 1) The enzyme could be inhibited by a compound produced in the liver during ischaemia. 2) The enzyme could be deactivated by removal of a modifier, such as citrate, or a substrate such as ATP. 3) The enzyme could be converted

to a less active from e.g. by phosphorylation and/or depolymerisation.

Direct effects of ATP or citrate on the activity of the enzyme cannot be responsible for the changes observed, since optimal concentrations of both these substances are provided in the assay.

A change in the state of polymerisation and/or phosphorylation of the enzyme seems to be the most likely explanation for the reduction in activity observed. The optimum conditions for phosphorylation of acetyl-CoA carboxylase in vitro are relatively high concentrations of ATP in the presence of magnesium ions (Carlson and Kim 1974 a) so that the 50% decrease in the concentration of ATP which occurs within the first minute of hypoxia (Hems and Brosnan, 1970) would not assist this process. AMP which shows a 3-fold increase in concentration within the first minute of ischaemia (Hems and Brosnan, 1970) has been shown to inhibit both lipogenesis (Harris and Yount, 1975) and acetyl-CoA carboxylase (this study). This does not seem to be the entire explanation for the reduction in the initial activity of acetyl-CoA carboxylase observed, however, since the inhibition by AMP was not reduced by incubation of the liver homogenate with citrate and magnesium ions (results not shown). On the other hand the total activity of the enzyme (i.e. after treatment with citrate and magnesium) in the ischaemic livers was similar to that in normal livers. The concentration of AMP in the high speed supernatant of liver after 2 min of ischaemia can be calculated to be about

0.1 mM i.e. sufficient to cause approximately 10% inhibition, and a further 10-fold dilution of the supernatant occurred during the assay.

Adenosine-3',5'-monophosphate which has been suggested to inhibit fatty acid synthesis at the acetyl-CoA carboxylase step (Allred and Roehrig, 1973; Klain and Weiser, 1973) would not cause the inhibition seen here, since the concentration of this nucleotide does not increase in the liver in response to hypoxia (D.A. Hems, unpublished data).

From these considerations it would seem most likely that the rapid reduction in the activity of acetyl-CoA carboxylase, which occurs in response to hypoxia, in the liver, is brought about by two mechanisms 1) an increase in phosphorylation and/or depolymerisation of the enzyme protein and 2) inhibition of the enzyme by the increased tissue concentration of AMP. This increase in enzyme phosphorylation probably occurs in response to a change in concentration of one or a group of tissue intermediates which remain to be identified. The tissue concentrations of AMP which are known to occur in hypoxia viz. about 1 mM within 2 min of the onset of ischaemia in the fed rat (Hems and Brosnan, 1970) are sufficient to cause nearly 30% inhibition of acetyl-CoA carboxylase activity in the intact cell.

The activity of acetyl-CoA carboxylase in the obese (ob/ob) mouse liver showed a similar reduction in activity in response to ischaemia as that observed in the lean mouse liver. The rate of onset of the reduction of the initial

activity of the enzyme was however slower in the obese mouse liver, so that by 30 sec the enzyme showed only 30% reduction compared to 60% in the lean mouse liver.

This observation suggests that the control of acetyl-CoA carboxylase activity, in the obese (ob/ob) mouse liver, shows normal responsiveness to at least one stimulus. The lack of an inhibitory effect on lipogenesis in the obese (ob/ob) mouse liver by certain hormones (known to inhibit the process in the lean mouse liver) seems therefore to be due to a reduced sensitivity to specific extracellular effectors and not a generalised resistance to catabolic control.

6.5. Inhibition of hepatic acetyl-CoA carboxylase by AMP and ADP

The rapid reduction in the activity of acetyl-CoA carboxylase, seen in response to hypoxia, raises the possibility that some of the metabolites which show changes in concentration under these conditions may be modifiers of the enzyme. One such group of molecules are the adenine nucleotides. AMP and ADP were found to inhibit acetyl-CoA carboxylase at both 0.5 and 5 mM. Adenine, inosine, xanthosine, IMP and XMP only caused noticeable inhibition at the higher concentration. Therefore the inhibition of acetyl-CoA carboxylase by AMP and ADP could not have been due to the formation of these breakdown products in the liver homogenates. The degree of inhibition of the enzyme by AMP was not altered by other nucleotide monophosphates. As the assay was only 3 min in duration these data clearly demonstrate a short-term inhibitory effect of AMP and ADP on acetyl-CoA carboxylase. This inhibition could be competitive with ATP since increasing the concentration of triphosphate above that normally present in the assay reduced the inhibition due to both AMP and ADP (data for ADP not shown).

