THE LIPID METABOLISM IN LIVER AND PLASMA OF GENETICALLY OBESE (ob/ob) AND NON-OBESE MICE

by

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ABSTRACT

Lipoproteins (LP) in the plasma of mice were characterized by agarose-gel column chromatography, polyacrylamide-gel disc electrophoresis and preparative ultracentrifugation. Mice homozygous for the recessive gene ob exhibited hyperlipoproteinaemia compared to non-obese mice, largely owing to an increase in cholesterol in high density LP. Only one other major LP class was found in mice, very low density LP, which had a similar concentration and lipid class composition in plasma from both types of mice. Fatty acid (FA) profiles of tissue and plasma lipid classes were analyzed and differences between ob/ob and non-obese were most marked in the liver.

The rates of hepatic FA synthesis and esterification of plasma free FA were enhanced in ob/ob mice, as shown by experiments with (U-^{14}C)-glucose and (^{14}C)-palmitate. Plasma triglyceride (TG) turnover was increased in ob/ob mice, calculated from the time course of incorporation of ^{14}C from injected albumin-bound (U-^{14}C)-palmitate into plasma TG, and the rate of removal from plasma of murine very low density LPTG-(^{14}C)-FA or of an injected soybean oil emulsion.

The activity of the enzyme ATP-citrate lyase was measured in epididymal and scapular adipose tissue of mice. An increased hepatic activity, but a decreased activity in adipose tissue, was found in ob/ob mice.

The control of carbon supply for FA synthesis de novo was investigated in the perfused mouse liver. The total rate, measured by the

incorporation of ^{3}H from $^{3}H_2O$ into FA, closely resembled that found *in vivo* in the liver of mice fed *ad lib*. Perfusions with L-(U-^{14}C)-lactate and D-(U-^{14}C)-glucose showed that medium glucose at concentrations less than about 17mM was not a major carbon source of newly-synthesized FA, whereas lactate (10mM) markedly stimulated FA synthesis and contributed up to 50% of the carbon required for this process. The total rate of FA synthesis, and the contribution of glucose carbon to FA synthesis, were directly proportional to the initial hepatic glycogen content. The fraction of the tritiated lipid which was released into the perfusion medium was 0.19 and 0.25 of that in the liver in perfusions of non-obese and *ob/ob* mouse livers respectively. The major products of FA synthesis were saturated FA in TG and phospholipids. Cholesterol synthesis, also measured with $^{3}H_2O$, utilized a maximum of one-quarter of the acetyl residues required for FA synthesis. Oleic acid (about 0.4mM) inhibited the rate of FA synthesis in perfusions of livers from non-obese mice.

Alterations in hepatic FA metabolism and plasma TG turnover in *ob/ob* mice are discussed in terms of their role in genetic obesity. The control of hepatic FA synthesis in relation to events in carbohydrate metabolism are discussed, especially with respect to carbon sources of newly-synthesized FA, and the effect of glucose, glycogen and lactate in stimulating the rate of this process independently of their role as carbon precursors. In conclusion, it is suggested that enhanced rates of FA synthesis, esterification and LP secretion by the liver have a major role in the excessive TG accretion in adipose tissue of *ob/ob* mice.

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PUBLICATIONS

Some of the work described in this thesis has been published in the following papers:

D.Michael W.Salmon & Douglas A.Hems(1973)"Plasma Lipoproteins and the Synthesis and Turnover of Plasma Triglyceride in Normal and Genetically Obese Mice."Biochem.J. 136,551-563.

D.Michael W.Salmon,Neil L.Bowen & Douglas A.Hems(1974)"Fatty Acid Synthesis in the Perfused Mouse Liver."Biochem.J. 142,611-618.

"The road of excess leads to the palace of wisdom" (ca.1793)

William Blake.From The Marriage of Heaven and Hell,Proverbs of Hell.

CONTENTS

CHAPTER 2 (continued)

ABBREVIATIONS, SYMBOLS, CONVENTIONS AND DEFINITIONS.

Units and Physical constants.

c.p.m.	counts per minute
Ci	Curie (2.22×10^{12} d.p.m.)
d.p.m.	disintegrations per minute
E	extinction (absorbance)
g	acceleration due to gravity (981 cm s^{-2})
i.u.	international unit
k	rate constant
K_D	partition co-efficient (dimensionless) (= Ve-Vo/V_i)
log	logarithm (base 10)
ln	logarithm (base e)
u	micro (10^{-6}x)
ug	microgram (me)
ug-at	microgram (me) atom
umol	micromole
mM	millimolar (concentration)
M	molar (concentration)
mol	mole
mol.wt.	molecular weight
ml	millilitre
n	nano (10^{-9}x)
nm	millimicron (10^{-9}m)
P	probability of an event being due to chance alone

RTA	relative total activity (defined in Section 4.2.1)
RQ	respiratory quotient (= CO_2 produced (ml)/O_2 utilized (ml) at S.T.P.)
SD	standard deviation
S.E.M.	standard error of estimate of mean value
S.T.P.	standard temperature and pressure (25°C, 760mm.Hg)
Vo	void volume (ml)
Ve	elution volume (ml)
V_i	imbibed volume (ml)

Chemicals and Lipoproteins

ATP	adenosine-5-triphosphate
butyl PBD	5-(4-biphenylyl)-2(4-t-butylphenyl)-1-oxa-3,4-diazole
c-AMP	adenosine-3:5-monophosphate
CE	cholesteryl ester (3β-acyl-5-cholestene)
CEFA	cholesteryl ester fatty acid (acyl component of CE)
CoA & acyl CoA	coenzyme A and its acyl derivatives
DG	diglyceride (diacyl glycerol)
EDTA	ethylene-diamino-tetra-acetic acid
FA	fatty acid
FAME	fatty acid methyl ester
FC	free cholesterol (5-cholesten-3β-ol)
FFA	free fatty acid
HDL	high density lipoprotein (density 1.075-1.21 g ml^{-1})
IDL	intermediate density lipoprotein (density 1.05-1.075 g ml^{-1})
LCFA	long-chain fatty acid ($>C_{14}$)
LDL	low density lipoprotein (density 1.006-1.05 g ml^{-1})
MG	monoglyceride (monoacyl glycerol)
NAD+	nicotinamide-adenine dinucleotide, oxidized
NADH	nicotinamide-adenine dinucleotide, reduced
NADP+	nicotinamide-adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
P_i	orthophosphate (inorganic)
PCA	perchloric acid
PL	phospholipid
PLFA	phospholipid fatty acid

TC	total cholesterol (FC+CE)
TG	triglyceride (triacyl glycerol)
TGFA	triglyceride fatty acid
tris	2-amino-2-hydroxy-methylpropane-1,3-diol
VLDL	very low density lipoprotein (density less than 1.006 g ml^{-1})

Other abbreviations

g.l.c.	gas-liquid chromatography
t.l.c.	thin-layer chromatography
u.v.	ultraviolet

Nomenclature of Fatty Acids

When individual fatty acids are referred to (as after separation by g.l.c.), they are described by the number of carbon atoms in the molecule, followed by the number of double bonds per molecule (e.g.octadecadienoic acid is referred to as 18:2)

Trivial name	g.l.c. identification	recommended name*
palmitic acid	16:0	hexadecanoic
stearic acid	18:0	octadecanoic
palmitoleic acid	16:1	hexadec-9-enoic
oleic acid	18:1	octadec-9-enoic
linoleic acid	18:2	octadec-9,12-dienoic
linolenic acid	18:3	octadec-9,12,15-trienoic

*IUPAC rules for Nomenclature of Organic Chemistry.

Pure Appl. Chem. 11 1 (1965) Rule C4.

CHAPTER 1.

INTRODUCTION

1.5.3. The proportional contribution of (^{14}C) precursors in blood to hepatic triglycerides.

1.5.4. The study of hepatic lipid metabolism in the isolated, perfused mouse liver.

CHAPTER 1.

INTRODUCTION

1.1. Obesity in humans.

1.1.1. The incidence and pathological consequences of obesity.

Biochemical research on obesity has largely been motivated by the high incidence of this apparently dysfunctional condition in humans. Obesity is widely considered to be harmful to health, and statistics obtained from insurance companies are frequently quoted in support of this assertion (e.g. Metropolitan Life Insurance Statistical Bulletin, 1943), although the accuracy of the representation of the overweight population in such statistics has been questioned (Keys, 1955; Chlouverakis, 1973).

Recent surveys indicate a positive correlation between obesity and glucose intolerance, hyperlipaemia and a predisposition to diabetes (Ostrander et al, 1965; Keen & Jarrett, 1970; Sims et al, 1973). Hypertriglyceridaemia, hypercholesterolaemia and abnormal glucose tolerance have been observed in 46%, 17%, and 26% of obese patients respectively (University Group Diabetes Program, 1970). Although such pathological conditions may predispose to coronary heart disease (the major cause of mortality in western countries), Carlson & Bottinger (1972) concluded that elevated concentrations of plasma TG and/or TC were the major factors predisposing to coronary heart disease, and not obesity per se.

Weight reduction has long been considered to be an obvious way to avoid undesirable consequences of obesity, but such treatment has not been shown to necessarily decrease the mortality risk (Beem, 1959; Hood et al, 1963). Indeed, dieting regimes frequently result in a cycle of 'fasting-refeeding', which itself may be deleterious to health (U.S. Public Health

Service Publication No. 1485, 1966).Work on experimental animals has suggested that such episodes increase food intake and weight gain (Szepesi et al, 1973).

1.1.2. Definition of obesity.

It is essential in the present context to distinguish between obesity and "overweight" (Keys, 1955).Obesity is characterized by an altered body composition such that there is an absolute or relative excess of body TG. This feature is well illustrated by the occurence of obesity in rodents (section 2.1.), such that they require a higher intake of a diet of fixed composition in order to maintain their body weight near to that of their non-obese counterparts, but at this weight they still have a higher % body TG and lower % body protein (Alonso & Maren, 1955).This situation resembles that in humans (Sims et al, 1973) and other animals (Blaxter, 1971) in which the intake (metabolizable energy/body surface area) of a diet high in carbohydrate required to maintain a constant weight increased after a period of fat deposition.This was not the case when a diet high in fat was used in the same experiments.A related phenomenon is the marked difference between individuals of 'normal' weight who accumulate fat by increasing the intake of a mixed diet or by increasing the % dietary fat (Passmore et al, 1963; Blaxter, 1971; Sims et al, 1973; Lemonnier et al, 1971).As a result of such experiments (it may be thought) obese individuals of 'normal' weight may be identified.

Thus obesity may be defined by the presence of the following characteristics: (1) an absolute or relative increase in the % body TG, (2) at a given level of intake of a diet high in carbohydrate and protein there are differences in the utilization of these dietary components in comparison with that in the non-obese state, (3) an increase in the % fat

in the diet results in a greater increase in weight gain than in the non-obese state (i.e. higher proportion of weight gained to intake of metabolizable energy).These characteristics have been reviewed in detail by Sims et al (1973) and Hill (1971).

1.1.3. Biochemical consequences of obesity in humans.

Obesity in humans leads to a wide range of biochemical sequelae, the inter-relationships of which are poorly understood, but some of which may be implicated in the pathogenesis of the condition.This field has been reviewed by Bortz (1969) and Gordon (1971).Although no single metabolic alteration has been found to be ubiquitous in obesity, probably the most studied is "insulin-resistance" - the lack of action of insulin with respect to a number of its normal physiological actions - in muscle and adipose tissue (e.g. acceleration of the conversion of glucose to CO_2 and FA; amino acid accumulation by muscle).An increased plasma insulin:glucose ratio is a common observation in obese subjects.A number of changes which may affect insulin resistance are also associated with obesity.These are hormonal, metabolic and cytological : an increased cortisol production rate (Cope & Black, 1959; Sims et al, 1973) and decreased growth hormone levels (Beck et al, 1964; Hunter et al, 1966; Fedele et al, 1970), increased turnover rates of plasma TG and FFA (Birkenhager & Tjabbes,1969; Nestel & Whyte, 1968; Issekutz et al, 1967) and in spontaneous obesity there is adipocyte hyperplasia (Hirsch & Knittle, 1970) associated with a decreased response to hormones normally affecting adipocyte c-AMP levels (reviewed by Bjorntorp & Ostman,1971).

1.1.4. Relationship of the present work to obesity research.

There are a number of obvious limitations in the use of humans to investigate obesity (Chlouverakis, 1973; Sims et al, 1973), not least of

which is their variable genetic background. In recent years there has been an increasing interest in the use of genetically obese rodents to investigate metabolism in obesity more extensively under more controlled conditions. Such animals are regarded as appropriate for such studies as they show many of the abnormalities seen in the spontaneously obese human (cf. section 1.3.), especially since the latter condition is heritable to some extent (see section 1.1.2.; Astwood, 1962). Genetically obese mice were used in the present experiments in order to investigate aspects of lipid metabolism in liver and plasma and their possible roles in obesity.

1.2. Relative importance of various organs as sites of fatty acid synthesis.

An initial step in the study of mechanisms leading to the accumulation of TG in adipose tissue is to elucidate the organ of origin of the synthesized FA.This has been subject to grave methodological difficulties.Experiments in vivo should attempt to take into consideration both the possibility of recycling of precursors, or products, and the differential metabolism of precursors, between tissues.In vitro, the achievement of consistent de novo FA synthesis has proved particularly difficult.Large variations in rates and responses to hormonal stimuli have been reported.It became apparent from studies (Barrett et al,1938; Stetten & Grail,1943) in which deuterium from $^{2}H_2O$ was incorporated into FA during the reductive metabolic steps of synthesis (Schoenheimer & Rittenberg,1937),that two major organs involved in FA synthesis were adipose tissue and liver.Stetten & Grail (1943) revealed a major difficulty in assigning relative rates of FA synthesis to these two tissues, since the half-times for replacement of de novo FA were shown to be markedly different between adipose tissue and liver.From such experiments it was suggested that the liver was the major site of FA synthesis.
In 1950, Pihl & Bloch proposed that FA deposited in adipose tissue may have been transported from the liver as TG in blood.The possibility that adipose tissue might have a high biosynthetic activity was suggested by Rosenfeld (1902), who showed that this tissue has a high R.Q.,and was first demonstrated by Shapiro & Wertheimer (1948) in experiments in which pieces of tissue were incubated in medium containing $^{2}H_2O$.Favarger & Gerlach (1955) showed that in mice killed within 12 minutes of intravenous injection of (^{14}C)-glucose, more

(^{14}C)-FA were found in adipose tissue than in liver.This and further work, frequently using (^{14}C)-glucose, has been reviewed by Favarger (1964) and Jeanrenaud & Renold (1961), who suggested that the liver contributed only a small fraction of newly-synthesized FA to adipose tissue.This, still widely held, conclusion is subject to criticism,partly due to the relatively low rates of blood glucose utilization by the liver at normal blood glucose concentrations, as discussed later.Also, measurement of the incorporation of radioactivity from (^{3}H),(^{14}C)-precursors as parameters for comparison of rates of FA synthesis between tissues may be unsatisfactory because of the differential utilization by tissues (Shreeve et al,1967; Lamdin et al,1969), the possibility of recycling of products in blood (e.g.via the Cori, alanine or glycerol cycles) and the uncertainty of the intracellular specific radioactivity of the precursor.

In a review by Jeanrenaud (1968), it was concluded that the respective contributions of liver and adipose tissue to FA synthesis varied with the species under consideration.In the rat and mouse, more than in any other mammal, it has been considered that adipose tissue synthesizes its own FA, largely on the basis of the large insulin dependance of FA synthesis in these species and the relatively high activity of enzymes associated with this process (Ball, 1966; Saggerson et al,1970; Halestrap & Denton,1973).Again, some observations in vivo are not entirely in accord with this concept. Thus the products of FA synthesis in adipose tissue are saturated acids; using an in vivo incubation technique, these were not observed to be converted (elongated and desaturated) to the glyceride composition of the bulk of adipose TG (Hollenberg,1966).

Even if adipose tissue represents a major site of FA synthesis in the young, lean animal, this may not be the case in animals in which obesity (of various origins) is developing. Rates of FA synthesis from (^{14}C)-glucose or (^{14}C)-acetate have been found to decrease as the adipose cell expands with the increased accumulation of TG. (Yen et al, 1971). This has been observed in states of increased TG accretion induced by age (Benjamin et al, 1961; Matthew & Hubbard, 1971), prolonged feeding of high carbohydrate diets, experimental obesity induced by injection of goldthioglucose or hypothalamic lesions (Bates et al, 1955a) or genotype (Christophe et al, 1961; Jansen et al, 1967; Stauffacher et al, 1968).

The above considerations illustrate the need for further systematic study of the site(s) of FA synthesis, especially in obesity.

1.3. Characteristics and experimental use of genetically obese (G.O.) rodents.

1.3.1. General characteristics of genetically obese rodents.

Many genetically different forms of inherited obesity have been described in rodents. Some of these are transmitted through dominant, and others through recessive, inheritance. Examples of the former are A^{iy} and A^{vy} present in the yellow obese mouse (Cuenot, 1905; Danforth, 1927); and of the latter, *ob* in the mouse (Ingalls *et al*, 1950) and *fa* which is the only gene reported to produce obesity in rats (Zucker & Zucker, 1961). Other forms of genetic transmission reported to produce obesity have been classified as inbred, hybrid or polygenic (Bray & York, 1971). The 'sand rat' and the 'spiny mouse' may show obesity when fed a high-carbohydrate laboratory diet; however, obesity is not common in their natural desert environment (Haines *et al*, 1965; Gonet *et al*, 1965).

Much of the physiological, biochemical and genetic research on these animals has been reviewed by Hellman (1965), Bray & York (1971) and Stauffacher *et al* (1971). In the present context, the *ob/ob* mouse is the main interest, since mice homozygous for this gene were the subject of experiments in much of the present work. No biochemical abnormality directly attributable to a genetic mutation has yet been identified in *ob/ob* mice, or in most other forms of genetic obesity. Rather, many of the biochemical features described in these animals have elements in common with other forms of obesity, such as experimentally-induced (through hypothalamic lesions or goldthioglucose injections) or nutritionally-induced obesities.

1.3.2. Metabolic alterations in G.O. mice.

1.3.2a. Alterations in hepatic metabolism in G.O. mice.

Since the early work of Zellman (1952) on the liver of obese humans, obesity has been associated with hepatomegaly and an increased hepatic TG content.

In contrast to adipose tissue, the livers of GO mice have consistently been shown to have a higher FA biosynthetic activity from a wide range of precursors than that of non-obese mice, both *in vivo* and *in vitro*. Jansen *et al* (1967) reported that *ob/ob* mice incorporated approximately twice as much ^{14}C from (U-^{14}C)-glucose, given as a glucose meal, into hepatic FA/g liver than did non-obese mice when the animals were fed a balanced laboratory diet, but not when a high carbohydrate diet was given. Shreeve *et al* (1964,1967) showed that the same parameter was greater in *ob/ob* mice after intraperitoneal injection of a trace dose of (1-^{14}C)-glucose. Similar findings were reported by Christophe (1970) after the injection of (1-^{14}C)-acetate. In all these instances, more radioactivity was recovered in the carcass than in hepatic FA about one hour after isotope administration. In contrast, Shreeve *et al* (1967) and Lamdin *et al* (1969) showed that more ^{3}H was recovered in hepatic FA than in the carcass of *ob/ob* mice after intraperitoneal injection of DL-lactate-(2-^{3}H), glycerol-(2-^{3}H), DL-malate-(2-^{3}H) and Succinate-(2,3-^{3}H) or than in comparable tissues in non-obese mice. Increased incorporations of radioactivity from isotopically labelled precursors into hepatic FA have also been reported in New Zealand obese mice, KK mice and *fa/fa* rats (Bray & York, 1971).

The calculation of rates of FA synthesis is not possible from these data because of considerations previously mentioned (1.1.1.), the lack of any estimate of precursor*s.r.a. or time course of isotope incorporation.However, investigators of FA synthesis in ob/ob mice have unanimously concluded that the liver of these animals shows more marked alterations in FA synthesis than does adipose tissue, and is likely to be the major site of accelerated synthesis.

The activities of enzymes postulated to contribute reducing equivalents utilized in the biosynthesis of FA (1.2.2.) have been reported to be increased in livers of ob/ob mice.These enzymes show approximately the same proportional activities as found in non-obese mice.Martin et al (1973) reported that 6-phosphogluconate dehydrogenase, malic enzyme and $NADP^+$-linked isocitrate dehydrogenase all showed an increase in ob/ob mice, relative to lean mice.However, Hellman (1967) reported that there was not a comcomitant rise in the NAD^+-linked isocitrate dehydrogenase, which may be a rate-limiting reaction in the rate of the tricarboxylic acid cycle (Marr & Weber,1971). The provision of cytoplasmic acetyl CoA may also be enhanced in the liver of ob/ob mice since ATP-citrate lyase also shows an increased activity (Spencer & Lowenstein,1966).A rate-limiting role of acetyl-CoA carboxylase has been postulated (1.4.2.).The specific activity of both this enzyme and FA synthetase complex were shown to be raised in ob/ob mouse livers (Chang et al,1967; Nakanishi & Numa,1971).As in fasted and fasted-refed normal rats, the change in specific activity of the carboxylase was brought about through both alterations in the rate of synthesis and degradation of enzyme protein.This is in contrast to alloxan-diabetic rats, in which a decrease is effected through

*specific radioactivity

decreased synthesis only (Nakanishi & Numa,1970,1971; Majerus & Kilburn,1969).

Thus many of the alterations noted in the rates of hepatic processes are similar to those mentioned previously in normal animals, in which high rates of hepatic FA synthesis had been induced (1.4.). In ob/ob mice, however, these processes show a diminished adaptability to changes in nutritional status.Thus after 24h. of food deprivation, the incorporation of ^{14}C from (^{14}C)-glucose into hepatic FA was still 50% of that found in the fed state, whilst non-obese littermates showed a 90% reduction in incorporation (Jansen et al,1967).On re-feeding ob/ob mice with a high carbohydrate diet after a period of starvation, a lack of adaptation has been observed in the activities of several enzymes: ATP-citrate lyase (Spencer & Lowenstein,1966), acetyl CoA carboxylase (Maragoudis et al,1972) and certain enzymes associated with glycolysis and the generation of NADPH (Kaplan & Fried,1973).Furthermore, ob/ob mice show an unusual response to a high-fat diet; Lemonnier et al(1971) found a lack of a depression in hepatic de novo FA synthesis and a high rate of dietary TG accretion by adipose tissue, which resulted in a 50% increase in the rate of weight gain, compared to that observed on a high carbohydrate diet. It has been suggested that this lack of adaptation is due to their fasting hyperinsulinaemia (1.3.3.) but the adrenal cortex is probably also involved.

1.3.2b. Metabolism in adipose tissue of G.O. mice.

Much of the interest in the search for metabolic alterations in ob/ob mice has centered around adipose tissue.This has arisen, as indicated by Hellman(1965), on the basis that adipose tissue was thought to be the major site of the conversion of carbohydrate to FA in the normal animal.The reasoning behind such a conclusion has been considered previously (1.1.1.).However, Bray & York (1971) have pointed out out that many of the metabolic alterations observed in the adipose tissue of G.O. mice, including ob/ob mice, appear to result from obesity per se.In addition to the insulin insensitivity of adipose tissue (1.3.3.), it has been reported that this tissue shows relative insensitivity in vitro to adrenalin with respect to FFA release.However, normal elevations in plasma FFA concentrations were observed after intravenous injection of adrenalin or after starvation (Abraham et al,1971).It may be relevant that a feature of some G.O. mice, including ob/ob mice and yellow obese mice, is the lack of elevation in plasma FFA levels after cold exposure, accompanied by a fall in body temperature (Kamioka,1965; Van der Kroon,1966).Such a response resembles that of rodents entering hibernation.A metabolic alteration of adipose tissue which has been suggested to be important in the development of obesity in the ob/ob mouse is the occurrence of glycerokinase activity in this tissue (Treble & Mayer,1963;Lochaya et al,1963).The significance of this observation has been questioned since Shreeve et al(1967) were unable to detect an abnormally high incorporation of isotope from intravenously injected (^{14}C,^{3}H)-glycerol into CO_2 or FA by adipose tissue.Jansen et al(1967) doubted the significance of such activity due to the high incorporation of (U-^{14}C)-glucose into glyceride-glycerol in adipose tissue, which

might have been expected to be less if this tissue were capable of significant glycerol phosphorylation.The occurence of glycerokinase activity in adipose tissue may be secondary to obesity in rodents since Kasemsi et al(1971) reported such an observation in goldthioglucose-treated mice.

1.3.3. Alterations of endocrine glands in G.O. mice.

Mice which are homozygous for the recessive gene ob have certain similarities to the obese human with adult-onset diabetes. Such characteristics are frequent hyperglycaemia, fasting and postprandial hyperinsulinaemia (Bleisch et al,1952; Christophe et al, 1959; Stauffacher et al,1967; Abraham et al,1971) and hypertrophy of the islets of Langerhans (Gepts et al,1960).It has been mentioned previously that these animals show a lower conversion of glucose to FA in adipose tissue (1.1.1.); in addition thereis a diminished response to insulin by this tissue with respect to glucose to CO_2(Christophe et al,1961).Until recently, it was thought that all forms of rodent obesity were accompanied by increased basal and stimulated serum insulin levels (Bray & York,1971).However, Cameron et al(1973) have recently reported that obesity in the spiny mouse is not associated with abnormally raised serum insulin levels, either in the basal state or after a glucose load.The ob/ob mouse, however, has been shown to have an exremely marked hyperinsulinaemia which becomes more severe with age (Westman,1968; Abraham et al,1971).

Alterations in the functions of other endocrine glands have been widely implicated in the pathogenesis of genetically

transmitted rodent obesity.Hypertrophy of the adrenal cortex has been reported in this state.Similar observations have been made in experimentally-induced obesity, such as after goldthioglucose injection or hypothalamic lesions.Adrenal cortex hypertrophy has been considered to be implicated in the obesity of ob/ob mice, since restriction of food intake ameliorates the condition and adrenalectomy prevents weight gain (Hellerstrom et al,1962; Solomon & Mayer, 1973).The blood glucose level in ob/ob mice has been correlated with the degree of adrenal hypertrophy (Van der Kroon,1966).The obesity of yellow obese and NH mice appears similar to that seen in humans with Cushing's syndrome.In these animals, adrenalectomy prevents both the development of obesity and islet hypertrophy.

It has been suggested that the endocrine abnormalities reported in ob/ob mice may originate in an altered secretion of one or more trophic hormone(s) from the pituitary (Bray & York,1971).The basis for this suggestion rests largely on the observation that hypophysectomy halts further weight gain in these mice (Spiers,1952). However, no levels of any trophic hormone either in the pituitary or in blood have as yet been reported.It is clear that the investigation of the adrenal-gonadal-pituitary axis would be of great interest in relation to the development of obesity in general and in relation to ob/ob mice in particular.

1.3.4. Experimental use of G.O. rodents.

Few studies on G.O. rodents have concentrated on investigating possible sites of a genetic mutation expressed as a metabolic 'error' (c.f. glycogen storage diseases).The aim of the majority of research

on these animals has been to investigate altered metabolism in the obese state by description of biochemical changes in a particular G.O. rodent species which are typical of obesity in general.Current evidence concerning the nature of metabolic alterations (1.3.2.) and the lack of evidence for a metabolic 'lesion' in G.O. rodents, has led to a view that genetic obesity may be occasioned by an altered, genetically-determined intensity of endocrinological and/or nervous stimuli producing kinetic alterations of normal metabolism.

Thus, in the ob/ob mouse, it may be considered that metabolic alterations are produced as the consequence of endocrinological changes (1.3.3.), especially the marked hyperinsulinaemia and adrenal hypertrophy.However, the potential involvement of other factors in producing metabolic alterations should not be neglected in their interpretation.

1.4. Factors controlling the rate of fatty acid synthesis in mammals.

Factors thought to control the rate of fatty acid synthesis in liver and adipose tissue have frequently been classified as long-term -the most intensively studied of which are dietary and hormonal influences -or short term,usually intracellalar modifiers of enzyme activity.

1.4.1. Dietary and hormonal influences.

Earlier work in this field has been reviewed by Fritz(1961) and Masoro(1962).Whilst much of the work was done using liver slices,in which rates of fatty acid synthesis are markedly lower than in more recent perfusion preparations,it was possible to elucidate some major dietary effects on fatty acid synthesis.Thus Masoro et al (1950) and Brice & Okey (1956) observed that liver slices from rats fed a high-fat diet showed low rates of FA synthesis from (^{14}C)-glucose or (^{14}C)-acetate. Medes et al (1952) first noted the exceptionally high rates of FA synthesis in liver slices from rats refed carbohydrate after a period of food deprivation.Tepperman & Tepperman (1958,1961) carried out an extensive study of this phenomenon,termed 'hyperlipogenesis'.From such experiments came correlations of the rate of FA synthesis with events in carbohydrate metabolism (e.g.the level of hepatic glycogen,and the activity of the pentose pathway).

Earlier than these experiments were indications that insulin might be associated with the regulation of FA synthesis.Drury (1940) and Stetten et al (1944,1946) demonstrated low rates of FA synthesis in diabetic rats in a series of in vivo experiments employing D_2O. When insulin was injected into alloxan-diabetic rats prior to sacrifice (Osborn et al,1953; Felts et al,1951; Chernick & Chaikoff,1951), or added to medium perfusing the isolated rat liver (Haft & Miller, 1956,1958) the hepatic capacity for FA synthesis was restored.The effects of insulin on hepatic FA synthesis have been reviewed by Nikkila (1969), who has pointed out that the responses to insulin observed in the diabetic animal cannot be extrapolated to the normal animal or to states of hyperglycaemic hyperinsulinaemia (e.g.adult-onset diabetes with obesity).

1.4.2. Intracellular control of fatty acid synthesis.

A more recent approach has been the study of the intracellular control of FA synthesis.Two major mechanisms by which hormones may change the rate of intracellular processes have been widely considered.A hormonal stimulus may alter the specific activity of enzymes which catalyze rate-controlling steps in the process; e.g.through alterations in enzyme protein turnover rates or through inter-conversion of different forms of an enzyme.Alternatively, hormones may alter the rate of a process indirectly, by acting on another process (which may be kinetically or spatially distinct) such that changes in metabolite levels, including redox potential or energy charge (Atkinson,1967), are effected in the region of the process under consideration. These approaches have been discussed in connection with the control of FA synthesis by Lowenstein (1970).A major objective is to elucidate

rate-controlling steps; this has proved a difficult problem in relation to the control of FA synthesis.This may be attributable, in part, to the controversial nature of pathways associated with the process.Three areas have been the subject of investigation: (i) the processes whereby the reducing equivalents are made available for the reductive steps of FA biosynthesis, (ii) the pathways by which acetyl CoA may be delivered to the cytoplasmic site of synthesis, and (iii) the potential rate-controlling properties of acetyl-CoA carboxylase, the first committed step in FA synthesis.An extensive discussion is outside the scope of the present work, but reviews on the above-mentioned areas of interest have been made by Ball (1966), Flatt (1970), and Rous (1971) on the generation of reducing equivalents, Lowenstein (1968) on the provision of cytoplasmic acetyl CoA, and Numa et al (1965) and Lowenstein (1970) on acetyl CoA carboxylase.

1.5. Scope of the present experiments.

1.5.1. Liver and plasma triglyceride kinetics.

Consideration of the altered hepatic metabolism reported in ob/ob mice (1.3.3.) raised the possibility that there might also be an altered lipid metabolism in the plasma of these animals, since the liver is known to be the sole site of formation of both the apoproteins and the majority of lipids constituting plasma lipoprotein complexes. It is known that the metabolism of plasma lipoproteins is subject to several forms of genetic and dietary modifications . For several reasons it was of interest to investigate rates of hepatic secretion and turnover of endogenous plasma lipoprotein TG. As plasma TGFA can be hydrolyzed and assimilated by adipose tissue and muscle (Robinson, 1970), its metabolism in ob/ob mice is of interest in relation to the deposition of TG in adipose tissue. If TGFA accumulated in adipose tissue of ob/ob mice was derived from FA synthesized de novo in the liver, then considerable transport of FA via plasma would be required. A simple calculation shows that hepatic synthesis and secretion would be of considerable magnitude. If the difference between the rate of weight gain of ob/ob and non-obese mice is considered to be entirely due to TG deposited in adipose tissue, then from the growth-curves of mice bred in the Imperial College colony, the difference is 20g. between 4-12 weeks of age. This represents an average rate of TG accretion of $15 mg.h^{-1}$ or 1μmol TGFA $min^{-1} mouse^{-1}$. This is about three times the turnover rate of plasma TG which has been estimated in rats (0.30μmol TGFA $min^{-1}(60g.)^{-1}$; Baker & Schotz, 1964).

An additional interest of an enhanced plasma TG turnover rate in ob/ob mice would be the possibility of impairment of insulin-dependent glucose uptake from blood (1.1.) through competition between glucose and TG (or FA derived from their hydrolysis) in tissues (e.g. by mechanisms analogous to those proposed for the 'glucose-FA cycle'; Randle et al,1963).

A number of metabolic alterations in ob/ob mice (1.3.) suggested that there might be an increased turnover of plasma TG in these animals. In addition to enhanced rates of hepatic de novo LCFA synthesis (1.3.3.), Dade & Hems (1972) and Elliot et al (1974) have reported an increased rate of turnover of FFA and glycerol in the blood of ob/ob mice. The metabolism of these substances is closely related to that of hepatic and plasma TG (Fig.5.1) In view of the hyperinsulinaemia of the ob/ob mouse (1.4.3.), it was relevant to consider the relationship of serum insulin levels to plasma TG turnover rates. Nikkila (1969) has reviewed evidence that insulin may increase plasma TG efflux in rats (especially in the insulin-deficient state). Owing to the nature of the experiments, it has proved difficult to differentiate insulin effects from those of glucose alone. The relationship of TG turnover rates to serum insulin levels appears to be influenced by metabolites such as glucose, FFA and glycerol (Thorell et al,1966; Mietinen et al,1966) and has been the subject of much discussion (Nikkila,1969; Bierman et al,1970).

An approach which has been used in attempting to estimate the rates of endogenous TG turnover in humans (Farquhar et al,1965), dogs (Gross et al,1967), rabbits (Havel et al,1962) and rats (Laurell,

1959; Baker & Schotz,1964) is to calculate rates of influx and/or efflux from complex time-courses of the incorporation of radioactivity into plasma TG after a single injection (or short infusion) of ($^{3}H/^{14}C$)-palmitic acid or ($^{3}H/^{14}C$)-glycerol.This approach necessitates a number of assumptions (usually in terms of a hypothetical model) and may be subject to extensive mathematical treatment in order to obtain a solution.In the experiments of Farquhar et al (1965), Gross et al (1967) and Havel et al (1962), VLDL-TG were endogenously labelled with ^{14}C or ^{3}H and isolated for subsequent reinjection.By this method, an estimate of the rate of efflux of VLDL-TG from plasma may be obtained requiring a minimal number of assumptions (e.g.that injected (^{14}C)-VLDL-TG entirely resembles native lipoproteins).

In the present experiments, time-courses of the incorporation of (U-^{14}C)-palmitic acid (injected as a FFA-albumin complex) into plasma TG has been established in ob/ob and non-obese mice.From these data, rates of secretion of endogenous TG have been estimated.In another series of experiments, (^{14}C)-VLDL-TG were prepared after injection of (U-^{14}C)-palmitic acid into donor mice.The labelled lipoproteins were injected into recipient mice, and the rate of disappearance of ^{14}C from plasma (^{14}C)-TG established.

In addition, the intravenous exogenous TG tolerance of ob/ob and non-obese mice was investigated, using a stable soya-bean oil emulsion, Intralipid,employing the method described by Boberg et al (1969).The use of this test has previously been largely confined to the investigation of TG kinetics in humans with hypertriglyceridaemia.

1.5.2. Plasma lipoproteins.

Plasma lipids circulate as plasma lipoprotein (LP) complexes, of which a number of moieties have been identified.These have been classified according to a wide range of physico-chemical criteria which have recently been summarized by Lopez-S (1971).The most widely used criteria have been according to hydrated density or flotation rate on ultracentrifugation (expressed as $g.ml^{-1}$ or negative Svedberg sedimentation co-efficient S_f.) and according to their electrophoretic mobility (usually relative to the migration of plasma globulins).On the basis of ultracentrifugal criteria, electrophoretic behavior on paper and N-terminal amino acid residue composition, four main groups of LP have been characterized in human serum: chylomicra, VLDL, LDL, HDL.Current concepts of the structure and metabolism of human plasma LP have been reviewed by Scanu (1965), Alaupovic (1968) and Schumaker & Adams (1969), and discussed in two recent symposia (Fredrickson,1969; Smellie,1971).

Although the study of human plasma LP is advanced, the direct application of such knowledge to other species requires careful consideration in view of the considerable species variation in the distribution, composition and physico-chemical properties of plasma LP that have been reported (Koga,1969; Nichols,1969).

In the present experiments, murine plasma LP have been analysed by three techniques in order to determine their lipid distribution and content.This was achieved by preparative ultracentrifugation, agarose gel column chromatography and polyacrylamide-gel disc electrophoresis.Techniques whereby adequate resolution of

plasma LP may be achieved by agarose-gel column chromatography have only recently been described (Sata et al,1970; Bowden & Fried,1970). An advantage of these techniques is that a continuous record of lipid and protein components of plasma LP, separated according to size only, is obtained. Polyacrylamide-gel disc electrophoresis of plasma LP (Narayan,1966) afforded improved resolution of smaller, more highly charged LP (LDL and HDL).

1.5.3. The proportional contribution of (^{14}C)-precursors in blood to hepatic triglycerides.

Although there is evidence that the liver is almost the sole source of endogenous plasma TG, the precursors of plasma TG have been shown to be highly variable - depending on both the species and the nutritional state. In man, after an overnight fast, the main precursors were plasma FFA (Havel,1961; Friedberg et al,1961; Eaton et al,1965) and a similar situation obtained in the rabbit (Havel et al, 1962). However, in the rat, an important source of plasma TGFA after a glucose load was hepatic de novo synthesized FA and PL (Baker & Schotz,1964; Nikkila et al,1966). Recently, Barter & Nestel (1973) have reported evidence suggesting that a greater proportion of plasma VLDL-TG is delivered from either preformed liver TG or newly-synthesized liver FA in obese subjects compared to lean controls. It was of relevance in the present work to establish the situation with respect to ob/ob mice. Such investigations in vivo, however, are subject to some complexity. It was desirable to attempt experiments under conditions of near-maximal de novo FA synthesis and it has been widely thought that this may be achieved some time after the administration of a glucose load. The chronology of events following

such a load has been shown to be variable, depending on conditions of administration, especially with respect to FA synthesis and hepatic TG secretion (reviewed by Nikkila,1969).Baker & Huebotter (1973) have demonstrated that delays in initiation of FA synthesis after a glucose load may occur in starved rats.

In the present experiments, an initial attempt has been made to estimate the contribution of plasma glucose to hepatic lipid formation after a glucose load.Time courses of incorporation of ^{14}C from (U-^{14}C)-glucose into hepatic and plasma lipids (LCFA,TG,PL) were established from 0-180 minutes, following injection.Rates of lipid formation have been estimated from the most rapidly rising phases of incorporation.

In view of the multicompartmental nature of hepatic TG metabolism, (Baker & Schotz,1964,1967; Farquhar et al,1965,Havel et al, 1962), it is not possible to employ similar techniques to estimate the contribution of plasma FFA to plasma TG.Rather, one of two methods have been used.Radioactive FFA has been infused over long periods into human subjects until the hepatic and plasma TG pools reached isotopic equilibrium, whenthe fraction (s.r.a.* of plasma FFA)/ (s.r.a.plasma VLDL-TG) was taken as an indication of the contribution of plasma FFA to plasma VLDL-TG (Birkenhager & Tjabbes,1965; Barter & Nestel,1973).Alternatively, an estimate may be made for the rate of hepatic esterification of an injected pulse dose of (^{14}C)-FFA determined 2-5 minutes after injection(Stein & Shapiro,1959; Baker & Schotz,1967; Schotz & Oliverona,1966; Schotz et al,1964. ; Havel et al,1962).It is the latter approach which has been attempted in the present experiments.This required the assumption that only a small

* specific radioactivity

quantity of hepatic TG is degraded in that organ in the fed state. This has been justified by experiments both *in vivo* (Baker & Schotz, 1964 ,1967) and in the isolated, perfused liver (Topping & Mayes,1972).

1.5.4. The study of hepatic lipid metabolism in the isolated, perfused mouse liver.

Results of experiments *in vivo* to be described (Chapter 3) indicated that newly-synthesized FA in the liver contributed to the TG output of the *ob/ob* mouse liver.Furthermore, blood glucose appeared to contribute less than half the carbon required for synthesis of such FA, even in conditions of marked hyperglycaemia.It therefore appeared that the current understanding of the precursors utilized for hepatic LCFA synthesis (Tepperman & Tepperman,1970) required some expansion in order to account for these observations.It was considered that perfusion of the isolated liver was eminently suitable for such investigations, because of the high degree of control over liver perfusate composition and the superior biosynthetic rates compared to other liver preparations (Windmueller & Spaeth,1966) which were afforded by this technique.Several prerequisites existed in conducting the present investigations: a technique whereby total rates and the proportional contribution of C-precursors to the total rate of LCFA synthesis measured simultaneously should be used.Secondly, a medium of known composition should be used, but should not be limiting with respect to basic hepatic functions (e.g. oxygen consumption and carbon dioxide output or glucose uptake).Thirdly, it was desirable that a system in which medium metabolite concentrations could be held relatively constant should be employed in order that the

concentration dependence of precursors of FA synthesis might be established.A constant specific radioactivity of medium precursors was also desirable in order to calculate absolute rates of LCFA synthesis. The present experimental conditions were designed to meet these requirements.

Although the possible sources of reducing equivalents and substrates required for FA synthesis have been extensively investigated in adipose tissue (1.4.2), there is little information concerning the quantitative contribution of various pathways to these processes in the intact liver.Such studies are of special interest in the liver, since several biosynthetic processes proceed simultaneously and may compete for substrates, ATP and reducing equivalents.In the present work, the dependence of the total rate of FA synthesis on perfusate glucose concentration (0-30mM) was investigated using $^{3}H_2O$ (Jungas, 1967; Foster & Bloom, 1963).In addition, the contribution of medium glucose-C and lactate-C to this process over a range of glucose concentrations has been established and the potential participation of glycogen-glucose carbon in FA synthesis has been considered.

CHAPTER 2.

ANIMALS, MATERIALS and METHODS.

2.1. Mice used in the present experiments.

2.2. Sources of materials and equipment.

2.3. Preparation and treatment of lipid extracts.

2.3.1. Extraction of total lipids from tissues, plasma and perfusates.

2.3.2. T.l.c. of lipids.

2.3.3. Isolation of total fatty acids and non-saponifiable lipid fractions from lipid extracts.

2.3.4. Lipid estimation techniques.

2.4. Separation and assay of non-lipid metabolites.

2.4.1. Glucose.

2.4.2. Hepatic glycogen.

2.4.3. L-lactate.

2.5. Characterization of plasma lipoproteins.

2.5.1. Ultracentrifugation.

2.5.2. Agarose-gel column chromatography.

2.5.3. Polyacrylamide-gel disc electrophoresis.

2.6. Gas liquid chromatographic analysis of lipids.

2.7. Liquid scintillation counting of ^{14}C alone, ^{3}H alone and ^{14}C with ^{3}H.

2.7.1. Determination of ^{14}C in lipids and glucose.

2.7.2. Determination of ^{3}H in lipids, plasma and perfusates.

2.7.3. Determination of ^{14}C with ^{3}H.

2.8. Determination of the turnover rates of exogenous and endogenous plasma triglycerides.

2.8.1. Measurement of exogenous triglyceride tolerance.

2.8.2. Measurement of influx rates of endogenous plasma triglycerides.

2.8.3. Preparation and injection of (^{14}C) VLDL triglycerides.

2.9. Investigation of hepatic synthesis and secretion of lipids synthesized from (U-^{14}C) glucose in blood.

2.10. Assay of the activity of ATP-citrate lyase (E C 4.1.3.8.) in mouse adipose tissue and liver.

2.11. Perfusion of the isolated mouse liver.

2.11.1. Perfusion apparatus.

2.11.2. Composition of perfusion medium.

2.11.3. Operating procedure.

2.11.4. Measurement of O_2-uptake.

2.11.5. Sampling during the course of perfusions.

CHAPTER 2.

ANIMALS, MATERIALS AND METHODS.

2.1. Mice used in the present experiments.

The autosomal recessive gene ob arose spontaneously in the highly inbred C57BL/6J strain at the Jackson Memorial Laboratory, Bar Harbor, U.S.A. (Ingalls et al,1950) and was introduced into local mixed colonies (not pure strains) in Edinburgh (uniformly dusty brown) and Birmingham (albino and dusty brown).The original stocks of mice used in the formation of the colony at Imperial College were obtained from these colonies and all the mice used in the present experiments were bred from these stocks over a period of 3-6 years prior to experimentation.All mice were fed Oxoid breeding diet (Oxoid Ltd., London S.E.1.) until 6-8 weeks of age, when they were fed Thompsons rat cake (Pilsbury's Ltd.,Birmingham and Heygate & Sons, Northants.). Obese mice fed ad lib (whose feed intake is 7-9g/day) are referred to as ob/ob.At 3 months and 6 months of age, non-obese mice weighed an average of 28g and 33g respectively, whereas ob/ob mice weighed about 60g and 90g respectively.Obese mice on a restricted diet (ob/ob-RD) were selected at age 6-8 weeks and allowed 5g food each morning.From 3-12 months old they weighed 30-40g (Eastcott,1972).Such mice may be expected to have a body composition consisting of a higher % fat and a lower % protein than non-obese mice (Alonso & Maren,1955).Non-obese mice were homozygous (ob^+/ob^+) or heterozygous (ob/ob^+).All mice were maintained under a 12 hour light/dark cycle at uniform temperature of $20\pm3^{\circ}C$ until they were removed for experimentation.

2.2. Sources of Chemicals.

Solvents and reagents ('AnalaR' grade) were obtained from Fisons Ltd.(Loughborough,U.K.),Hopkin & Williams(Romford,Essex,U.K.) or BDH Chemicals Ltd.(Poole,Dorset,U.K.) unless otherwise stated.Alcohols were from James Buuoughs Ltd.(London,S.E.11.,U.K.).Sodium L-lactate and lipids (for use as chromatographic standards) were from Sigma(London) Chemical Co.(London,S.W.6.,U.K.).All chemicals containing radioactive isotopes were from The Radiochemical Centre(Amersham,Bucks.,U.K.).Enzymes,ATP,NAD^+, NADH,phophoenolpyruvate and CoA were obtained from Boehringer Corporation (London) Ltd.(London,W.5.,U.K.).Bovine serum albumin ('Pentex',Fraction V) was from Miles Laboratories(Kankakee,Illinois,U.S.A.) and in a recrystellized form (for use as a reference protein) from Sigma(London) Chemical Co. Intralipid (10%) was obtained from Vitrum(Stockholm,Sweeden).One litre of this emulsion contains fractionated soya bean oil (100 g),egg lecithin (12 g) and glycerol (25 g).The scintilator butyl-PBD was obtained from CIBA Ltd.(Basle,Switzerland) and Hopkin & Williams.

The anaesthetic 'Avertin' was obtained from Winthrop Laboratories (Surbiton,Surrey,U.K.) and 'Nembutal' from Abbott Laboratories Ltd. (Queenborough,Kent,U.K.).

Agarose beads (1% Bio-gel A 50m,50-100 mesh)were obtained from Bio-Rad Laboratories (Richmond,California,U.S.A.).Silica gel G for t.l.c. was from Anderman Ltd.(London,S.E.1.,U.K.) and was manufactured by Merck Ltd.Liquid and stationary phases for g.l.c. were from Pye Unicam (Cambridge,U.K.)

Plastic cannulae for liver perfusion were made from 'Portex' plastic intrvenous cannulae,and obtained from Portland Plastics Ltd. (Hythe,Kent,U.K.).Silicone rubber tubing ('Silescol') was from Esco (Rubber) Ltd.(London,W.1.,U.K.).

2.3. Preparation and treatment of lipid extracts.

2.3.1. Extraction of total lipids from tissues, plasma and perfusates.

Blood was obtained by cardiac puncture of mice under diethyl ether anaesthesia. Serum was obtained by centrifugation, after allowing a clot to form for about one hour at room temperature. Plasma was prepared by the addition of trisodium citrate to whole blood (20 µl 16% Na_3-citrate/ml whole blood) followed by centrifugation at 3,000r.p.m. at 4°C for 10 min. Tissue samples (liver, adipose tissue, heart and small intestine) were frozen in liquid N_2 immediately after excision.