The inactivation of the enzyme by adenine nucleotides can be considered with regard to the known characteristics of hepatic acetyl-CoA carboxylase. On the evidence so far, the enzyme can exist in an active polymerised form and as an active dephospho-protein (Carson and Kim, 1973; Carlson and Kim 1974 a, Lee and Kim, 1977). The relationship

between the reversible polymerisation and phosphorylation processes is not clear. Following treatment with citrate and magnesium for 30 min, maximal activity is attained, presumably because the enzyme is both polymerised and dephosphorylated. Inhibition of this form of the enzyme (total activity) was observed with AMP to about the same degree as for the initial activity. This is compatible with the simple and prevailing view that both assays reflect activity of the same enzyme form (which is generated from inactive depolymerised and phosphorylated forms of the enzyme on incubation with citrate and magnesium).

In the extramitochondrial phase of the liver parenchymal cell AMP is a particularly sensitive indicator of the adenine nucleotide status as it undergoes larger proportional changes during anoxia than does ATP or ADP.

A linear relationship was found between the percentage inhibition of the enzyme and log AMP concentration, with 50% inhibition occurring at an AMP concentration of 4.6 mM. The inhibition was partially competitive with ATP with a Ki AMP of 4.1 mM under the assay conditions used. The apparent Km for ATP was 0.39 mM showing fairly close agreement with the value of 0.36 mM obtained by Carlson and Kim (1974 b).

The simplest interpretation of these observations is that of competition between the substrate analogue, AMP, and the substrate, ATP, for the active site, with the overall interaction further complicated by the inhibition

of the enzyme by high concentrations of ATP.

The inhibition of hepatic acetyl-CoA carboxylase by AMP seems likely to be operative *in vivo*. The AMP concentration in the liver varies from about 0.1-0.2 mM in the aerobic liver of fed animals, to about 1 mM in anoxic conditions (Brosnan *et al*, 1970; Hems and Brosnan, 1970). Over this concentration range inhibition of the enzyme by AMP would gradually increase switching off fatty acid synthesis as ATP availability declined and AMP accumulated. Such inhibition would probably be most significant in relation to the state of oxygenation of the liver, to which the tissue concentration of AMP is extremely sensitive.

This effect of AMP on the activity of acetyl-CoA carboxylase, would explain the inhibition of fatty acid synthesis by AMP which has been observed in liver slices and isolated hepatocytes (Harris and Yount, 1975).

The control of acetyl-CoA carboxylase activity by adenine nucleotides would also be relevant in other states where fatty acid synthesis is decreased and the concentration of AMP increased, e.g. starvation, after alcohol treatment and in response to the provision of free fatty acids. The activation of free fatty acids produces long-chain acyl-CoA and AMP, both of which are inhibitors of acetyl-CoA carboxylase. These effectors could provide the explanation for the short-term (and possibly the long-term) inhibition of *de novo* fatty acid synthesis which occurs in the liver when preformed long-chain fatty acids are available (Mayes and Toppin, 1974; Toppin and Mayes, 1976).

The findings of Akerboom *et al* (1978) that addition of oleate to isolated hepatocyte incubations caused a change in the state of adenine nucleotide phosphorylation in the mitochondrial fraction only is not consistent with the above hypothesis however since acetyl-CoA carboxylase is a cytoplasmic enzyme.

Inhibition of acetyl-CoA carboxylase by ADP would have the same physiological significance in controlling fatty acid synthesis as that described for AMP except that the changes in the concentration of ADP in the liver tend to be smaller in magnitude than those of AMP concentration.

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