Extraction of lipids from serum, LP fractions or tissues was carried out according to Folch et al (1957). Samples were extracted with 20 vols chloroform methanol (2:1 v/v) v/v or w/v for serum and perfusates or tissues respectively. Samples were left at room temperature over night, homogenized with a vortex mixer and filtered under vacuum. After the addition of 0.2 vols 0.1M KCl and shaking, the aqueous phase was removed and the infranatant washed with a synthetic upper phase (chloroform/methanol/0.1M KCl/H_2O, 3:48:47:1 by vol). The chloroform phase was evaporated to dryness either under vacuum at 30-40°C or, in experiments where ^3H_2O was used, under N_2 at 70°C. Lipid extracts were stored in chloroform solution to minimize autoxidation of polyenoic FA.

2.3.2. T.l.c. of lipids.

Lipid classes were separated by t.l.c. Neutral and non-polar lipids were separated from each other and PL by the double solvent development technique of Freeman & West (1966) on 0.25 mm thin layers of silica

gel G made up in 0.01% aqueous rhodamine 6G (Christophe & Mathjius, 1967).The plates were developed sequentially in two solvent systems: diethyl ether/benzene/ethanol/acetic acid (50:40:2:0.2 by vol) and n-hexane/benzene (94:4 v/v).In experiments where lipids were eluted, white plates were employed and standard lipids only detected by spraying with 0.1% methanolic rhodamine 6G.Separated lipids were identified under u.v. at 254nm.Where necessary, lipids with polarities less than (R_f greater than) FFA were eluted with Et_2O/light petroleum (b.p.40-60^{o}C)/formic acid (50:50:1 by vol) and PL with chloroform/methanol/H_2O (100:50:1 by vol), as described by Bickerstaffe & Annison (1970).

FAME, prepared by trans-esterification of TG, PL and CE (2.6.1.), were separated according to their degree of unsaturation by argentation t.l.c. (Malins,1966).Thin layers (0.25mm) of silica gel G, made up in 10% $AgNO_3$ were prepared and activated for 90 min. at 110^{o}C. They were pre-run in diethyl ether before use.After spotting samples, the plates were developed in benzene/hexane (1:1 v/v).Bands were detected after spraying with 0.1% methanolic rhodamine 6G and brief visualization (to prevent excessive photocatalysis of silver oxide formation) under u.v. at 254nm.At least four bands were generally identifiable (by comparison with standard spots): FAME containing saturated acyl components were not retarded by Ag-complex formation and ran just behind the solvent front.Monoenoic, dienoic and polyenoic FAME were found in a descending order down the plate.Methyl esters of polyenoic FA were found near the origin.

Major classes of PL were separated on silica gel G and developed with a solvent system of chloroform/methanol/acetic acid/H_2O

(85:15:10:3.7 by vol) and detected with a phosphate reagent spray (PCA/ammonium molybdate/HCl/H_2O, 85:15:10:3.7 by vol) as described by Banburski & Axelrod (1951). This technique was used to check the purity of PL fractions which were subsequently assayed for radioactivity.

2.3.3. Isolation of total fatty acids and non-saponifiable lipid fractions from lipid extracts.

Long chain fatty acyl esters in washed, dried lipid extracts were hydrolysed to release their constituent FA. 2ml 30% NaOH and 1ml EtOH were added to less than 50mg lipid in a stoppered glass tube, and heated at 80°C for 3 hours in a water bath. 5ml H_2O were added and allowed to cool. Non-saponifiable lipids, consisting almost entirely of free sterols (FS) were extracted with 5ml n-hexane. The supernatant was removed and the aqueous phase acidified with 2ml 8.3N HCl. FFA were extracted three times with n-hexane. The completion of hydrolysis and subsequent extractions were tested by hydrolysis of total lipid extract of liver from mice which had been previously injected intravenously with (^{14}C)-palmitic acid bound to albumin (2.8.2). More than 95% of the ^{14}C radioactivity found in the total lipid extracts was extracted after hydrolysis as FFA. In similar experiments, t.l.c. of the total lipid extract, the hexane extract of the subsequently acidified hydrolysate (by the method of Freeman & West, 1966), and the hexane extract of alkaline hydrolysate, was carried out. More than 98% of the radioactivity obtained in the hexane extract of alkaline hydrolysate was present as FS, and the hexane extract of acidified hydrolysate contained less than 1% radioactivity present in any fraction other than FFA. The sum of the radioactivity measured in both hexane extracts and in the residual aqueous phase accounted for 100% of that present in the total lipid extract.

2.3.4. Techniques for the determination of lipids.

Glycerides were determined enzymatically as glycerol (Pinter et al,1967) after saponification (Albrink,1959).Washed and dried extracts of tissues,serum or perfusates,or native serum (containing less than 1.0 µmol glyceride-glycerol) were saponified with 0.5 ml 0.5 N ethanolic KOH for 30 min at 70°C,after which FFA were precipitated as their magnesium salts by the addition of 1 ml 0.15 M $MgSO_4$.Less than 0.4 µmol glcerol was added to a cuvette containing 40 µmol phospho-enol pyruvate,0.2 mg pyruvate kinase (15 U/mg),0.2 mg lactate dehydrogenase (36 U/mg),18 µmol ATP,0.4 µmol $NADH_2$,6 mM $MgCl_2$ and 2 mM KCl in 0.1 M triethanolamine-hCl buffer,pH 7.6.The reactions were initiated by the addition of 20 µg glycerokinase and the total assay volume was 3.11 ml. The number of µmol $NADH_2$ oxidized was calculated from the ΔE 340 nm, since it was known that each µmol glycerol in the cuvette gave rise to the oxidation of 1 µmol $NADH_2$,and that the molar extinction co-efficient of $NADH_2$ (1 cm light path) was 6.22.Under the present saponification conditions,PL were hydrolysed to 1- and 2-glycerophosphates,stable in alkaline solution (Artom,1957).Glycerokinase does not act on these two products,and there is no interference from PL (Eggstein,1966).No change in absorbance at 340 nm was obtained when PL were subjected to the above procedures.

Alternatively,triglycerides were determined spectrophotometrically after t.l.c. (Snyder & Stephens,1959).In this method,acyl hydroxamates, formed after the reaction of TG with alkaline,ethanolic hydroxylamine at 70°C,react with ethanolic ferric perchlorate to give a purple derivative with maximum absorbance at 600 nm.

Total cholesterol was determined in total lipid extracts by the ferric chloride method of Rosenthal et al (1959).FC and CE were determined by the same method after separation by adsorption t.l.c.

PL were measured as P_i (Martin & Doty,1949),after refluxing total lipid extracts with 60% (w/v) PCA.Egg-yolk lecithin was used as a lipid standard to check the efficiency of oxidation.

FFA were determined spectrophotometrically after reaction of their copper soaps with sodium diethyldithiocarbamate (Lauwerys,1969).

2.4. Separation and assay techniques for non-lipid metabolites.

2.4.1. Glucose.

Glucose, in perchloric acid extracts of blood, plasma or perfusates (6% PCA:sample, 1:1 v/v), was separated from other major acid solutes (according to Jacin & Mishkin,1965) by t.l.c. on 0.25mm layers of silica gel G impregnated with 0.02M borate buffer (pH 8.0) and were developed in butan-1-ol/acetic acid/H_2O (5:4:1 by vol).The following R_f values were found for a number of major blood solutes: glutamine 0.28, alanine 0.38, glucose 0.45, glycerol 0.53 and lactic acid 0.62.Glucose was detected by spraying standard spots only (run simultaneously with the samples at both sides of the plate) with a diphenylamine/aniline spray (prepared by adding ortho-phosphoric acid until a clear solution was obtained to 2% w/v, diphenylamine in 2% (v/v) aniline in ethanol). Blue spots appeared in the presence of mono- or oligo-saccharides after heating at 100°C for 30 min.Other components were detected by spraying standard spots with 0.5% $KMnO_4$ in 1M NaOH, followed by heating at 70°C for 5-10 min.Radioactivity was measured as described.

Glucose was determined using a glucose oxidase method (Krebs et al,1964) .2.5ml of a solution containing 12.5mg glucose oxidase (20i.u./mg), 4.0mg peroxidase (36i.u./mg) and 0.5ml 1% o-dianisidine in 95% ethanol per 100ml buffer containing 0.5M phosphate and 0.1M tris pH 7.3, were incubated with 1.0ml H_2O or PCA extract.The absorbance of the resulting oxidized dianisidine was measured at 440nm.

2.4.2. Determination of hepatic glycogen.

Liver samples were frozen in liquid N_2 immediately after excision, and 50-250 mg weighed and extracted in 5ml 30% KOH and briefly boiled to break up the liver. They were occasionally stored in this state for 3-4 days before further heating at 100°C for one hour in a water bath. The contents of the digest were transferred to 50ml centrifuge tubes and 15ml EtOH added (Good et al, 1933). The glycogen was allowed to precipitate overnight at 40°C and then centrifuged at 1×10^4 r.p.m. for 10 min. The supernatant was discarded and the glycogen pellet dissolved in water using a homogeniser. Suitable aliquots, containing 0.05-0.3μmol glycogen-glucose were pippetted into tubes containing 0.05ml diazyme (C.F. Boehringer Ltd.: a mixture of α(1→4) and α(1→6) glucosidase) in acetate buffer, pH 4.8, and less than 0.3ml sample diluted to 1ml with H_2O and incubated at 37°C for 30 min. The hydrolysed glycogen samples were then assayed for glucose (2.4.1). More than 95% of added standard glycogen was recovered through this procedure, and was included routinely as a check on the hydrolysis conditions.

2.4.3. Determination of the concentration of L-lactate in perfusates.

L-lactic acid was determined in PCA extracts of perfusates (perfusate: 6%, 1:1 v/v) by the enzymatic procedure of Hohorst (1963). Less than 0.25µmol L-lactate was added to a cuvette of 1cm light path containing 0.6mmol hydrazine sulphate, 1mmol glycine, 15µmol EDTA and 3µmol NAD^+ at pH 9.5, in a total assay volume of 3.11 ml. When E_{340} remained constant with time the reaction was initiated by the addition of 10µl (50ug) lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase from pig heart, 360u/mg). After about 40 min, the E_{340} again remained steady, and the ΔE_{340} was calculated. Presuming that each µmol lactate gave rise to the formation of 1µmol $NADH_2$ and that the molar extinction co-efficient of $NADH_2$ in a 1cm light path was 6.22, the µmol lactate in the cuvette was calculated. Cuvettes containing no lactate and standard known amounts of lactate were subjected to an identical procedure. The ΔE_{340} obtained in the cuvette containing no lactate was subtracted from that obtained in the sample cuvettes before calculation of µmol $NADH_2$ formed.

2.5. Characterization of plasma lipoproteins in mice.

In an initial attempt to separate serum LP classes, electrophoresis on cellulose acetate strips was employed (Chin & Blankenhorn, 1968).The resolution of fractions was not satisfactory, despite the adequate separation of human plasma LP in parallel experiments. Acceptable resolution of murine plasma LP classes was, however, achieved with other methods, as follows.

2.5.1. Ultracentrifugation.

The density of 2-3ml plasma was adjusted by the addition of calculated volumes of concentrated salt solutions (Havel et al,1955). Plasma was centrifuged at 1.05×10^5g for 22 hours at four successive densities: 1.006 (Gustafson et al,1965), 1.050, 1.075 (Faloona et al, 1969) and 1.21g/ml, using the 3 x 5ml swing rotor (59589) of the MSE 65 ultracentrifuge at 15-20^{o}C.The upper fractions were collected from centrifuge tubes.Recovery of plasma lipid in the combined fractions was roughly 90%.

2.5.2. Agarose-gel column chromatography.

Agarose beads (2% w/v) were washed with 10 volumes distilled H_2O to free the agarose of sodium azide in which it was supplied as a preservative.A column 45 x 2.5cm was prepared and equilibrated with a buffer containing 0.4M NaCl, 1M potassium phosphate, 0.2M EDTA and one drop of toluene per litre, by passing 5 bed volumes through the column. (Bowden & Fried,1970; Margolis,1967).2ml of d<1.21 LP was layered onto the column under eluant.Elution was at a rate of 10-15ml/h, and fractions

of 5-9ml were collected by a fraction collector.The eluant was continuously monitored for protein and turbidity at 254nm.The separation properties of the column were evaluated with a variety of particles, and particle weights of LP species were estimated from a calibration graph.Human LP complexes behaved on this column as described by Sata et al (1970).Recovery of serum lipids in the sum of all the fractions was 60-90% of that present in whole serum.

2.5.3. Polyacrylamide-gel disc electrophoresis.

Serum LP lipids were pre-stained with Sudan Black B before electrophoresis, as described by McDonald & Ribeiro (1959).Electrophoresis was carried out on 30µl serum, according to Narayan et al (1966).Samples were applied under a sucrose sample layer, drawn into narrow bands in a 7.5% acrylamide/riboflavin spacer gel (pH 6.7)and separated in a 'main' gel of 3.75% acrylamide. pH 8.9.The solutions required to prepare the above gels were made up as described by Davis (1964).Bands were temporarily fixed in 7.5% acetic acid at 2°C and sketched before they rapidly faded.

2.6.1. Preparation and separation of fatty acid methyl esters.

Major lipid classes were separated by t.l.c. and the bands marked during visualization under u.v., and the silica to which they were absorbed scraped directly into 100ml r.b. flasks. Transesterification (or methylation in the case of FFA) was achieved under reflux with 25ml methylation solvent (methanol/benzene/conc. H_2SO_4; 20:10:1 by vol.) for 90 min. The products were extracted with 25ml H_2O and 25ml light petroleum (b.p. 40-50°C). The aqueous infranatant was removed and the supernatant washed three times with water. The final supernatant was dried with a small quantity of anhydrous Na_2SO_4. Solvent was removed under N_2 and the FAME taken up in about 0.25ml light petroleum before injection of 2-5µl onto the column. Methyl esters derived from the cholesterol ester fraction were separated from free sterols if these interfered with the g.l.c., by t.l.c. using as the development solvent cyclohexane/chloroform (1:1 v/v).

The Pye 104 gas chromatograph was operated under the following conditions:

liquid phase	:10% polyethylene glycol adipate (PEGA)
solid support	:celite 650 (60-80 mesh)
column dimensions	:5' x $\frac{1}{4}$" (glass)
oven temperature	:200°C ± 0.05°C
detection temperature	:250°C
gas flow rates	:carrier (Ar) 60ml/min
	H_2 60ml/min
	air in excess of 200ml/min
detection	:flame ionization

Peaks were identified by comparison of the retention time with standard peaks. Under the conditions described, 16:0-18:3 gave equal area/weight responses (± 1%) as checked by comparison with quantitative mixtures.

At the start of the analyses, 0.1mg heptadecanoic acid and 0.1mg triheptadecanoin were added to the homogenate, as internal standards with which to check the recoveries of FFA and TGFA through the entire procedure. 17:0 did not occur as a natural component of any of the extracts. The mean recovery through the whole procedure was variable (50-100%). However, the % recovery was the same for all FAME, measured as determined by chromatography of a mixture of FAME of known composition. This mixture contained 20% by weight of the methyl esters of each of the following FA: palmitate, palmitoleate, stearate, oleate, linoleate and linolenate, and was obtained from Sigma (London) Ltd.

2.7. Liquid scintillation counting of ^{14}C alone, ^{3}H alone and ^{14}C with ^{3}H.

2.7.1. Determination of ^{14}C in lipids and glucose.

Lipid classes were separated by t.l.c. and the bands scraped into 20ml. counting vials, as described previously (2.3.2.).Unstained (^{14}C)-glucose bands were eluted into vials with 0.5ml. water, followed by 5ml. 2-methoxyethanol and 10ml. liquid scintillation fluid (6g. butyl P.B.D./litre toluene).Radioactivity in each vial was measured in a Packard Tri-Carb liquid scintillation spectrometer.The d.p.m. present were computed from recorded c.p.m. by a channels ratio method (Baillie,1960), and checked with ^{14}C-hexadecane as an internal standard.The efficiency of counting of ^{14}C was roughly 70-75% for lipids and 40% for aqueous samples.Recovery of ^{14}C-labelled lipids after t.l.c., which was assessed by comparing the d.p.m. recovered (in all bands) from the t.l.c. plate and d.p.m. in the total lipid extract prior to separation, was approximately 90%.

2.7.2. Determination of ^{3}H in lipids, plasma and perfusates.

Tritium, present as $^{3}H_2O$ in plasma or cell-free perfusates, was measured by the addition of 10µl.sample to 1ml.2-methoxyethanol and 10-15ml. scintillation fluid.The radioactivity in each vial was measured by a similar method to that described for the determination of ^{14}C (2.1.7.).^{3}H in total lipid extracts or total FA extracts was measured after the dried sample had been dissolved in scintillation fluid.Lipid classes were separated by t.l.c. and, having a R_f greater

than that of MG, were detected by spraying with 0.1% methanolic rhodamine 6G but PL were not sprayed (2.3.2.).Scintillation fluid was added directly to the silica to which lipids were absorbed, and the radioactivity measured except in the case of PL.^{3}H in PL was measured by the addition of 4% Cab-O-Sil in scintillation fluid to each vial when a thixotropic gel, in which the silica to which the PL were absorbed, was formed (Snyder & Stephens,1962).Such a procedure is necessary, as PL, unlike other lipid classes, were not eluted from silica into toluene.^{3}H, unlike ^{14}C, in solid samples remaining at the bottom of the vial were not accurately counted, owing to the low energy of the B-particles emitted from the tritium nucleus.The efficiency of counting of ^{3}H was 30-35% for lipid samples and approximately 20% for samples containing 2-methoxyethanol.

2.7.3. Determination of ^{14}C with ^{3}H.

Samples were prepared for counting in an identical manner to that described for measurement of ^{14}C and ^{3}H alone.The method for the simultaneous assay of ^{14}C with ^{3}H was performed as described by Hendler (1964).Counts were registered in three different channels using a Packard Tri-Carb liquid scintillation spectrometer.Two of these channels excluded 99.9% of all counts arising due to the presence of tritium.The scintillations arising from emission of higher energy β-particles (^{14}C) were monitored in the two other channels, which were arranged such that the efficiency of counting of ^{14}C could be calculated by a channels ratio method.This channels ratio was also related to the proportion of counts due to ^{14}C appearing in the window accepting ^{3}H counts, and also to the efficiency of counting of ^{3}H. By the use of suitable standard vials containing equal quantities of

Fig. 2.1. Discriminator settings and calibration curves for double-isotope liquid scintillation spectrometry.

The procedure followed was essentially that described by Hendler (1966) as applied to the Packard Tri-Carb liquid spectrometer. In (a), the six discriminator potentiometers were adjusted to produce three windows (A,B,C), whose widths were defined by the criteria of Hendler (1966). Essential features were that <0.01% of counts due to ^{3}H appeared in A or B, and that in an unquenched sample R_1=0.3. In (b) is shown the calibration curves required for the calculation of d.p.m. due to ^{14}C and ^{3}H from c.p.m. registered in three windows.

o: shows the relationship between the ratio R_1 and the ratio R_2, determined with samples containing (1-^{14}C) hexadecane of knwn radioactivity (d.p.m.) and quenched with increasing quantities of chloroform.

o: shows the relationship between R_1 and the efficiency of counting of ^{14}C, also determined using quenched samples of (1-^{14}C) hexadecane.

Δ: shows the relationship between R_1 and the efficiency of counting of ^{3}H determined with samples containing both (1-^{3}H) hexadecane and (1-^{14}C) hexadecan of known radioactivity, and variously quenched by the addition of chloroform.

By reference to these curves, d.p.m. due to ^{3}H and ^{14}C in samples of unknown radioactivity were caculated as follows:

d.p.m. ^{14}C = (c.p.m. in window A) x 100/efficiency of counting of ^{14}C

d.p.m. ^{3}H = c.p.m. in C - (c.p.m. in A x R_2) x 100/efficiecy of counting of ^{3}H

(a) Discriminator settings relative to the energy spectrum of ^{3}H and ^{14}C.

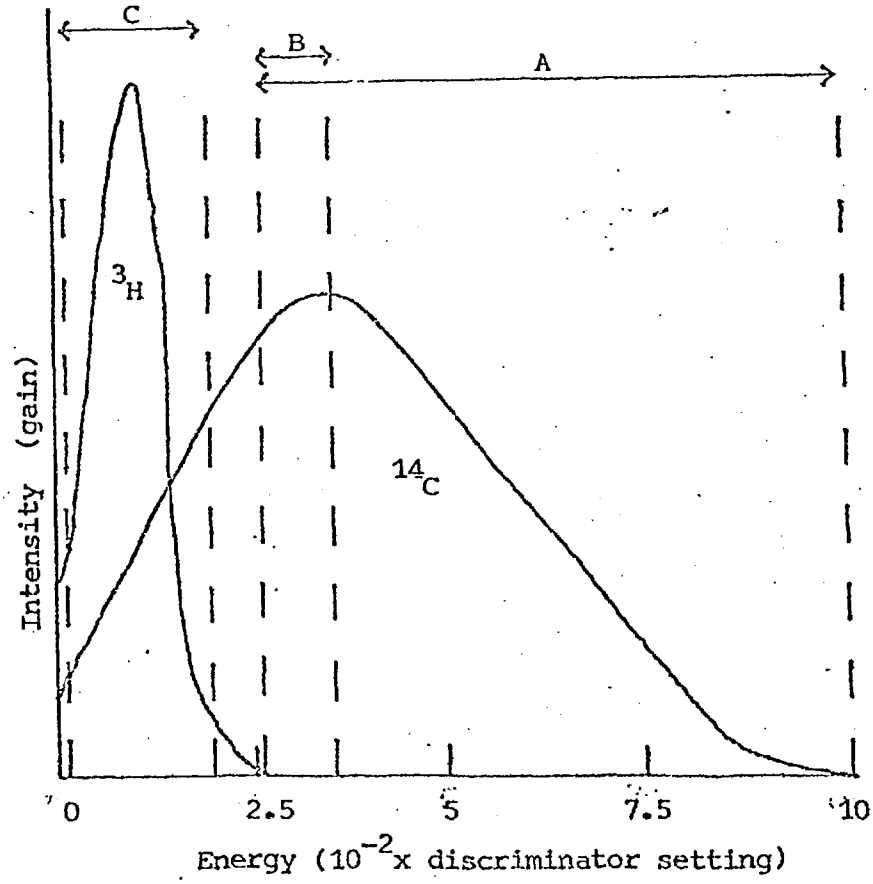
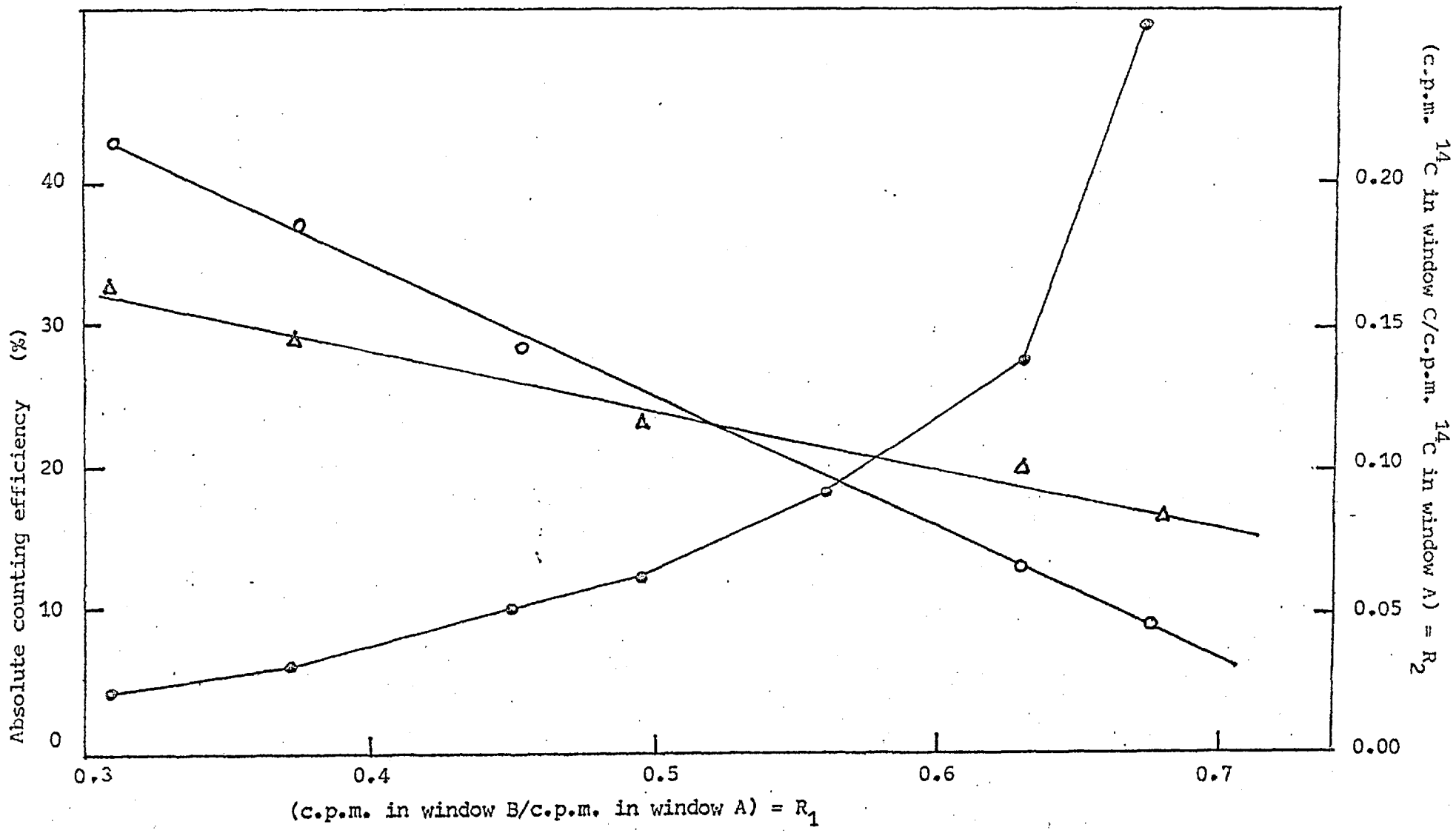


Fig. 2.1(b) Calibration curves for double-isotope liquid scitillation spectrometry.



d.p.m. ^{14}C and/or ^{3}H variably quenched with chloroform, a calibration curve was constructed depicting these three relationships (Fig.2.1.). The equations of the three lines were established and a computer was programmed for the calculation of d.p.m. and efficiencies from recorded c.p.m.The efficiency of counting of ^{14}C and ^{3}H in lipid extracts was 35-40% and 25-30% respectively.Aqueous samples containing 1ml.2-methoxyethanolwere counted with efficiencies of approximately 20% for both ^{14}C and tritium.It is noteworthy that the method was unsuitable for the counting of highly-quenched samples.Calculation of (c.p.m. ^{14}C in C/c.p.m. ^{14}C in A) was inaccurate when R_1 exceeded 0.6.The counting of (^{14}C,^{3}H)-PL separated by t.l.c. required modification of the method described for counting ^{3}H-PL, since Cab-O-Sil appeared to create quenching such that the quenching-correction methods used in this procedure were inappropriate.It was therefore necessary to elute PL from the silica (2.3.2.) and add scintillant, after evaporation of the eluant, before counting.

2.8. Determination of the turnover rates of exogenous and endogenous plasma triglycerides.

2.8.1. Measurement of exogenous triglyceride tolerance.

Mice were anaesthetized with Avertin (2.5%V/v; 20ml./kg.body weight).After about 15 minutes a stable TG emulsion,Intralipid (10% fractionated soyabean oil,w/v) was injected into a tail vein.Non-obese mice received 0.1ml. and ob/ob mice 0.13ml., representing a TG load ten times that of the endogenous plasma TG pool.Over the next 30 min., serial blood samples (50-75 µl.) were withdrawn from the tip of the tail into heparinized capillary tubes (Radiometer,Copenhagen),cooled in ice and centrifuged.Intralipid TG in plasma were measured within a few hours by nephelometry, after suitable dilution with 0.9%NaCl, against a standard curve(Lewis et al, 1972).

2.8.2. Measurement of influx rates of endogenous plasma triglycerides.

A solution of (U-^{14}C)-palmitic acid was prepared (Friedberg,1960) containing (U-^{14}C)-palmitic acid (>200mCi/mmol, 10-20uCi/ml.) bound to bovine serum albumin (Armour Pharmaceutical Co.Ltd.,Eastbourne,U.K.) Albumin was dialysed before use against a bicarbonate-buffered saline (Krebs & Henseleit,1932).Mice were anaesthetized with 2.5% (v/v) Avertin (20ml./kg.b.wt.) and, 15 min. later, 100µl. of the above solution was injected into a tail vein.A series of blood samples were taken from the tip of the tail, into heparinized capillary tubes, removing 50-100µl. at each time.The tubes were sealed and centrifuged at roughly 1,000r.p.m. and 4^{o} for 30 min.Plasma (50µl.) was removed with a syringe and needle.Lipid extracts were prepared and treated

as described above, for the determination of ^{14}C in lipids (2.7.1.); carrier lipids (PL, FFA, FC, DG, TG, and CE) were added to extracts to facilitate separation.

2.8.3. Preparation and injection of (^{14}C)-VLDL triglycerides.

Groups of 4-5 mice (aged 6-10 m.) were injected intravenously with 5-10uCi of (U-^{14}C)-palmitate.After 30 min., they were anaesthetized with diethyl-ether; blood was removed by cardiac puncture and citrated plasma was prepared (2.3.1.).Samples of serum were pooled, and VLDL were prepared by ultracentrifugation (2.5.1.).The major lipid classes in a small sample of the preparation of LP were separated by t.l.c., and their content of ^{14}C determined (2.3.2., 2.7.1.).Freshly prepared LP (0.2ml. containing about 10^3 d.p.m. in TG) was injected into the tail vein of mice aged 2 months.After various times, blood was obtained by cardiac puncture and the ^{14}C in serum TG was determined after t.l.c. of lipid extracts (2.3.2., 2.7.1.).When the interval between injection of VLDL and sampling was less than 4 min., mice were anaesthetized with Avertin (2.8.1.) before injection, to facilitate accurate timing.In the remaining experiments, VLDL was injected into conscious mice, which were bled eventually under diethyl-ether anaesthesia.

2.9. Investigation of hepatic synthesis and secretion of lipids synthesized from (U-^{14}C)-glucose in blood.

Conscious, fed mice, aged 12 weeks, recieved (U-^{14}C)-glucose intraperitoneally (1mmol containing 4.4-6.6 x 10^7 d.p.m. in 1ml.). After a time interval, mice were lightly anaesthetized with diethyl-ether/air. Plasma was obtained by centrifugation (2.3.1.) and lipid extracts of plasma and liver prepared as described previously. Major lipid classes and total LCFA were isolated from these extracts (2.3.2., 2.3.3.) and their content of ^{14}C assayed by liquid scintillation spectrometry (2.7.1.). Glucose was isolated from plasma and its concentration measured (2.1.7.). The ^{14}C content of plasma glucose was measured as previously described (2.4.1.). From these measurements the s.r.a. of glucose in plasma was calculated, and rates of lipid synthesis calculated as described in 3.4.2.

2.10. Assay of the activity of ATP-citrate lyase (EC 4.1.3.8.)in mouse adipose tissue and liver.

Mice were killed by cervical dislocation.Samples of adipose tissue from the epididymal and interscapular regions were removed and rinsed in 0.25M sucrose.Samples of liver were taken at the same time and processed in an identical manner.Values for the activity of ATP-citrate lyase in livers of various species, including ob/ob mice, are reported in the literature and it was thought that simultaneous assay of the hepatic enzyme would provide a check on the validity of the assay system.Tissue samples were blotted, weighed and homogenized in 3 vols. 0.25M sucrose and 1mM dithiothreitol.A high-speed supernatant fraction was prepared, essentially as described by Shrago et al (1969).The homogenate was centrifuged initially at 3.2×10^4g for 10 min.The supernatant was removed from the floating fat cake and from sedimented cell debris, nuclei and mitochondria with a pasteur pipette, transferred to cooled centrifuge tubes, centrifuged at 1.05×10^5g for 60 min, filtered through glass-fiber filters and assayed within two hours of preparation.

The assay of ATP-citrate lyase activity was carried out by the malate dehydrogenase method of Srere (1959).In this method, oxaloacetate - a product of the ATP-citrate lyase reaction - is reduced to malate by $NADH_2$ in the presence of malate dehydrogenase.Each µmol of oxaloacetate produced is converted to malate with the simultaneous oxidation of 1µmol $NADH_2$.This is monitored by the decrease in extinction of $NADH_2$ at 340nm.The complete reaction mixture contained 150umol tris-HCl; pH 7.5; 9µmol $MgCl_2$; 9µmol ATP; 0.3µmol CoA; 30µmol glutathione; 60umol citrate; 0.3µmol $NADH_2$; 100µg malate dehydrogenase (5.5i.u./µg) and

high-speed supernatant protein, in a total volume of 3.11ml.The reaction was carried out in a glass cell of 1cm light path, and the temperature was 31°C.The change in E_{340} with time was measured using a Unicam SP 800 spectrophotometer equipped with an extension chart recorder.The complete reaction mixture, with ATP but without citrate, gave a change in E_{340}/min when supernatants were present.However, if citrate was added without ATP, a negligible ΔE_{340}/min was measured.Thus the reaction was usually started by first recording ΔE_{340}/min obtained after the addition of ATP, then adding citrate. ΔE_{340}/min obtained after the addition of ATP was subtracted from that obtained with the complete reaction mixture.Reactions were carried out at two different concentrations of high-speed supernatant protein (less than 1mg/3ml), as recommended by Spencer & Lowenstein (1966).

2.11. Perfusion of the isolated mouse liver.

The perfusion technique was essentially that described by Hems et al (1966), which was based on the method of Miller et al (1951). Perfusion of the isolated mouse liver was described by Elliott et al (1971); some further modifications have been introduced in the present study.

2.11.1. Perfusion apparatus.

The arangement of the apparatus, housed entirely in a cabinet thermostatically controlled at 37°C, is shown in Fig.2.2.The perfusion medium was placed above a magnetic stirrer in a reservoir, from which it was pumped to the top of a corrugated glass gas-exchanger, at the entrance to which was a nylon filter.A thin layer of medium passed down the inner surface of the lung where it was exposed to one atmosphere of O_2:CO_2 (95:5 v/v).Oxygenated medium then passed to a constant pressure head arrangement, the overflow from which was returned to the reservoir for mixing and recycling.The medium from the constant head was connected to 15cm of silicone tubing (i.d.1mm), terminating in a female Luer adaptor, the flow through which was controlled by a roller clamp.

The portal venous cannula was a 1cm length of polythene cut to a bevelled point (o.d. 0.75mm, i.d. 0.5mm), connected to a plastic female Luer fitting.(Intravenous cannula from Portex Ltd.)A 10cm length of silicone tubing (i.d. 1mm) was connected to this fitting by means of a plastic adaptor.This tube terminated in a double-ended male Luer fitting.The inferior vena cava cannula consisted of a 1cm

Fig.2.2. Apparatus for perfusion of the isolated mouse liver.

The apparatus was basically that described by Hems et al (1966) for the perfusion of the rat liver,but several modifications were introduced in order to accomodate several requirements of the smaller mouse liver, as described in the text.The mouse was placed on a polystyrene tray at the side of the main reservoir,and the input pressure was equivalent to about 20 cm of medium;the end of the output tube from the liver was placed about 7 cm below the liver.The entire apparatus was maintained at 37^{o} with the aid of a thermostat.

Key: P pump;L artificial 'lung';F filter;M mouse;I infusion pump; o oxygenator (O_2/CO_2,95:5 v/v passed through first bicarbonate-saline then distilled H_2O).

length of plastic tubing (o.d. 1.02mm, i.d. 0.6mm) obtained from an intravenous cannula (Portex Ltd.), from which the Luer fitting, with which it is supplied, has been removed but retaining the plastic connection to the tube.10cm silicone tubing (o.d. 1.0mm) was joined directly to this remaining portion of the connector.Prior to the start of operating procedure, the tube from the portal venous cannula was connected through an adaptor to a bottle containing bicarbonate-saline, continually gassed with O_2:CO_2 (95:5 v/v).Frequently two sets of apparatus were operated simultaneously, in the same cabinet.

2.11.2. Composition of the perfusion medium.

The standard medium of total volume 60ml, consisted of 40ml bicarbonate-saline (Krebs & Henseleit,1932) containing 3.5% bovine serum albumin (Fraction V; Armour Pharmaceutical Co.,Eastbourne,Sussex): dialysed against four changes of bicarbonate-saline for two days, and 20ml (packed cell volume) washed, aged, human erythrocytes which were added to the medium five min before the start of liver perfusion. Human erythrocytes were prepared from whole human blood stored at 4°C. in A.C.D. anticoagulant medium (2g disodium citrate and 3g D-glucose in 100ml H_2O; 440 ml whole blood was added to 100ml anticoagulant medium) for 4-5 weeks.(Aged, donor blood was kindly supplied by St. George's Hospital, Hyde Park Corner, London S.W.1.)Washed erythrocytes were prepared from A.C.D. blood by centrifugation at 3,000 r.p.m. for ten min three times in bicarbonate-saline (bicarbonate-saline: erythrocytes, 4:1 v/v).The haematocrit of the medium was 25-30% throughout all perfusions, except where stated.

Additions of substrates or hormones were made as a single dose, except in the case of FFA-albumin complexes which were added as a priming dose initially and the concentration maintained by infusion. FFA were bound to albumin by the drop-wise addition of 5M NaOH to 50umol FA and warming in a water bath at 70°C until a clear soap solution was formed. 15ml 0.9% NaCl was added. When cool, 25ml 20% bovine serum albumin was introduced and the solution adjusted to pH 7.4 with HCl, with continual agitation. The solution was made up to 50ml with 0.9% NaCl to give a final FA content of 10µmol/ml, and further dilutions were made with 0.9% NaCl as required. The final solution was optically clear at 4-37°C. Where necessary, this was infused into the medium reservoir at a rate of 50µl/min, using a "Delta" pump (Watson-Marlow, Falmouth, Cornwall, U.K.) to maintain perfusate FA concentrations at 0.6±0.2µmol/ml cell-free perfusate.

2.11.3. Operating procedure.

Mice were anaesthetized by interperitoneal injection of sodium pentabarbitone (Nembutal, Abbott Laboratories Ltd.). Both ob/ob and lean mice received a dose of 2ml/kg total body weight. No heparin was used throughout the entire procedure.

The mouse was placed in a supine position on a polystyrene board (15 x 15cm) and a mid-line incision was made the full length of the abdomen. Loose ligatures were placed round the inferior vena cava, anterior to the right renal vein, and around the hepatic portal vein, about 1cm from the point of entry to the liver. A cannula, connected to a tube through which was flowing constantly oxygenated bicarbonate-saline under a pressure of about 20cm. of medium, was

inserted into the hepatic portal vein, and the ligature tightened. The cannula was fixed into place by implantation in a small piece of plasticine which was pinned to the board.At this point the liver immediately cleared of blood and assumed a pale olive colouration (Fig. 2.3.).An incision was made in the inferior vena cava below the renal vein and slow flow was allowed.The diaphragm and ribcage were quickly removed, and a ligature lightly placed around the inferior vena cava, just below the heart.A cannula was inserted through the right auricle into the vena cava and held in place in a similar manner to that inserted into the hepatic portal vein.The ligature just anterior to the right renal vein was tightened and bicarbonate-saline started to flow out of the tube from the inferior vena cava cannula.The platform carrying the mouse was then placed on a stand beside the medium reservoir in the cabinet.The perfusate was then changed to erythrocyte-containing medium, when the liver assumed an even red-brown colouration similar to that seen *in vivo*This appearance could be maintained for at least six hours, during which time no exudate or drying of the liver lobes could be discerned.Care was taken to prevent drying of the liver by the use of saline-soaked tissue supported on a gauze arch over the organ.

The output tube, from the vena cava cannula, was placed in a side arm of the reservoir such that its end was about 7cm below the liver.Thus, allowing for input cannula resistance and output negative pressure, the input pressure was equivalent to about 20cm medium.Flow rates of medium through livers of both *ob/ob* and non-obese mice were 1.1-1.6ml/min/g.

Fig. 2.3. Cannulation of the hepatic portal vein and vena cava of the mouse prior to liver perfusion.

Mice were anaesthetized with Nembutal and abdominal incisions were made as described in the text. Loose ligatures were placed round the hepatic portal vein and inferior vena cava, just anterior to the right renal vein. A plastic cannula (1 cm long, i.d. 0.5 mm, o.d. 0.75 mm) through which gassed bicarbonate-saline buffer was flowing under a pressure of 20 cm H_2O, was inserted into the hepatic portal vein, tied into place and secured with plasticine. The inferior vena cava was cut below the renal veins, the ribcage opened and a ligature places round the vena cava just below the heart. A plastic cannula (1 cm length, o.d. 1.02 mm, i.d. 0.6 mm) was inserted into the vena cava through the right auricle. This was also tied into place and secured with plasticine. The ligature just anterior to the right renal vein was tied and a flow of bicarbonate-saline (about 4 ml/min) was allowed to pass through the liver.

2.11.4. Measurement of O_2-uptake.

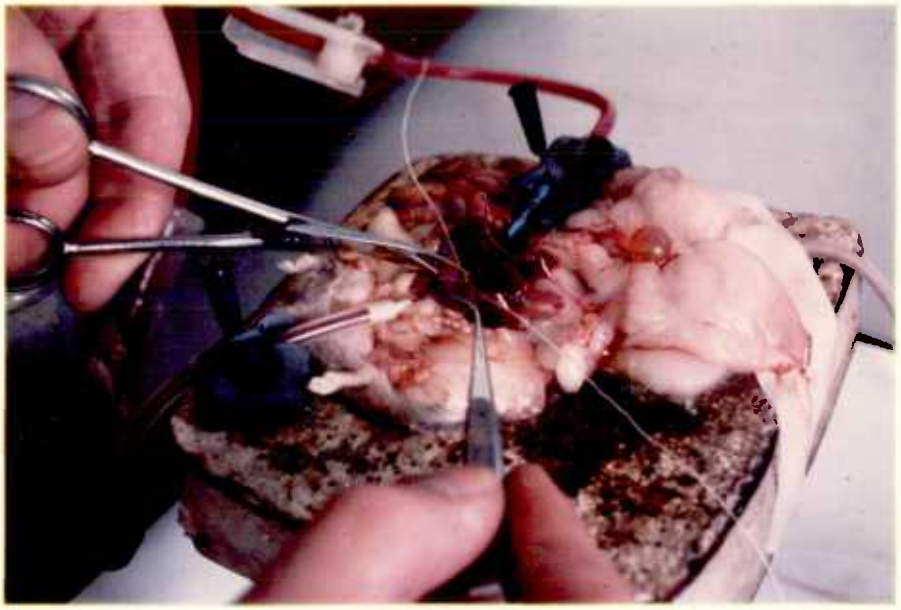
Uptake of O_2 by the perfused liver was measured in some perfusions by connecting the output tube to a flow-through cell, containing a pO_2 electrode connected to a Beckman gas analyser. The pO_2 of the input medium was 320-370mm Hg. (This is less than the pO_2 of the gas mixture because of inequilibration of the medium with the gas mixture in the lung, and the porosity to O_2 of the silicone tubing carrying the medium to the liver.) The pO_2 of the output medium was 30-40mm Hg when livers from non-obese mice were perfused with standard medium containing added glucose only. The amount of O_2 bound to Hb in the output medium was calculated from the O_2-dissociation curve for human, aged erythrocytes at pH 7.4 given by Valtis & Kennedy (1964). The amount of O_2 in solution was calculated from a solubility co-efficient of 0.024ml/ml at 37°C, 760mm Hg. The O_2-uptake of livers from non-obese mice, perfused with standard medium to which only glucose was added, was 6-9µmol O_2/min/g wet liver.

2.11.5. Sampling during the course of perfusions.

Samples of medium taken during the course of perfusions did not generally exceed 9ml in total volume during three hours of perfusion. Medium samples were centrifuged at 3,000r.p.m. for five minutes to sediment erythrocytes, and the cell-free supernatant stored below -10°C for subsequent analysis of glucose and lipids. Medium samples taken for the assay of lactic acid were directly mixed with 6% PCA (whole medium/PCA; 1:1 v/v), centrifuged and the supernatant stored frozen.

Fig. 2.4. Liver biopsy during perfusion of the mouse liver.

In order to sample the liver during perfusion, individual lobes were removed by tying the base of the lobe with fine silk thread (in the photograph below the right half of the median, bifurcted lobe of the liver of an ob/ob mouse). The thread was tightened and the lobe was cut away leaving a small stub above the ligature. Liver samples were immediately frozen in liquid N_2.



Liver samples were taken during the course of perfusions after tying the base of the lobe with fine thread; the lobe was cut away and immediately frozen in liquid N_2 (fig.2.4.).The order of sampling of liver lobes was generally such that each half of the median (bifurcated) lobe (situated each side of the gall bladder) was taken sequentially as the first two samples, usually at 60 min and 120 min, after the start of perfusion.The large left lateral lobe and the smaller right and caudate lobes were taken to provide two final samples.Potential metabolic differences between liver lobes were checked by simultaneous sampling, and also by changing the order of sampling in a few perfusions (Section 4).

CHAPTER 3

RESULTS OF EXPERIMENTS IN VIVO

3.1. Levels and lipoprotein distribution of serum lipids.

3.2. Composition of tissue lipids.

3.2.1. Fatty acid composition of major lipid classes in plasma, liver and adipose tissue.

3.2.2. Composition of liver lipids

3.3. The fate of injected (^{14}C)-palmitic acid and turnover of plasma triglycerides.

3.3.1. Fate of injected (^{14}C)-palmitic acid

3.3.2. Rates of influx of triglycerides into plasma.

3.3.3. Removal of (^{14}C)-VLDL-TG from plasma.

3.3.4. Intravenous triglyceride tolerance in mice.

3.4. Hepatic formation and secretion of lipids synthesized from blood (U-^{14}C)-glucose.

3.4.1. Incorporation of ^{14}C from (U-^{14}C)glucose into liver lipids.

3.4.2. Calculation of rates of hepatic fatty acid synthesis from blood (U-^{14}C)glucose.

3.4.3. Formation of plasma triglycerides from blood (U-^{14}C)-glucose.

3.5. Activity of ATP-citrate lyase (EC 4.1.3.8) in liver and adipose tissue.

CHAPTER 3.

RESULTS OF EXPERIMENTS IN VIVO.

3.1. Levels and lipoprotein distribution of serum lipids.

In serum from ob/ob mice, the concentration of cholesterol was higher than in non-obese mice; this increase was accounted for by increases in both FC and CE (Table 3.1), such that the ratio (CE/TC) was almost identical in serum from both types of mice. Most of the cholesterol in serum from both ob/ob and non-obese mice was in the last major lipoprotein fraction (K_D=0.64) obtained on agarose-gel column chromatography of d<1.21 LP (Fig.3.1). This corresponds to the HDL fraction obtained after similar chromatography of rat plasma LP (Bowden & Fried, 1970). The separation of plasma LP according to their hydrated densities, in a single experiment using pooled plasma from six mice, also showed that about 80% of plasma TC was located in an HDL (d>1.075) fraction. Polyacrylamide-gel disc electrophoresis of mouse plasma, prestained with sudan black B, showed that the extra lipid in ob/ob mouse plasma (shown in the above-mentioned experiments to be largely CE) was in a band not visible in gels on which plasma from non-obese mice had been separated (Fig.3.2), which may have been HDL_1, according to the nomenclature of Narayan (1967).

The concentration of glycerides was not markedly increased in plasma from ob/ob mice, fed ad lib (Table 3.1). There was less change in plasma glyceride concentrations in ob/ob than in non-obese mice throughout the day (Table 3.2), although a greater fall in plasma

Table 3.1. Concentrations of lipids in serum of fed and starved mice.

Blood was obtained from female mice, aged 12 weeks, under light diethyl ether anaesthesia. The methods of lipid analysis and other experimental details are given in Chapter 2. The concentrations of FFA, which are taken from the data of Abraham et al (1971) for mice of the same age and sex and from the same colony as those used in the present experiments, are included for comparison. Results are expressed as means ±S.E.M., of the numbers of observations indicated in parenthesis.

Nutritional state of mice	Mean body weight g	FFA	TG	CE	TC	PL
		μmol/ml serum				
non-obese, fed ad lib.	27.9	0.26±0.03(12)	0.68±0.08(13)	1.61(2)	*2.02±0.23(10)	1.89±0.41(3)
non-obese, 24h starved	26.2	1.02±0.10(12)	0.54±0.10(6)	1.71(2)	**2.72±0.24(6)	1.06(2)
ob/ob, fed ad lib.	64.1	0.29±0.06(6)	0.82±0.09(13)	2.06(2)	*2.64±0.25(10)	2.58±0.50(3)
ob/ob, 24h starved	59.6	0.89±0.11(6)	0.47±0.08(5)	2.25(2)	**3.96±0.45(6)	1.14(2)

* and** $P<0.02$ non-obese vs. ob/ob

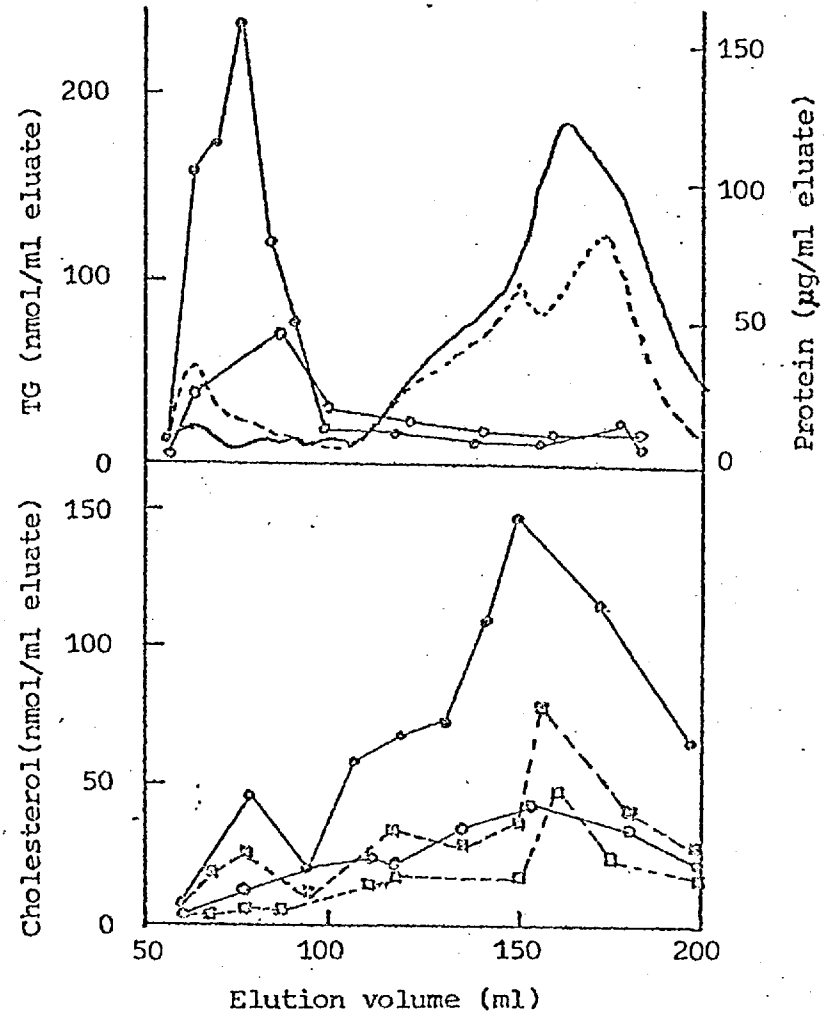


Fig. 3.1. Separation of plasma lipoproteins by agarose-gel column chromatography.

Agarose-gel column chromatography was carried out by passing 2.5 ml of d<1.21 LP, prepared by ultracentifugation of serum from female mice, fed ad lib. and aged 6 months, through a column of Bio-gel A50m (50-100 mesh, 45cm x 2.5cm). The separation properties of the column were attested by the use of standard marker particles and compounds of known molecular weight, as described in Fig. 3.3. Results are from a single analysis of pooled serum (about 4 ml) from more than 12 mice. No fractions were omitted from analysis, although they were pooled for lipid measurements. Fractions were analysed individually
(a) for protein: (——), non-obese mice; (-----), ob/ob mice and pooled for lipid determination. Open and closed symbols denote analyses of serum from non-obese and ob/ob mice respectively.
(a) TG: (○, ●)
(b) CE: (○, ◐); and FC: (□, ▣).

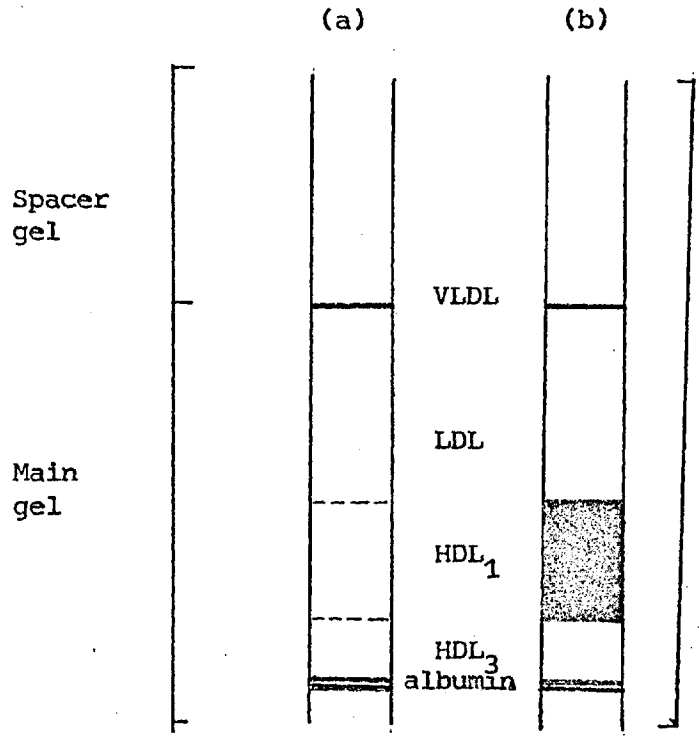


Fig.3.2. Separation of serum lipoproteins by polyacrylamide-gel disc electrophoresis.

Serum lipoprotein lipids were prestained with Sudan black B and separated by polyacrylamide-gel disc electrophoresis, as described in Section 2.5.3. The diagram is representative of results that were obtained three times each in serum from fed non-obese and ob/ob mice. The designations for the bands (VLDL, LDL, HDL_1 and HDL_3) are derived from Narayan (1967).

(a) denotes analyses of serum from non-obese mice, and
(b) analyses of serum from ob/ob mice.

Table 3.2. Concentrations of triglycerides in the blood of mice at various times throughout the day.

Blood was obtained fom female mice, aged 12 weeks and fed *ad lib.* At the times indicated, groups of three mice (non-obese and *ob/ob*) were lightly anaesthetized with diethyl ether/air and blood obtained by cardiac puncture. Triglycerides were determined enzymatically as glycerol (Pinter *et al*, 1967) after saponification (Albrink, 1959). Other experimental details are given in Chapter 2.

Mouse type	Sampling time(h)	Number of observations	Plasma triglycerides μmol/ml plasma ±S.E.M.
non-obese	10.15	3	1.05±0.09
ob/ob	10.15	3	0.84±0.19
non-obese	14.15	3	0.73±0.03
ob/ob	14.15	3	0.84±0.09
non-obese	16.15	3	0.57±0.08
ob/ob	16.15	3	0.61±0.14

glyceride concentrations was observed in ob/ob mice after 24h of food deprivation.Agarose-gel column chromatography of pooled sera from 15 mice aged 6 months, did appear to show an increased TG content in the fraction containing particles of the largest size (Fig.3.1), but this was by no means certain owing to the variable recovery of serum lipids through the procedure.The elution volume at which TG emerged from the column was most accurately assessed from the corresponding protein peak, since each fraction was analysed individually to provide this measurement.Owing to the insensitivity of the lipid estimation techniques employed in this experiment, it was necessary to pool 2-3 fractions for lipid analysis.The protein component of the largest particle size LP (and major TG-containing LP) emerged from the column as a single peak (K_D=0.13), with no indication of an initial peak of K_D=0 (corresponding to chylomicra) such as has been observed in similar experiments employing human serum (Sata et al, 1970).This conclusion was validated by three experiments in which pooled plasma from ob/ob and non-obese mice was used (Table 3.3), which indicated that almost all plasma glyceride was associated with VLDL.

Most plasma PL was associated with HDL in both types of mice (Fig.3.1; Table 3.3) and no significant difference in the total plasma PL was noted between the two types of mice (fewer measurements were made of this parameter than of other lipid classes).

The quantity of lipids associated with LDL and IDL was small compared to that in VLDL and HDL (Table 3.3; Fig.3.1), as in other rodent species (Havel et al, 1955; Nichols, 1969).The observed higher content of TG and PL in IDL, and the smaller quantity of lipids in

Table 3.3. Lipid composition of lipoproteins in serum of mice.

Female mice, aged 12 weeks and fed ad lib., were lightly anaesthetized with diethyl ether/air, blood obtained by cardiac puncture and serum prepared at 15-20°C. Lipoproteins were fractionated according to their hydrated densities by preparative ultracentrifugation (Havel et al, 1955; Gustafson et al, 1965). Other experimental details are given in Chapter 2. The results are from a single analysis of pooled sera from five ob/ob mice and seven non-obese mice, except for the measurement of TG in VLDL, which are the means ±S.E.M. of three observations.

Density range g ml^{-1}	Type of mouse	TG	TC	PL
		μmol/ml original serum		
<1.006	non-obese	0.75±0.05	0.24	0.23
	ob/ob	0.86±0.04	0.27	0.50
1.006-1.050	non-obese	-	0.30	0.13
	ob/ob	-	0.02	0.07
1.050-1.075	non-obese	-	0.02	0.05
	ob/ob	-	0.14	0.11
>1.075	non-obese	-	0.86	1.78
	ob/ob	-	1.74	1.33

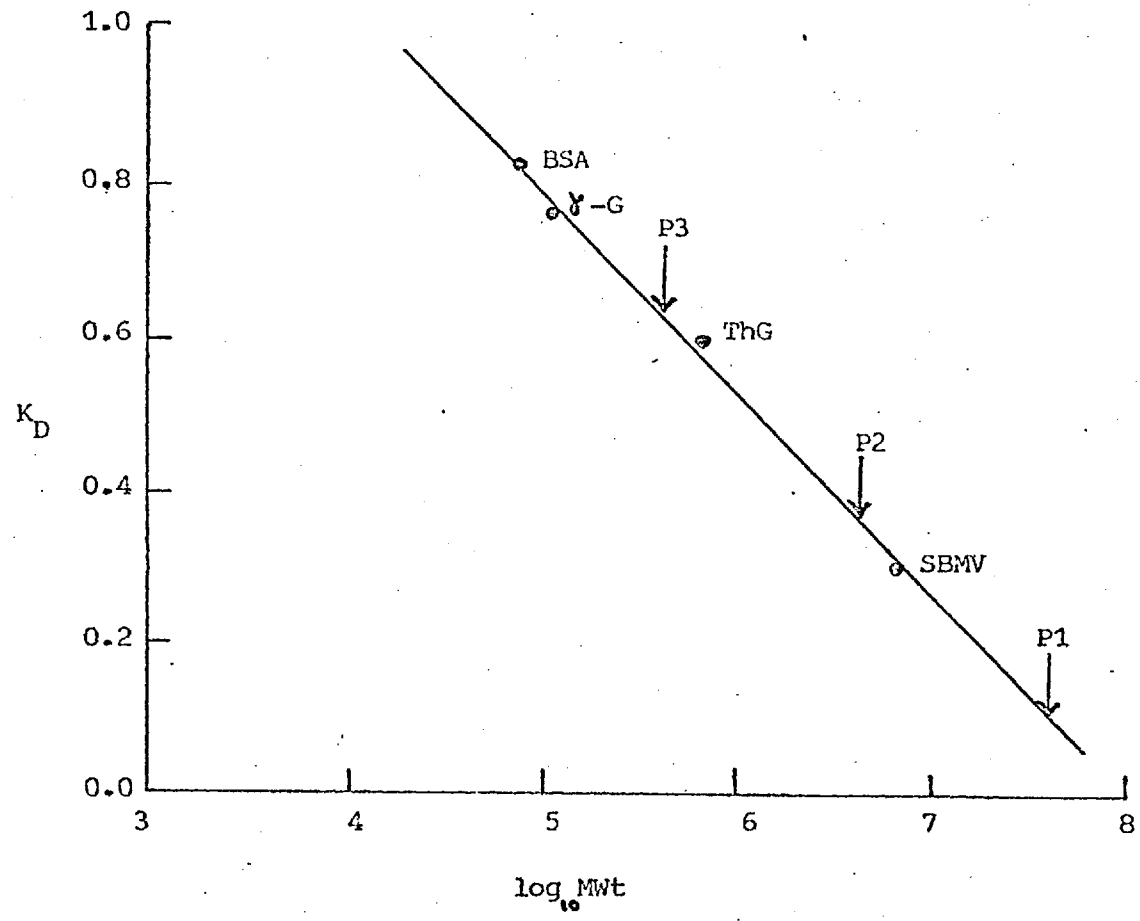


Fig. 3.3. Calibration of agarose-gel column with particles and substances of known molecular weight.

Particles and substances of known molecular weight were passed through a column of Bio-gel A-50m (50-100 mesh, 45cm x 2.5cm) and the elution volume (V_e) noted. The void volume (V_o), determined using a suspension of E.Coli, was 64ml. The total bed volume, determined with NADH, was 205ml. The partition coefficient (K_D) was calculated using the relationship:

$$K_D = (V_e - V_o)/(V_t - V_o)$$

Abbreviations used are BSA: bovine serum albumin; γ-G: γ-globulin; ThG: thyroglobulin; SBMV: southern bean mosaic virus. The arrows denoted P1, P2, and P3 refer to the elution maxima of the three major protein peaks obtained on chromatography of d<1.21 LP prepared by ultracentifugation of murine serum. V_e for the three peaks were the same for LP prepared from both non-obese and ob/ob mice. The K_D values for ThG and SBMV were taken from Bowden & Fried (1970).

LDL, in plasma from ob/ob mice, was inconclusive since only one separation of these density ranges of LP were performed, albeit on plasma from six mice. Nevertheless it is noteworthy that Faloona et al (1969) observed an increased IDL concentration in hyperthyroid rats. IDL has a similar range of hydrated densities as the HDL_1 (Narayan, 1966) and Lp(a) (Berg, 1963; Schultz et al, 1968), which have been detected as minor LP components of rat and human sera respectively. Agarose-gel column chromatography of d<1.21 LP from ob/ob mice (Fig. 3.1) also indicated that greater quantities of FC, CE and PL were associated with a particle of larger size than that of the major HDL fraction.

The particle weights of serum LP in mice were estimated by comparing the K_D of protein peaks associated with LP with a calibration graph prepared using standard proteins and marker particles (Fig.3.3). Those obtained for LP from ob/ob mice were similar to the corresponding particles in non-obese mice and rat plasma (Bowden & Fried, 1970).

3.2 Composition of Tissue Lipids.

3.2.1. Fatty acid composition of major lipid classes in plasma, liver and adipose tissue.

The % distribution of FAME (16:0-18:2) in major lipid classes in tissue from ob/ob mice and non-obese mice, fed ad lib and aged three months, are shown in Table 3.4(a).The most marked changes between the two types of mice were an increased % 16:1 and a decreased % 18:2 in hepatic TGFA and CEFA, plasma TGFA and epididymal adipose tissue TGFA.These changes in liver and adipose tissue resemble those in C57BL/6J ob/ob mice reported by Winand et al (1968), Christophe et al (1970), Lemonnier et al (1971) and Haessler & Crawford (1965); the changes in plasma TGFA and FFA were similar to those reported by Redding & Schally (1967) in goldthioglucose-obese mice.New aspects of the present study were analyses of plasma lipid classes and liver CEFA in ob/ob mice.A decreased % 16:0 was found in plasma PLFA and CEFA in ob/ob mice; this change was not found in any other lipid class. In ob/ob mice plasma and liver CEFA compositions resembled each other through an increased 16:1 content, but the decreased % 18:2 in hepatic CEFA was not reflected in plasma CEFA composition.In agreement with Christophe et al (1970) and Lemonnier et al (1971), ob/ob mice showed an increased relative desaturation of FA, particularly palmitic acid.

To aid the interpretation of Table 3.4(a), a scheme has been made of FA metabolism in order to illustrate some of the major metabolic processes, as shown in Fig.5.1 (see Chapter 5).Statistical comparisons were made of the changes in FA composition of lipid classes in relation to a number of these processes, using data from

Table 3.4(a)next page

Table.3.4(a) Percentage distribution of fatty acids in lipid classes in tissues and plasma of mice.

Total lipid extracts of tissues and plasma were prepared by the method of Folch et al (1957) and lipid classes separated by adsorption t.l.c. (Freeman & West,1966).Methyl esters of the acyl components of each lipid class were prepared and separated by g.l.c. on a column of 10% polyethylene glycol adipate on celite 650 at 190°C. Other experimental details are described in Chapter 2.Results are expressed as the % of the total FAME detected between 16:0 and 18:2.The significance of the differences between the FAME profile obtained in ob/ob and non-obese mice were tested by Student's 't' test.

Table 3.4(a)

Tissue		plasma				liver				adipose
Lipid class		TG	FFA	PL	CE	TG	FFA	PL	CE	TG
non-obese	No.obs.	6	5	9	5	6	3	9	7	4
	16:0	33.3±1.0	40.3±4.6	46.4±2.0	15.1±2.0	31.2±0.2	59.3±2.9	36.7±3.1	34.7±3.9	33.4±1.9
	16:1	8.8±1.2	9.6±0.8	1.3±0.4	9.1±1.0	5.4±0.5	4.9±0.3	2.6±0.5	10.5±1.1	9.6±1.0
	18:0	1.8±0.8	15.6±4.6	17.3±1.6	3.5±1.6	2.5±0.3	5.5±1.3	16.3±2.2	3.6±1.8	3.2±0.5
	18:1	37.2±2.1	28.5±2.6	16.2±2.2	22.9±1.8	44.1±0.7	10.7±4.1	19.9±3.5	30.7±4.4	36.7±3.0
	18:2	19.0±1.8	8.7±1.3	17.5±3.4	49.1±3.6	16.5±0.5	12.4±7.2	10.9±1.5	18.9±2.6	19.7±0.8
ob/ob	No.obs.	5	4	8	4	4	3	8	7	4
	16:0	33.1±2.5	42.8±5.4	35.5±1.5	11.1±1.1	35.3±2.8	37.7±3.3	33.7±1.3	33.0±2.7	34.0±0.7
	16:1	12.1±1.0	7.8±2.2	0.9±0.1	21.3±2.9	11.9±2.0	14.5±3.6	2.9±0.9	16.1±2.6	12.8±1.4
	18:0	4.2±0.3	13.8±1.2	19.4±2.2	1.1±0.1	2.0±0.5	5.5±1.3	23.1±3.9	8.0±3.4	2.7±0.7
	18:1	37.4±2.6	30.5±0.6	22.6±1.5	20.5±1.9	44.6±4.6	21.4±4.7	19.9±2.4	31.6±4.5	36.4±2.1
	18:2	14.0±1.5	7.6±1.1	18.9±1.4	48.2±4.1	6.7±0.4	14.7±1.3	13.6±1.0	13.3±1.3	14.7±1.0
ob/ob vs. non-obese	16:0	NS	NS	-	-	+	-	NS	NS	NS
	16:1	+	NS	NS	+	+	+	NS	+	+
	18:0	+	NS	NS	NS	NS	-	+	NS	NS
	18:1	NS	NS	+	NS	NS	+	NS	NS	NS
	18:2	-	NS	NS	NS	-	NS	+	-	-

NS not significant ($P>0.05$)

+/- $P<0.05$ ob/ob altered relative to non-obese in the direction indicated.

Table 3.4(b) Changes occurring in the % fatty acid composition of lipid classes in mice.

Changes occurring in the % FA composition of lipid classes reflecting some major processes in inter- and intra-tissue lipid metabolism (as outlined in Fig. 5.1) were tested for their significance by Student's 't' test, using data taken from Table 3.4(a). The analysis was carried out separately for both non-obese and ob/ob mice. + or - indicates an increase or decrease in % FA composition of the product lipid class with respect to the particular FA, relative to that in the precursor class ($P<0.05$), and NS indicates that the change was not significant ($P>0.05$). * indicates that a change was observed in ob/ob mice which was not the same as that in non-obese mice.

Comparison	Mouse type	16:0	16:1	18:0	18:1	18:2
adipose TG vs. plasma FFA	ob/ob	+	NS	+	−	−
	non-obese	+	NS	+	−	−
plasma FFA vs. liver TG	ob/ob	−	+*	−	+	NS*
	non-obese	−	−	−	+	+
plasma FFA vs. liver PL	ob/ob	NS*	−	+*	−	+
	non-obese	−	−	NS	−	+
plasma FFA vs. liver CE	ob/ob	NS	+*	-	NS	+
	non-obese	NS	NS	−	NS	+
plasma TG vs. liver FFA	ob/ob	NS*	NS	+	−	+*
	non-obese	+	NS	+	−	NS
liver PL vs. liver FFA	ob/ob	NS*	+	−*	NS	NS
	non-obese	+	+	NS	NS	NS
liver TG vs. plasma TG	ob/ob	NS	NS*	+*	NS	NS
	non-obese	NS	+	NS	NS	NS
plasma TG vs. adipose TG	ob/ob	NS	NS	NS	NS	NS
	non-obese	NS	NS	NS	NS	NS

Table 3.5 Lipid composition of livers of mice.

Livers were removed from mice under light diethyl ether/air anaesthesia and immediately frozen in liquid N_2. After extraction with chloroform/methanol (2:1 v/v) by the method of Folch et al (1957), lipids were analyzed for glycerides (as glycerol, after saponification), PL (as lipid P_i) and TC by methods described in detail in Chapter 2. All mice were 12 weeks old. Results are the mean ±S.E.M. of the number of observations in parenthesis.

Type of mouse	Nutritional state	lipid content (μmol/g fresh liver)		
		*glycerides	PL	TC
non-obese	fed ad lib.	31±3(9)	37±3(3)	11±1(3)
non-obese	24h starved	72±5(6)	-	-
ob/ob	fed ad lib.	72±7(8)	39±5(4)	11±1(3)
ob/ob	24h starved	39±4(6)	-	-

*Represents the sum of (MG+DG+TG)

Table 3.4, and are shown in Table 3.4(b).Evidence for some of the processes shown in Fig.5.1 is discussed in Chapter 5.

There was a marked similarity (in both types of mice) between FA distribution in plasma TG and adipose tissue TG, except that adipose tissue TG from non-obese mice showed an increased % 18:0 relative to plasma TG, possibly due to FA synthesis *in situ* in this tissue.Most of the changes found between *ob/ob* and non-obese mice in Table 3.4(b) occurred in the liver.Increased FA desaturation resulted in an increased % 18:1 and % 16:1 in liver TGFA relative to plasma FFA, in accordance with Nakagua & Uchiyama (1969) who showed that monoenoic FA were incorporated into hepatic TG twice as fast as saturated FA in normal rat liver.Unlike non-obese mice, hepatic PL from *ob/ob* mice contained increased % 18:0 and failed to show a change in % 16:0 relative to plasma FFA.In both types of mice it was notable that the same changes occurred in FA distribution between adipose FFA (or TGFA) and plasma FFA, such that plasma FFA was richer in 16:0 and 18:0 and poorer in 18:1 and 18:2 than adipose tissue.

3.2.2. Composition of liver lipids.

An increased hepatic TG content was observed in *ob/ob* mice, relative to non-obese mice (Table 3.5), although to a lesser extent than has been reported for other strains into which the gene *ob* has been incorporated, especially C57BL/6J (Bates *et al*,1955; Jansen *et al*, 1967; Christophe *et al*, 1970; Stein *et al*, 1970).Starvation brought about an increase in hepatic TG content in non-obese mice, but a decrease in *ob/ob* mice.The hepatic content of other lipid classes was not increased in *ob/ob* mice.

3.3. The fate of injected (^{14}C)-palmitic acid and turnover of plasma triglycerides.

The fate of injected (^{14}C)-palmitic acid in tissue lipids was investigated; these experiments permitted calculation of rates of esterification of plasma FFA in mice.Also, three types of experiments were carried out from which independent estimates of plasma TG turnover rates could be calculated.These included determination of the time course of the incorporation of ^{14}C from injected (U-^{14}C)-palmitic acid into plasma TG.From this experiment an estimate of the total rate of entry of endogenously synthesized TG into plasma was obtained. In a second group of experiments, rates of disappearance of (^{14}C)-VLDL-TG were investigated by injecting lipoproteins in which TG had been labelled by prior administration of (U-^{14}C)-palmitic acid to donor mice.Finally, a measurement of "TG tolerance" was made by injecting a TG emulsion, Intralipid (Lewis et al, 1972).

3.3.1. Fate of injected (^{14}C)-palmitic acid.

The distribution of ^{14}C in tissue lipids was investigated five minutes after an intravenous injection of (^{14}C)-palmitic acid.It was likely that more than 95% of the injected radioactivity would have disappeared from plasma FFA after this time (Elliott et al, 1974; Baker & Schotz, 1967).Tables 3.6 and 3.7 show that a major proportion of injected ^{14}C was found in hepatic lipids in both ob/ob and non-obese mice.Only small quantities of ^{14}C were found in extrahepatic lipids (Table 3.6), although it may be expected that the lipids of skeletal muscle (not measured in these experiments) would contain about 10%

Table 3.6 Radioactivity in tissue lipids five minutes after intravenous injection of (^{14}C)-palmitic acid into mice.

Female mice, aged 8 weeks and fed *ad lib.*, were either anaesthetized with Avertin (2.2.1.), or placed in a restraining cage, prior to injection with (U-^{14}C)-palmitate (anaesthetized mice) or (1-^{14}C)-palmitate (unanaesthetized mice). Five minutes later anaesthetized mice were killed by cardiac puncture and unanaesthetized mice were killed by cervical dislocation. Tissues were removed, total lipids extracted (Folch *et al*, 1957) and their content of ^{14}C measured (2.1.10.). The data refers to the ^{14}C present in the total tissue present in the mouse. The weight of adipose tissue was taken as 4% of total body weight in non-obese mice (about 1g) and 50% total body weight in *ob/ob* mice (about 25g). Results are expressed as %injected ^{14}C ± S.E.M., and the number of observations are in parenthesis.

Tissue	Mouse type	Mean tissue weight(g)	%injected radioactivity in total lipids	
			Avertin anaesthesia	without anaesthetic
liver	non-obese	1.07	23.3±2.2(3)	11.9(2)
	ob/ob	1.73	80.9±3.9(3)	24.8±3.4(3)
heart	non-obese	0.10	0.4(1)	0.6(2)
	ob/ob	0.15	0.8(1)	0.5(2)
adipose tissue (epididymal)	non-obese	-	1.2(1)	0.9(2)
	ob/ob	-	4.9(1)	7.0(3)
small intestine	non-obese	3.27	4.2(1)	-
	ob/ob	4.88	2.8(1)	-

Table 3.7. Incorporation of ^{14}C from (^{14}C) palmitate into liver and plasma triglycerides and phospholipids in mice.

In the experiments described in Table 3.6.,TG and PL were separated from total lipid extracts of liver and plasma by t.l.c. (Freeman & West,1966),and the content of ^{14}C in these two lipid classes determined.The data are expressed as the % of the injected radioactivity present in each lipid class in the total liver or plasma. Values for the mean plasma volume of mice were taken from the data of Abraham et al (1971).The mean % values are expressed ± S.E.M.,and the number of observations is in parenthesis.

Mouse type	tissue	anaesthetic	time after injection (min.)	% injected radioactivity in lipid class	
				PL	TG
non-obese	liver	Avertin	5	6.32±2.13(3)	16.92±0.99(3)
ob/ob	liver	Avertin	5	23.40±4.17(3)	57.53±8.03(3)
non-obese	plasma	Avertin	5		0.007±0.002(3)
ob/ob	plasma	Avertin	5		0.029±0.008(3)
non-obese	liver	none	5	4.53±1.80(3)	6.52(2)
ob/ob	liver	none	5	4.48±0.29(3)	13.26(2)
non-obese	liver	none	30	7.56(2)	13.17(2)
ob/ob	liver	none	30	5.52(2)	16.94(2)

of the injected radioactivity (Havel et al, 1962). Although epididymal adipose tissue from ob/ob mice contained less ^{14}C/g than in non-obese mice, about ten times as much was estimated to be present in the whole tissue (i.e. per mouse) (Table 3.6).

Rates of hepatic esterification of plasma FFA may be estimated by considering that the % of the injected dose of ^{14}C as (^{14}C)-palmitate incorporated into hepatic glycerolipids five minutes after injection (Table 3.7) represents the fraction of the total turnover of plasma FFA which is esterified during this time. From the data of Elliott et al (1974), turnover rates of plasma FFA are 4.5 and 6.7 μmol min^{-1} in Avertin anaesthetized non-obese and ob/ob mice respectively; the corresponding turnover rates in conscious mice have been calculated to be 1.1 and 2.2 μmol min^{-1}. Accordingly, rates of hepatic esterification are 0.39 and 0.12 μmol FFA min^{-1} in conscious ob/ob and non-obese mice respectively. The corresponding figures obtained under Avertin asaesthesia are greater, viz. 1.78 and 0.25 μmol FFA min^{-1}. These values probably underestimate the rate of the process, since the decline in radioactivity in plasma FFA shows complex kinetics, and is not first order during the whole of this time (Fredrickson & Gordon, 1959; Baker & Schotz, 1967 ; Elliott et al, 1974).

Avertin anaesthesia appeared to increase the rate of hepatic esterification, and this effect was greater in ob/ob mice presumably due to the administration of four times the dose of anaesthetic to these mice. It is relevant that other lipophilic agents (e.g. chloroform and diethyl ether) have been shown to increase the rate of hepatic esterification and decrease the rate of TG secretion into the plasma of rats five minutes after an injection of (1-^{14}C)-palmitic acid (Schotz

et al, 1964; Schotz & Olivecrona, 1966).However, irrespective of whether the mice were anaesthetized or not, the fraction of injected ^{14}C found in hepatic TG was greater in ob/ob than in non-obese mice, which is in agreement with the data of Winand et al (1968), obtained using liver slices.

3.3.2. Rates of influx of triglycerides into plasma.

After a trace dose of (U-^{14}C)-palmitic acid was injected, there was a delay of a few minutes before ^{14}C was detected in plasma TG (Fig.3.4).A similar delay of about ten minutes in other species has been reported (Laurell, 1959; Borgstrom & Olivecrona, 1961; Baker & Schotz, 1964; Havel et al, 1962; Gross et al, 1967).The amount of ^{14}C in TG rose to a maximum after 20-30 minutes.The decline in specific radioactivity of plasma FFA was probably very rapid in these experiments since the turnover of plasma FFA is very rapid in mice (Elliott et al, 1974).In addition, a number of very different fates of hepatic TG formed by the esterification of plasma FFA have been demonstrated, other than the secretion of VLDL-TG (Baker & Schotz, 1967).Hence calculation of the contribution of plasma FFA to plasma TG is not possible.Rather, these experiments permit calculation of the total rate of secretion of TGFA into plasma as follows: the incorporation of ^{14}C into plasma TG attained a relatively steady plateau, despite the administration of a single dose of (^{14}C)-palmitic acid.The most likely explanation for this is that it reflected release into plasma from an extravascular pool of (^{14}C)-TG which behaved as though it retained constant specific radioactivity, during the later phase of the experiment.This could be largely accounted for by recycling between (^{14}C)-FA in non-TG pools and TG in plasma.

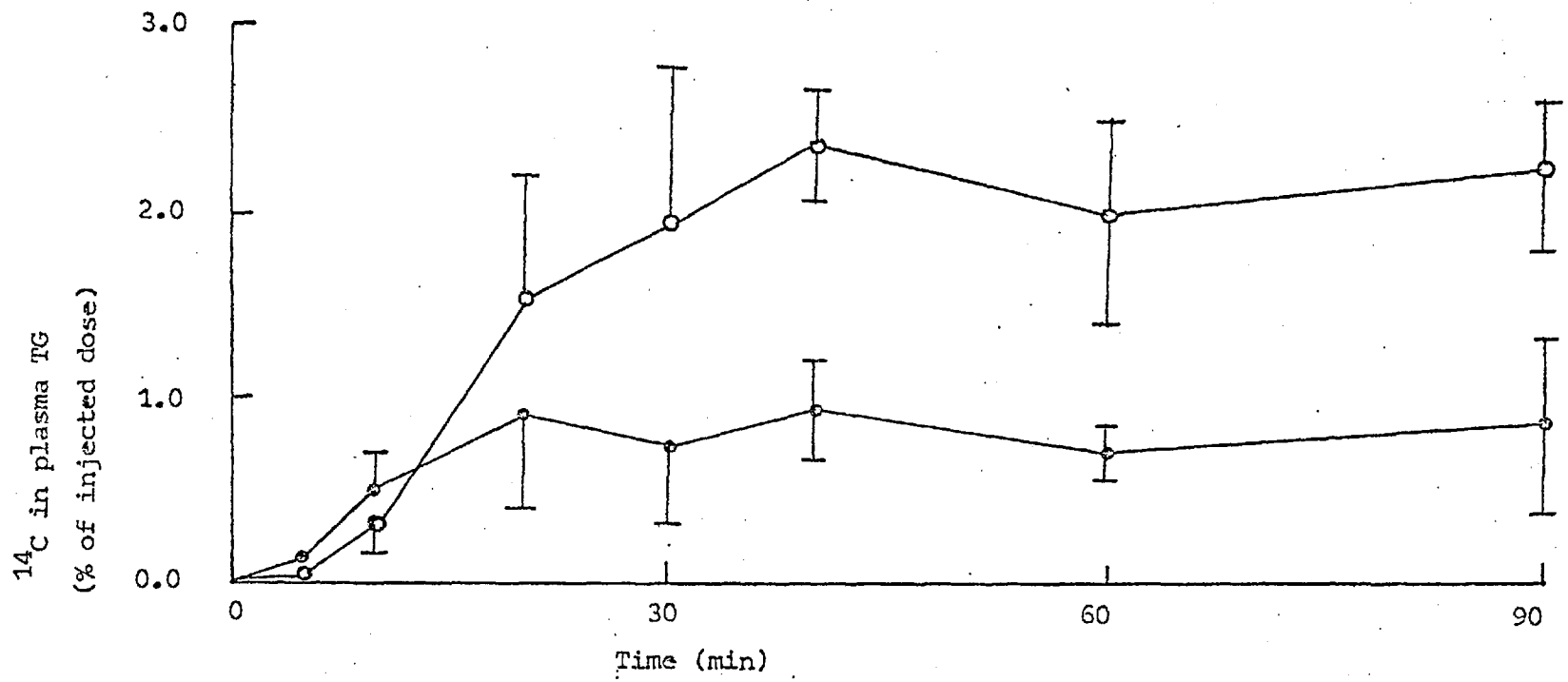


Fig. 3.4. Time-course of incorporation of ^{14}C from (U-^{14}C)-palmitate into plasma triglycerides.

Female mice, aged three months and fed ad lib., were injected intravenously with (U-^{14}C)-palmitate, as an average trace dose of 2.8 x 10^6 d.p.m. The content of ^{14}C in total plasma TG was determined after various times. Except for the 5 min point, the time course was followed in serial samples (50-100µl each obtained from a tail vein) of plasma from single mice. Other details are in the text. The values after 5 min are also shown in Table 3.7. Results are the mean of three to five measurements, in five non-obese (○) or ob/ob (●) mice, and bars represent the S.E.M.

Table 3.8. Calculated rates of entry of triglycerides into the plasma of mice.

The results shown in Fig. 3.4. have been used to calculate rates of TG secretion into plasma, as described in the text (3.3.2.) The designation '%D' refers to the percentage of the injected dose of (U-^{14}C)-palmitate.

calculation	mouse type	
	non-obese	ob/ob
plasma TG pool size(μmol TG)*	1.0	1.6
approximate specific radioactivity of plasma (^{14}C)-TG during steady phase in Fig 3.4: s (%D/μmol TG)	2.1	0.45
approximate initial rate of entry of (^{14}C)-TG into plasma during rising phase in Fig. 3.4 : r (%D/min)	0.13 (between 10 and 20 min)	0.08 (between 5 and 10 min)
calculated rate of entry of TGFA into plasma : 3r/s (μmol TGFA/min)	0.18	0.54

* From Table 3.1 and measurements of plasma volume in mice (Abraham et al, 1971)

Since the liver is thought to be almost the sole source of endogenously synthesized TG in plasma, and the % of the injected radioactivity in hepatic lipids remained relatively constant, both in the present work (Table 3.7) and other experiments (Baker & Schotz, 1964; Gross et al, 1967), it has been suggested that recycling may occur between liver and plasma TG (Farquhar et al, 1965; Havel et al, 1962; Baker & Schotz, 1964; Gross et al, 1967).However, Laurell(1959) and Fredrickson & Gordon (1958) have shown that considerable recycling of (^{14}C)-FA may occur via extra-hepatic tissues,blood and hepatic re-esterification.These possibilities have been illustrated in Fig. 5.1, and the topic is elaborated upon in the Discussion chapter.If it is assumed that TG from such an extra-vascular (^{14}C)-TG pool(s) was being released at a constant rate into an initially unlabelled plasma TG pool during the period 10-40 minutes (Fig.3.4), then the rate of release of TG may be calculated from the initial rising phase of incorporation of ^{14}C into TG, and the specific radioactivity of plasma TG, estimated from the steady phase of the time course (Fig.3.4).Such calculations of the rates of secretion of TG are shown in Table 3.8.From the nature of the calculation, they represent minimal estimates for the process in the present conditions.

3.3.3. Removal of (^{14}C)-VLDL-TG from plasma.

The assimilation of endogenously synthesized plasma TG was investigated by injecting murine VLDL in which TG had been labelled by prior administration of (U-^{14}C)-palmitic acid to groups of donor mice. Blood was obtained about 30 minutes after the injection of isotope,and VLDL prepared as previously described (2.2.3).Table 3.9 shows that more than 80% of VLDL-(^{14}C)-lipid was in TG.A fresh preparation was injected intravenously into mice.

Table 3.9. Distribution of radioactivity in different (^{14}C)-VLDL preparations obtained from donor mice, prior to injection into recipient mice.

0.1 ml (U-^{14}C)-palmitic acid (>200 mCi/mmol), bound to bovine serum albumin, was injected into ob/ob or non-obese mice (female, aged 6 months) under Avertin anaesthesia. 30 minutes later, blood was obtained by cardiac puncture and VLDL prepared from plasma. Lipids were extracted (Folch et al (1957) and separated by t.l.c. (Freeman & West, 1966). Other experimental details are given in Chapter 2.

	Donor mice			
	ob/ob		non-obese	
	A	B	C	D
Number of mice	5	5	4	4
Injected ^{14}C (10^{-7}.dpm/mouse)	1.22	1.26	1.22	1.77
Content of ^{14}C (10^{-4}.dpm/ml)				
30 minutes after injection in:				
pooled plasma	8.0	24.7	29.1	8.8
VLDL preparation	3.6	6.6	1.0	5.3
VLDL lipids:				
CE	-	-	-	-
TG	1.49	1.65	0.22	0.96
FC	-	-	-	-
FFA	0.16	0.05	0.13	0.04
PL	0.03	0.02	0.05	0.02
specific radioactivity of VLDL-TG (10^{-4}.dpm/umol)	1.60	1.55	1.55	0.89

The disappearance of (^{14}C)-TG from plasma was more rapid in ob/ob than in non-obese mice (Fig.3.5).Although the present results were not adequate to discern accurately the kinetic order of the process, a good correlation was obtained when linear regression analyses were performed on the logarithm of the % of the injected (^{14}C)-TG remaining in plasma (abscissa) with time (ordinate).Furthermore, VLDL-TG disappearance from plasma has been shown to exhibit first order kinetics in other similar exoeriments (Havel, 1965; Gross et al, 1967).Assuming that such kinetics pertained in the present instance, $t_{\frac{1}{2}}$ for the disappearance of VLDL-TG from plasma may be calculated.Non-obese mice showed a $t_{\frac{1}{2}}$ of 7.2 minutes and ob/ob mice a $t_{\frac{1}{2}}$ of 2.2 minutes.If it is assumed that the kinetics of removal of endogenous, native VLDL-TG were the same as those injected, then the turnover rate of endogenous VLDL-TG may be calculated according to the following equations.If (k) is the rate constant for the uptake of endogenous TG, then from the properties of a first order process,

$(k) = (1n2/t_{\frac{1}{2}})$

The turnover rate (T_r) is equivalent to the uptake at a steady TG concentration,If (p) is the plasma pool size of VLDL-TG (calculated from data given in Table 3.3 and measurements of plasma volume in mice reported by Abraham, 1973) then

$(k).(p) = T_r$

$T_r = (1n2/t_{\frac{1}{2}}).(p)$

Estimates of these parameters calculated according to the above method are given in Table 3.11.The turnover rates of plasma TG given in this table pertain to conscious mice since they were calculated from measurements made in such animals (although mice were anaesthetized during sampling).

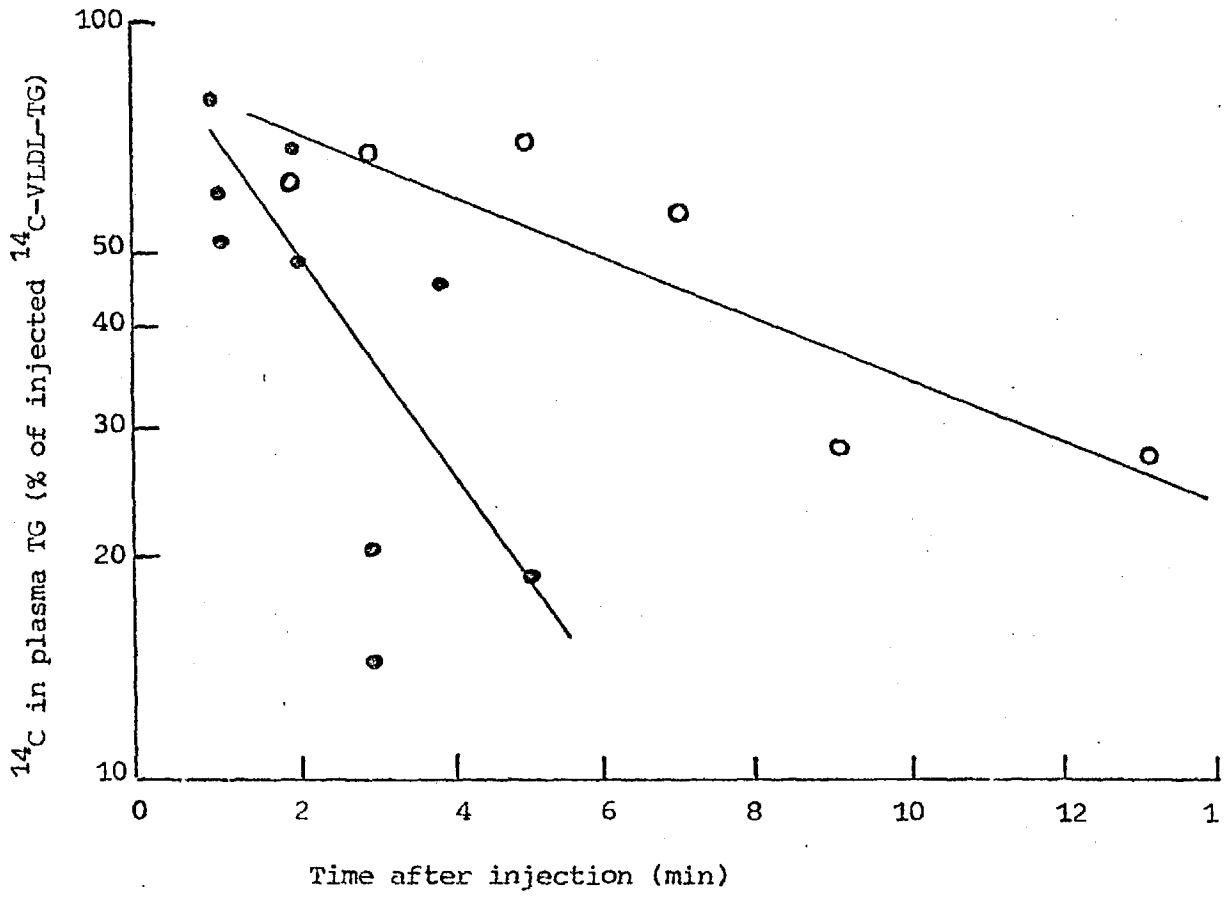


Fig. 3.5. Time-course of disappearance from plasma of ^{14}C in VLDL-(^{14}C)-triglyceride fatty acid.

Female mice, aged 8 weeks and fed ad lib., were injected intravenously with VLDL containing (^{14}C)-TGFA prepared from donor mice. VLDL from non-obese mice was injected into other non-obese (o) mice, and that from ob/ob mice into other ob/ob mice (●). Other details are in the text. After various times, ^{14}C in plasma TG was determined. Each result represents a single measurement from one mouse. The lines were fitted by linear regression analysis, taking 100% at zero time as one value. r was -0.88, $P<0.05$ for non-obese mice, and r was -0.77, $P<0.05$ for ob/ob mice.

3.3.4. Intravenous triglyceride tolerance in mice.

Fig.3.6 shows the elimination curve of injected Intralipid from the plasma of ob/ob and non-obese mice, aged three months and fed ad lib. on the standard laboratory diet (2.1.1).It can be seen from this figure, in which the logarithm of the plasma Intralipid TG concentration is plotted against time after injection, that a first order decline was followed in both types of mice between 4-20 minutes after injection. A rather more rapid decline was observed immediately after injection; a similar phenomenon was observed by Boberg et al (1969) in humans, in which an initial phase of zero order elimination was found, the cause of which remains unknown.The phase during which a first order decline was followed appeared to reflect physiological assimilation of TG, analogous to that of chylomicra; for example, the $t_{\frac{1}{2}}$ for the disappearance of injected TG emulsion was increased on starvation, in non-obese mice at least in the present experiments (Table 3.10).In addition, a number of similarities have been found between the metabolism in vivo of chylomicra and injected TG emulsions (Carlson & Hallberg, 1963; Hallberg, 1964, 1965; Gries et al, 1966).

Table 3.10 shows that the $t_{\frac{1}{2}}$ for the elimination of exogenous plasma TG was approximately twice as fast in ob/ob as in non-obese mice.Whilst the $t_{\frac{1}{2}}$ increased after 24h starvation in non-obese mice, this was not observed in ob/ob mice.The rapid clearance of TG in ob/ob mice was not slower in mice which had received a restricted diet (2.1.1) and starved for 24h prior to Intralipid administration.

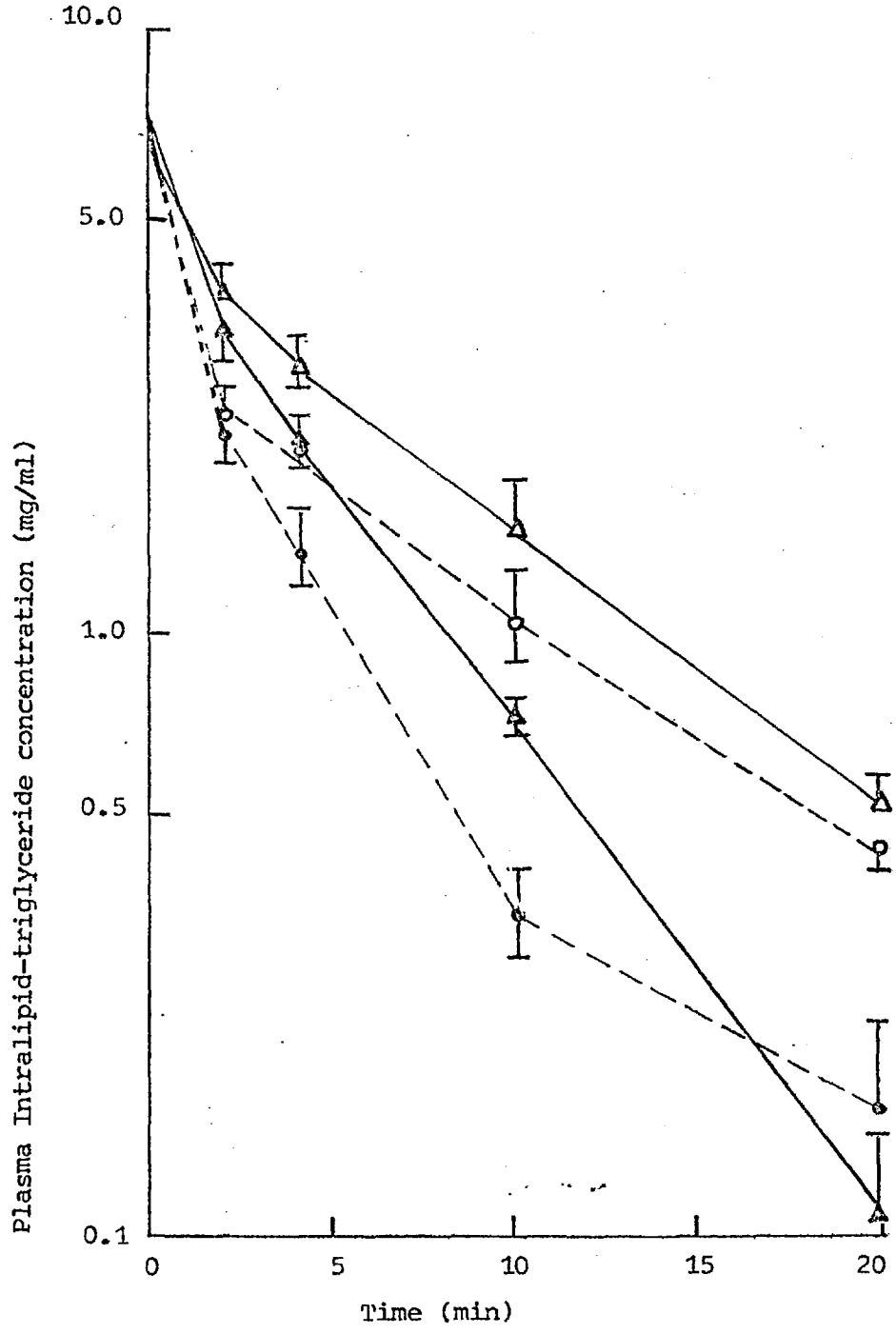


Fig. 3.6. Time-course of removal of injected Intralipid from plasma.

Female mice, aged 12 weeks, were anaesthetized with Avertin and a stable TG-PL emulsion, Intralipid (10% soybean oil, w/v) was injected intravenously. Non-obese mice received 0.10ml and ob/ob mice 0.13ml. Serial samples of blood (50-100µl each) were subsequently removed from a tail vein and Intralipid TG measured by nephelometry. Other details are in the text. Open and closed symbols represent non-obese and ob/ob mice respectively. (○,●) represents mice fed ad lib. and (△,▲) mice deprived of food for 24 h prior to injection.

Table 3.10. Half-times of removal of intravenously injected Intralipid from the blood of mice.

A stable emulsion of soybean oil, Intralipid (10% w/v) was injected into the tail vein of non-obese mice (0.1 ml) or ob/ob mice (0.13ml), which were anaesthetized with Avertin. The half time of its dissappearance from plasma ($t_{\frac{1}{2}}$) was calculated from the time course of removal in each mouse, which was followed in sequential blood samples by nephelometry (Lewis et al, 1972), and which exhibited first order kinetics (Fig. 3.6). Other experimental details are given in Chapter 2. Results are the average, or mean ± S.E.M. of the number of observations in parenthesis.

Nutritional state of mice	Age (months)	half-time of dissappearance (min)	
		non-obese	ob/ob
fed ad lib.	1	6.2(2)	3.2(2)
fed ad lib.	3	5.1±0.7(5)	2.5±0.2(6)
fed ad lib.	4	7.0(2)	3.5(2)
24h starved	3	7.0±1.5(3)	3.3±0.3(3)
24h starved	5	8.3(2)	4.0±0.3(3)
diet-restricted, 24h starved	6	-	2.7±0.2(3)
diet restricted, 24h starved	14	-	3.3(2)

Table 3.11. Calculated turnover rates and rate constants for plasma triglyceride fatty acids in mice.

The values for turnover rates (T_r) and rate constants (k) have been calculated by methods described in the text using data shown in other tables: T_r (secretion) is taken from Table 3.8., and $t_{\frac{1}{2}}$ for the uptake of VLDL-TGFA and Intralipid from data in Fig. 3.5. and Table 3.10. respectively. The experiments concerning the secretion of endogenously (^{14}C)-labelled plasma TGFA and the uptake of Intralipid were carried out using mice aged 12 weeks, and those concerning VLDL-TGFA assimilation using mice aged 8 weeks. All mice were females and fed ad lib.

Explanation of symbols: (a) μmol TGFA $min^{-1} mouse^{-1}$

(b) μmol TGFA min^{-1} (100g body weight)$^{-1}$

p plasma pool size.

$t_{\frac{1}{2}}$ half-time for the uptake of TGFA from plasma.

Mouse type	secretion			VLDL-TGFA uptake						Intralipid	
	body weight (g)	T_r (a)	T_r (b)	body weight (g)	$t_{\frac{1}{2}}$ (min)	k $(min)^{-1}$	p (μmol TGFA)	T_r (a)	T_r (b)	$t_{\frac{1}{2}}$ (min)	k $(min)^{-1}$
ob/ob	64.1	0.54	0.84	46.0	2.2	0.32	4.98	1.55	3.36	2.5	0.28
non-obese	27.9	0.18	0.65	25.6	7.2	0.10	3.12	0.31	1.21	5.1	0.14

3.4. Hepatic formation and secretion of lipids synthesized from blood (U-^{14}C)-glucose.

3.4.1. Incorporation of ^{14}C from (U-^{14}C)-glucose into liver lipids.

Rates of hepatic lipid formation from a single intraperitoneal dose of (U-^{14}C)-glucose (1mmol; about 25 μCi) were investigated. To correct for the variations in the injected radioactivity between experiments, the results from these experiments have been adjusted as if the injected ^{14}C was 10^8 d.p.m. All the results in this section have been corrected in the same manner. The specific radioactivity of blood glucose declined gradually between 0-90 minutes after injection from about 6.0×10^4 d.p.m. (μmol glucose)$^{-1}$ to 4.5×10^4 d.p.m. (μmol glucose)$^{-1}$ in both ob/ob and non-obese mice (Fig.3.7). The time course of incorporation of ^{14}C into total hepatic lipids, LCFA, TG and PL are shown in Fig.3.8(a,b). Some 90% of the ^{14}C that was incorporated into liver lipids was accounted for as TG and PL. After an initial lag period (corresponding to the first 40 minutes during which the blood glucose concentration was rising) (Fig.3.7), the incorporation of ^{14}C into hepatic TG was more rapid and more extensive in ob/ob than in non-obese mice. There was no difference between the two types of mice in the incorporation of ^{14}C hepatic PL, and the content of ^{14}C in this lipid class slowly increased during the experimental period. At later times (120-180 minutes after injection) the radioactivity in hepatic TG showed a second phase of increasing incorporation of ^{14}C. This is probably attributable to the incorporation of ^{14}C through an indirect metabolic route, and not directly from blood (U-^{14}C)-glucose. For example, this may have been (^{14}C)-glucose from hepatic glycogen, or

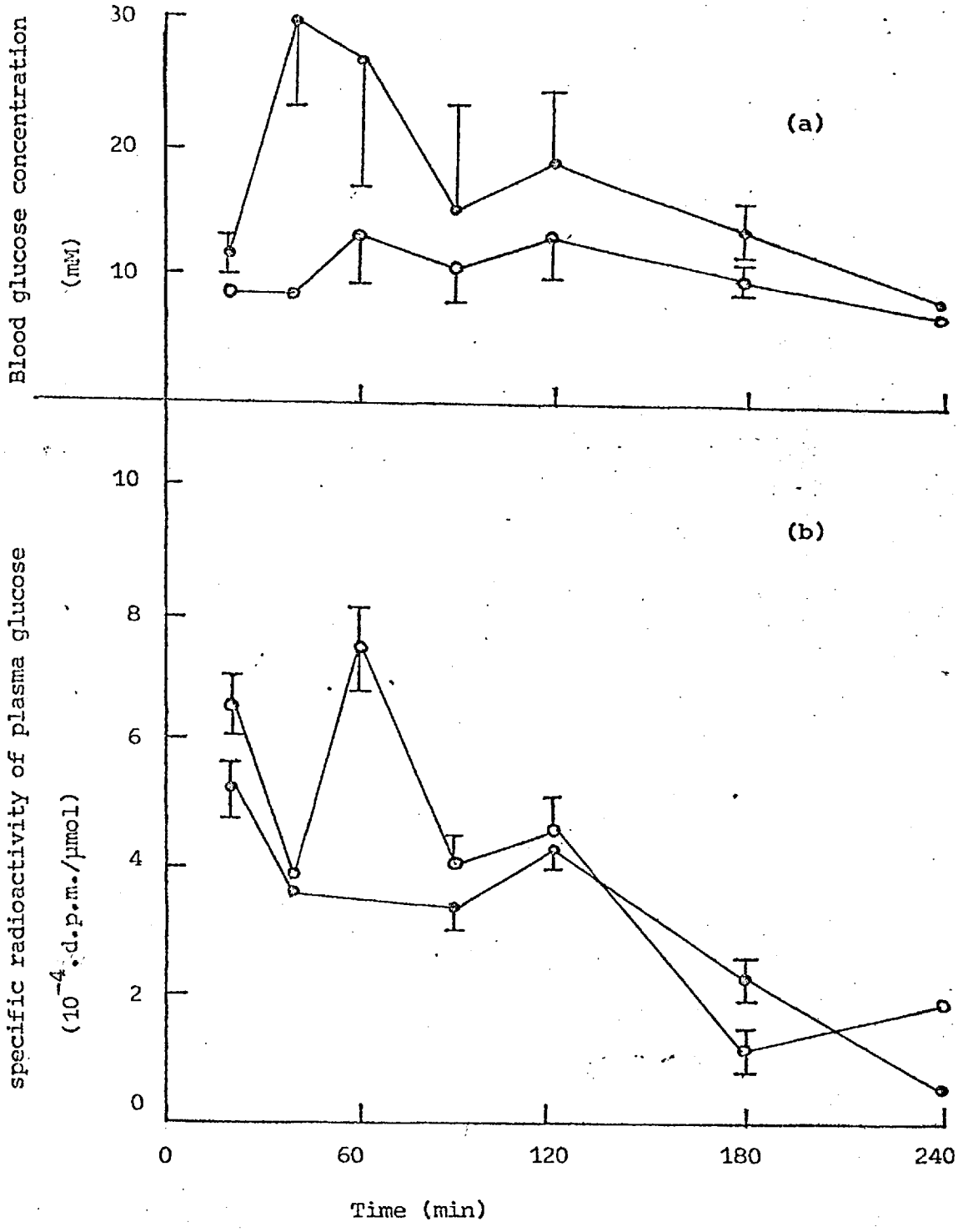


Fig. 3.7. Time-cousse of the concentration and specific radioactivity of blood glucose after injection of (U-^{14}C) glucose into mice.

Female mice, aged 12 weeks and fed ad lib. were injected intraperitoneally with 1 ml of 1 M (U-^{14}C) glucose (specific radioactivity 4.4-6.6 x 10^7 d.p.m./mmol) between 10.00-14.00 h. Other details are in the text. In (a) is shown the time-course of the concentration of glucose in blood and in (b) the time-course of the specific radioactivity of blood glucose. (●) denoted ob/ob mice and (○) non-obese mice. Each point represents the mean of 2-5 observations, and the bars are the S.E.M. when there were 3 or more observations.

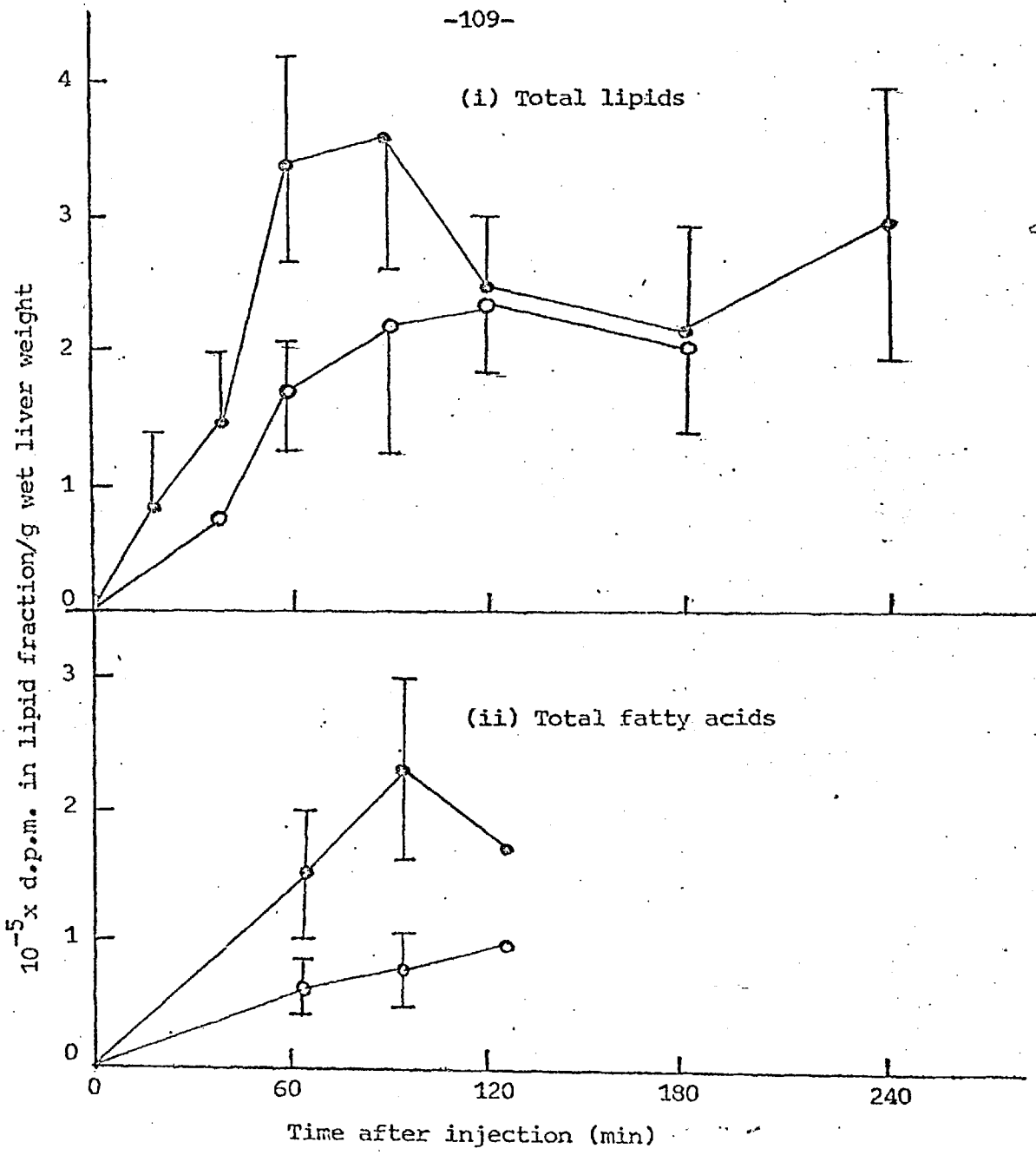


Fig.3.8(a). Time-course of the incorporation of ^{14}C from (U-^{14}C) glucose into total liver lipids and fatty acids.

1 ml of 1 M (U-^{14}C) glucose was injected intraperitoneally into conscious female mice, fed ad lib. and aged 12 weeks. After various times, ^{14}C was determin in total lipids (i) and total fatty acids (ii). The experiments are the same as those described in Fig. 3.7., and other details are in the text. The results are adjusted as if the injected dose of ^{14}C was 10^8 d.p.m., and are the means of three to five measurements, except in the case of the 120 min points for the total fatty acid analyses which are the mens of two measurements. The bars represent the S.E.M. Non-obese mice (o) and ob/ob mice (●).

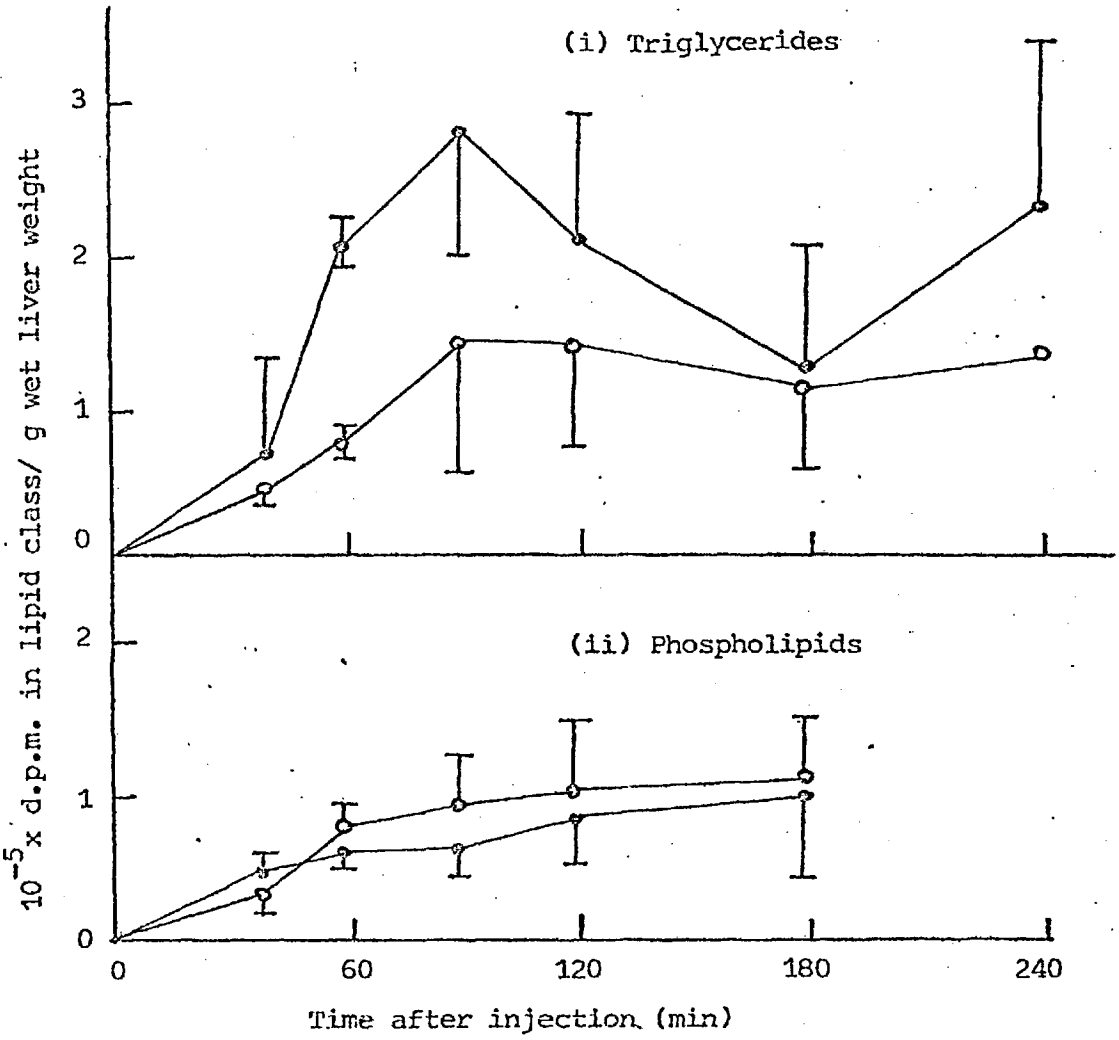


Fig. 3.8(b). Time-course of the incorporation of ^{14}C from (U-^{14}C)-glucose into liver triglycerides and phospholipids.

In the same experiments as those described in Figs.3.7 and 3.8(a), the ^{14}C from (U-^{14}C) glucose incorporated ito TG and PL was measured, after separation of the total lipid fraction into major lipid classes by t.l.c. Results have been adjusted as if the injected dose was 10^8 d.p.m., and are the means of three to five measurements; the bars represent the S.E.M. Non-obese mice (o) and ob/ob mice (•).

(^{14}C)-lactate formed from glucose in extrahepatic tissues (via the Cori cycle), or (^{14}C)-FFA recycling as previously described (3.3.2).

3.4.2. Calculation of rates of hepatic fatty acid synthesis from blood (U-^{14}C)-glucose.

Since the mice in the present study were ingesting a diet which was about 50% (w/w) digestible carbohydrate, it might be thought that a major proportion of FA were synthesized directly from blood glucose. In view of the evidence suggesting a marked increase in hepatic FA synthesis in ob/ob mice (1.3.3), it is pertinent to calculate the rate of FA synthesis from blood glucose, and the proportion of the total FA synthesis (per liver and per mouse) for which this might account. This is possible from the present data, partly because the administered glucose load (1 mmol) approximately corresponds to the quantity of dietary glucose which would have been ingested, on average, during the experimental period. Since the decline in the specific radioactivity of blood (U-^{14}C)-glucose was slow (Fig.3.7) due to the intraperitoneally-injected glucose passing slowly into the blood, and since the rising phases of the time courses were established (Fig.3.8), the rates of FA synthesis in the liver may reasonably be calculated from the average specific radioactivity of circulating (U-^{14}C)-glucose. This was taken to be 8×10^3 d.p.m. (μg atom glucose-C)$^{-1}$ (Fig.3.7). The results of such calculations are shown in Table 3.12. The rates of hepatic FA synthesis from (^{14}C)-glucose are similar to those obtained in normal mice in vivo, after feeding a glucose meal to mice starved overnight, calculated by a different method by Baker & Huebotter (1973). In Table 3.12, the rates of FA synthesis from blood glucose have been compared

Table 3.12. Rates of in vivo hepatic lipid synthesis in mice.

Rates of hepatic lipid synthesis from glucose in mice (12 weeks old, female and fed ad lib.) were calculated from the fastest rising phases of the time course of incorporation of ^{14}C into lipids after intraperitoneal injection of 1 ml of 1 M (U-^{14}C)-glucose (specific radioactivity 10^7 x 4.4-6.6 d.p.m./mmol), as described in Section 3.4.2. The total rate of FA synthesis was taken from unpublished data of T.Verrinder, E.A.Rath & D.A.Hems, which were obtained from experiments in which ^3H_2O was injected intraperitoneally into similar mice as described above, and the rate of FA synthesis calculated from the ^3H incorporation into liver FA. The results of these experiments and the fractional contribution of blood glucose to total hepatic FA synthesis were calculated as described in Section 4.2.1.

Process	Type of mouse		Ratio
	ob/ob(A)	non-obese(B)	(A/B)
Synthesis from glucose (μg-at C/min/liver) of:			
TG	2.10	0.29	7.2
PL	0.44	0.42	1.1
Total FA	0.76	0.18	4.2
Total lipid	2.89	0.79	3.7
Total rate of synthesis (RTA* ^3H/min/liver) of:			
TGFA	1.4	0.3	4.7
PLFA	1.1	0.6	1.8
Total FA	2.9	1.1	2.6
Calculated fractional contribution of injected glucose-C to the total utilized in FA synthesis:	0.22	0.14	

*RTA relative total activity (see Section 4.2.1)

with data obtained in experiments in which $^{3}H_2O$ was injected into similar mice, from which a measure of the total rate of FA synthesis may be obtained (4.2.1) (T.Verrinder & D.A.Hems, personal communication). It is clear from this table that even in a hyperglycaemic situation, less than half the hepatic FA synthesis in conscious mice may be accounted for by conversion of blood glucose to FA during the first 90 minutes following glucose administration.

3.4.3. Formation of plasma triglycerides from blood (U-^{14}C)-glucose.

In an attempt to measure the rate of formation of plasma TG from blood glucose, the incorporation of ^{14}C from (U-^{14}C)-glucose was followed. There was a delay in the appearance of ^{14}C in plasma TG (Fig. 3.9), especially in ob/ob mice. In common with other data obtained from these experiments, the data in Fig.3.9 have been adjusted to an injected dose of 10^8 d.p.m./mouse. The relatively high level of injected radioactivity was necessary in order to detect sufficient ^{14}C in plasma TG. The fastest rate of appearance of ^{14}C in plasma TG was observed during the period 40-60 minutes after injection, and was about 10^3 d.p.m. min^{-1} or 8×10^{-3} umol C_{16} FA min^{-1} (calculated as in 3.4.2). This value underestimates the rate of this process, since the observed rate of change of ^{14}C in plasma TG (Fig.3.9) represents the difference between the actual rate of secretion of (^{14}C)-TG and its disappearance from plasma which were probably both of the same order of magnitude. An increase in the rate of secretion probably resulted in a rapid increase in the rate of uptake, especially considering the high values for the rate constants (fractional turnover rates) with respect to the total plasma TG pool determined previously (Table 3.11).

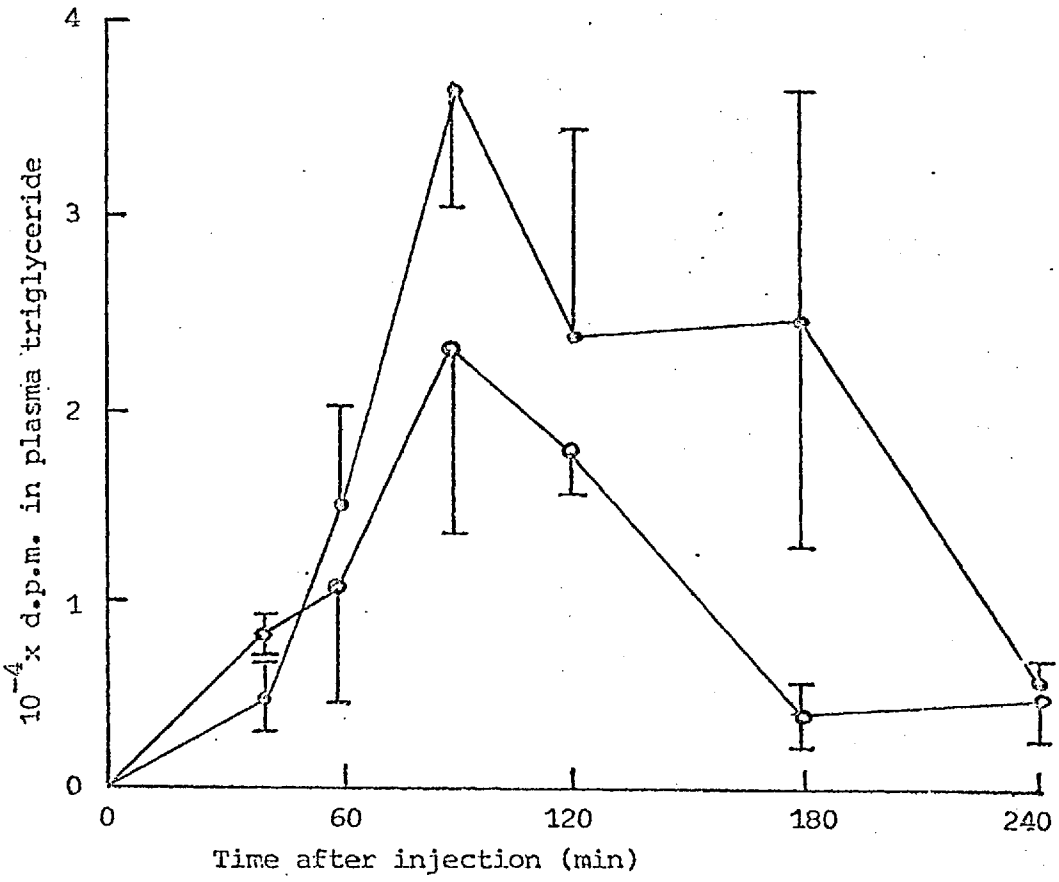


Fig. 3.9. Time-course of incorporation of ^{14}C from (U-^{14}C) glucose into plasma triglycerides.

In the experiments described in Fig.3.7., ^{14}C from (U-^{14}C) glucose incorporated into plasma TG was also measured. The results are the means of three measurements (bars indicate S.E.M.), except for the 120 min point in non-obese mice and the 40 and 90 min points in ob/ob mice, which are the average of two measurements (bars indicate half the range).
Non-obese mice (○) and ob/ob mice (●).

It is possible from the present data to attempt to estimate the fraction of plasma TG carbon derived from blood glucose (f) by application of the expression:

$$f = \frac{\text{specific radioactivity of plasma TG (d.p.m./}\mu\text{mol TG)}}{\text{specific radioactivity of plasma glucose (d.p.m./ug-at glucose-C)x51}}$$

The specific radioactivity of plasma TG was estimated by considering that the plasma TG concentration was the same as that shown in Table 3.1; however, the administration of a glucose load to rats at least, may lower plasma TG concentrations (Baker et al, 1968). The divisor of 51 is the number of carbon atoms in a molecule of tripalmitin. The fraction of plasma TG carbon derived from plasma glucose at 90 min. after injection is 2% in both ob/ob and non-obese mice. This low value is similar to that calculated by Haft (1973) from experiments in which $^{3}H_2O$ and (^{14}C)-palmitate were injected into rats.

3.5. Activity of ATP-citrate lyase (EC 4.1.3.8) in liver and adipose tissue.

Table 3.13 shows the activities of ATP-citrate lyase that were obtained in liver, epididymal and interscapular (brown) adipose tissue, expressed per mg high-speed supernatant protein. Although hepatic activities of the enzyme from ob/ob mice were four times those from non-obese mice, both were lower than those reported by Spencer & Lowenstein (1966). This may be accounted for by the factor of 1.59 which was applied by these authors to calculate activities at 37°C, whilst the present data were obtained at 31°C. Activities in epididymal adipose tissue from non-obese mice were similar to those found in rats fed ad lib. on a laboratory chow diet by Kornacker & Ball (1965), but those in adipose tissue from ob/ob mice were slightly decreased. No difference was found between the two types of adipose tissue assayed, a similar situation to that in normal rats (Kornacker & Ball, 1965).

Table 3.13. Activity of ATP-citrate lyase (EC 4.1.3.8) in adipose tissue and liver from ob/ob and non-obese mice.

Female mice, aged 12 weeks and fed ad lib., were killed by cervical dislocation, and high-speed supernatants of liver, epididymal and interscapular adipose tissue prepared essentially as described by Shrago et al (1969). The assay of ATP-citrate lyase activity was carried out by the malate dehydrogenase method of Srere (1959), and is described in detail in 2.10. The results are expressed as μmol citrate cleaved/h/mg high-speed supernatant protein, ±S.E.M. and the number of observations is in parenthesis.

Tissue	Activity of ATP-citrate lyase		P
	non-obese	ob/ob	
liver	0.29±0.10(3)	1.27±0.40(3)	< 0.05
epididymal adipose	4.54±1.03(5)	1.96±0.57(5)	< 0.05
inerscapular adipose	-	2.90±0.65(4)	-

CHAPTER 4

RESULTS OF EXPERIMENTS CONCERNING FATTY ACID SYNTHESIS AND ITS RELATIONSHIP TO CARBOHYDRATE METABOLISM IN THE PERFUSED MOUSE LIVER.

4.1 Glucose, glycogen and lactate metabolism in the perfused liver.

4.1.1. The maintenance of constant glucose concentrations in the perfusion medium.

4.1.2. Glycogen content of perfused mouse livers.

4.1.3. Relationships of glucose and lactate concentrations in the perfusion medium.

4.2. Relationships of carbohydrate metabolism to hepatic fatty acid synthesis in the perfused mouse liver.

4.2.1. Calculation of total rates of *de novo* synthesis of fatty acids and cholesterol from the incorporation of isotopes

4.2.2. Dependence on medium glucose and lactate concentrations of the total rate of fatty acid synthesis.

4.2.3. The dependence on medium glucose concentrations of the contribution of glucose-C to total long-chain fatty acid synthesis.

4.2.4. The dependence on medium glucose concentrations of the contribution of lactate carbon to total long-chain fatty acid synthesis.

4.2.5. The relationship of the initial hepatic glycogen content to the total rate of long-chain fatty acid synthesis, and the contribution of glucose carbon to this process.

4.2.6. Incorporation of radioactivity from isotopically-labelled precursors into 'lipid glycerol'.

4.2.7. The effect of oleate on the rate of fatty acid synthesis and the contribution of glucose carbon to this process.

4.3. Synthesis of cholesterol in the perfused mouse liver.

4.4. The secretion of lipids by the perfused mouse liver.

CHAPTER 4

EXPERIMENTS CONCERNING FATTY ACID SYNTHESIS AND ITS RELATIONSHIP TO CARBOHYDRATE METABOLISM IN THE PERFUSED MOUSE LIVER.

Livers of female mice, aged 12 weeks and fed *ad lib* as described in Section 2.12., were perfused according to the methods described in Section 2.11. All experiments in this section were carried out with the standard perfusion medium described in Section 2.11.2., except where stated.

4.1. Glucose, glycogen and lactate metabolism in the perfused liver.

4.1.1. The maintenance of constant glucose concentrations in the perfusion medium.

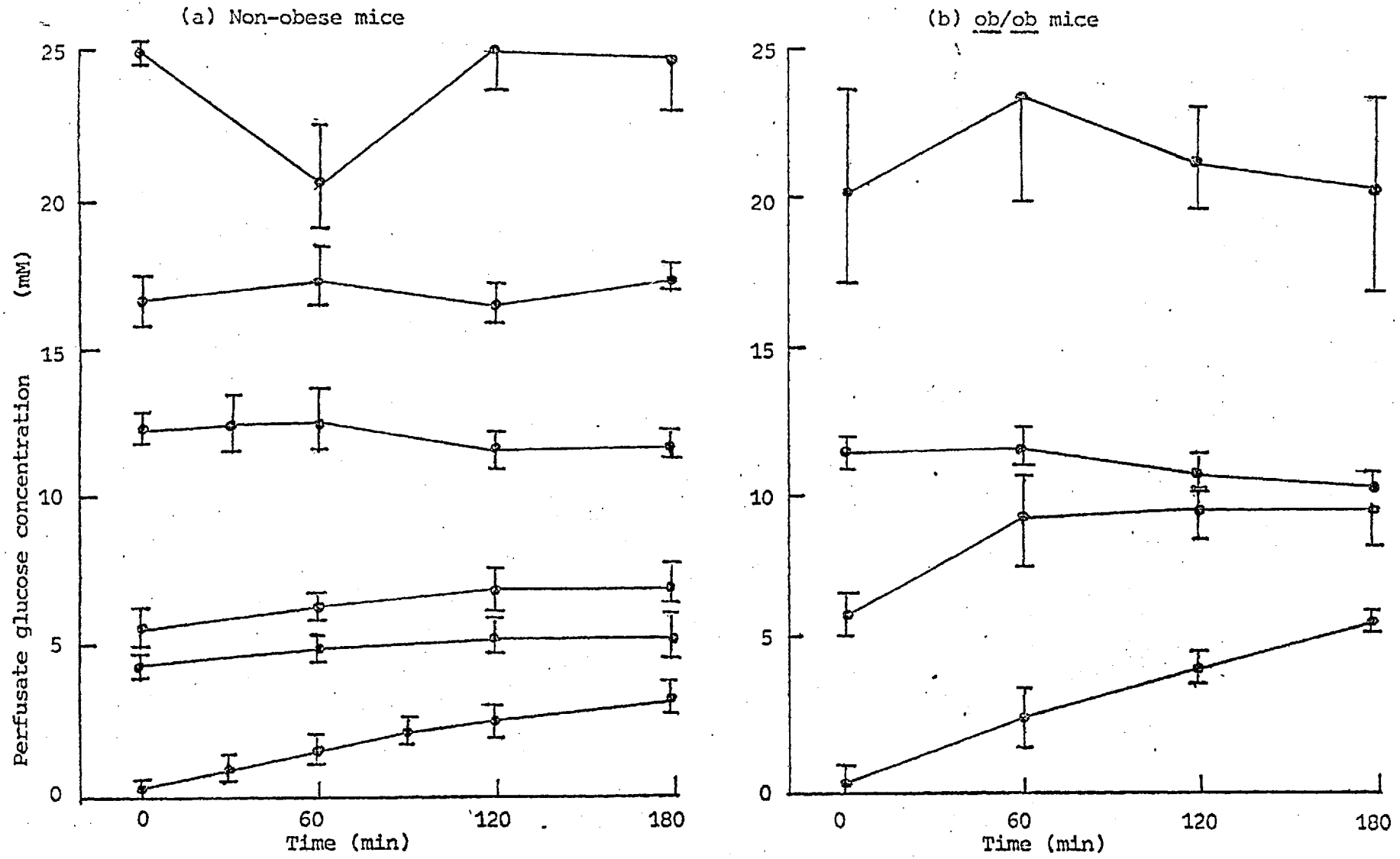
During perfusions of livers of fed rats, the perfusate glucose concentration tends towards a constant value, which is the same irrespective of whether the initial concentration is high or low ("autoregulation": Glinsmann *et al*, 1969).This characteristic was considered when the mouse liver was perfused under the present conditions.

Fig.4.1. shows the changes in glucose concentrations with time during perfusion of livers from non-obese mice, for six initial concentrations from 0-25mM.It can be seen that the use of a large medium/liver ratio (40 ml/g wet liver) has almost completely eliminated the autoregulatory maintenance of perfusate glucose concentrations.Between 5-15 mM the initial glucose concentration was maintained within ±1 mM for the three hour experimental period. Fig.4.1. also shows experiments in which livers from *ob/ob* mice

Fig. 4.1. next page.

Fig. 4.1. Time-course of changes in the medium glucose concentration during perfusion of the mouse liver.

Livers of mice (female,fed ad lib.) were perfused with 60 ml medium consisting of 45 ml bicarbonate-saline (Krebs & Henseleit, 1932),washed,aged human erythrocytes (25-30% haematocrit) and bovine serum albumin (3.3%),as described in Section 2.11. Glucose was added at the start of perfusion to produce a range of initial perfusate glucose concentrations.Average liver weights were 1.5g for non-obese mice and 2.3g for ob/ob mice.In most perfusions,sequential liver biopsies (50-200mg each) were taken after 60 and 120 minutes of perfusion.Each point is the mean of 3-7 perfusions,and the bars represent the S.E.M.


(a) Non-obese mice
(b) ob/ob mice
Perfusate glucose concentration (mM)
25
20
15
10
5
0
0
60
120
180
Time (min)

were perfused in an identical manner.The medium/liver ratio in these experiments was smaller (26 ml/g wet liver) than that for non-obese mouse livers, and the perfusate glucose concentration showed a slight tendency towards autoregulation.

In order to calculate absolute rates of LCFA synthesis from medium (U-^{14}C)-glucose, a relatively constant specific radioactivity of (^{14}C)-glucose was required.Using conventional techniques of rat liver perfusion, with a medium/liver ratio of about 7 ml/g wet liver, medium glucose concentrations can only be maintained above the autoregulatory point by glucose infusion, and below this point by the use of non-recycling media or large perfusate volumes.The constant perfusate glucose concentrations found using the mouse liver, as described above, resulted in relatively constant specific radioactivities of perfusate (U-^{14}C)-glucose, and thus allowed the calculation of quantitative rates of incorporation of precursor into product as described in Section 4.1.2.

4.1.2. Glycogen content of perfused mouse livers.

When livers were perfused with a standard medium containing less than 15 mM glucose, net glycogenolysis ocurred during the first hour, whereas perfusion with a medium containing glucose at concentrations in excess of 20 mM resulted in net glycogen accumulation (Fig.4.2.).The hepatic glycogen content was similar at 60 minutes to that at 180 minutes, indicating that a new steady state had been attained.The hepatic glycogen content after 180 minutes of perfusion varied according to the perfusate glucose concentration, such that a sigmoidal relationship existed between these two parameters (Fig. 4.2.).This phenomenon has been previously described by Glinsmann *et al* (1969) in rat livers perfused under slightly different conditions.

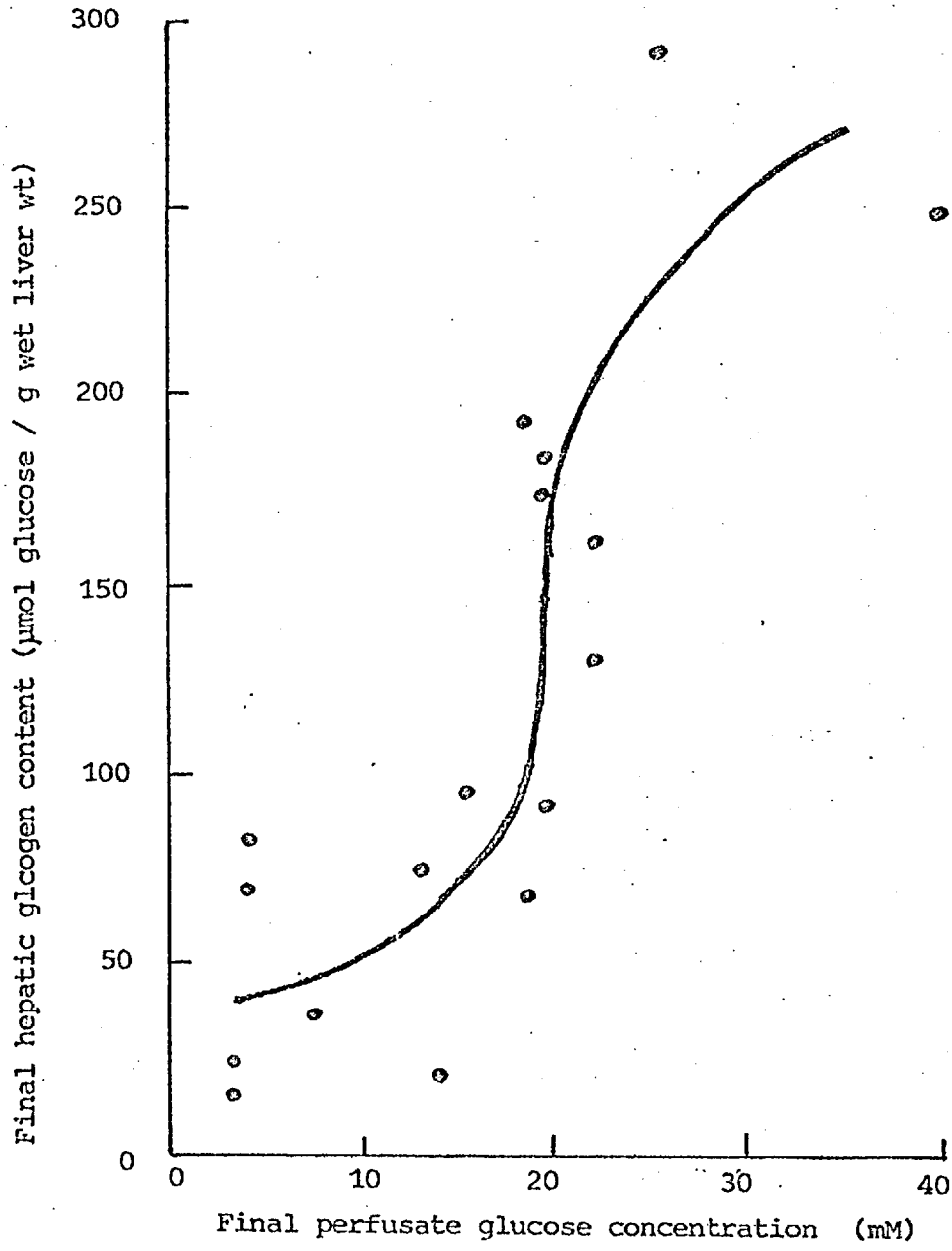


Fig. 4.2. Influence of the medium glucose concentration on the glycogen content of the perfused mouse liver.

Livers of non-obese mice, fed ad lib., were perfused as described in Fig. 4.1. and Section 2.11. After 3 h of perfusion, the glycogen content of the left lateral lobe was measured. For comparison, the hepatic glycogen content (µmol glucose/g wet liver) in untreated but anaesthetized animals was 255 ±12 and at the start of perfusion was 157 ±14 (means ± S.E.M. of 3 measurements). Other details are given in the text.

In one series of experiments it was desirable to produce a range of initial hepatic glycogen contents.High initial glycogen contents were achieved by cannulation of the hepatic portal vein with gassed bicarbonate saline containing 20 mM glucose, in an effort to minimize glycogen breakdown during operative procedure. Low initial glycogen contents were produced by a period of pre-perfusion with an erythrocyte-free medium containing no glucose, conditions which are known to be conducive to rapid hepatic glycogen depletion (Glinsmann et al, 1969).

For comparison, glycogen levels were measured in vivo.The content of glycogen in the left lateral lobe of livers removed from mice under pentobarbital anaesthesia at the same time of day as preperfusion operations were performed (11.30-12.30 h) was 255±12(3) µmol glycogen-glucose/g wet liver.During the cannulation of the hepatic portal and inferior vena cava veins,(using a cannula for the former through which bicarbonate-saline, gassed with O_2/CO_2 (95:5 v/v) but containing no glucose, was flowing), a loss of 100 µmol glycogen-glucose/g wet liver occurred.Thus the mean post-operative glycogen content of the left lateral lobe was 157±14 µmol(3) glycogen-glucose/g wet liver.The comparable value for livers from ob/ob mice was 291±41 µmol/g wet liver.(mean of three observations).

4.1.3. Relationships of glucose and lactate concentrations in the perfusion medium.

In perfusions in which no exogenous lactate was added to the medium, lactate concentrations in the whole perfusate were initially 1.5-2.0 mM (Fig.4.3.) and rose to 2.0-3.0 mM after 60-180 minutes of perfusion, except when perfusate glucose concentrations were greater than 20 mM, when perfusate lactate concentrations were 4.5-5.5 mM after this time (Fig.4.3.).Little of the lactate initially

Fig. 4.3. over page.

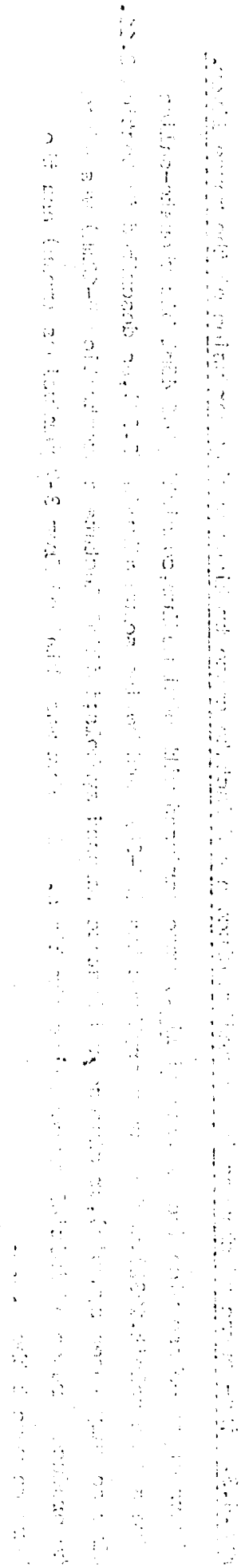
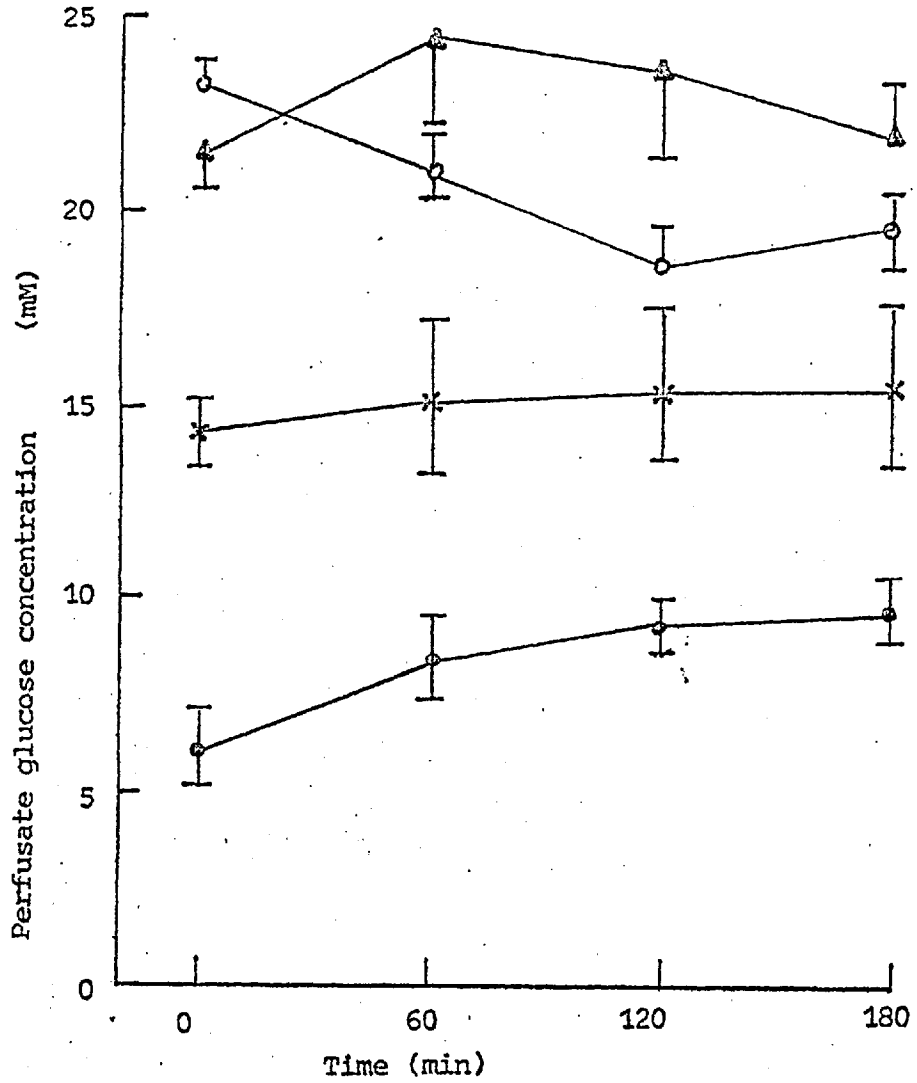
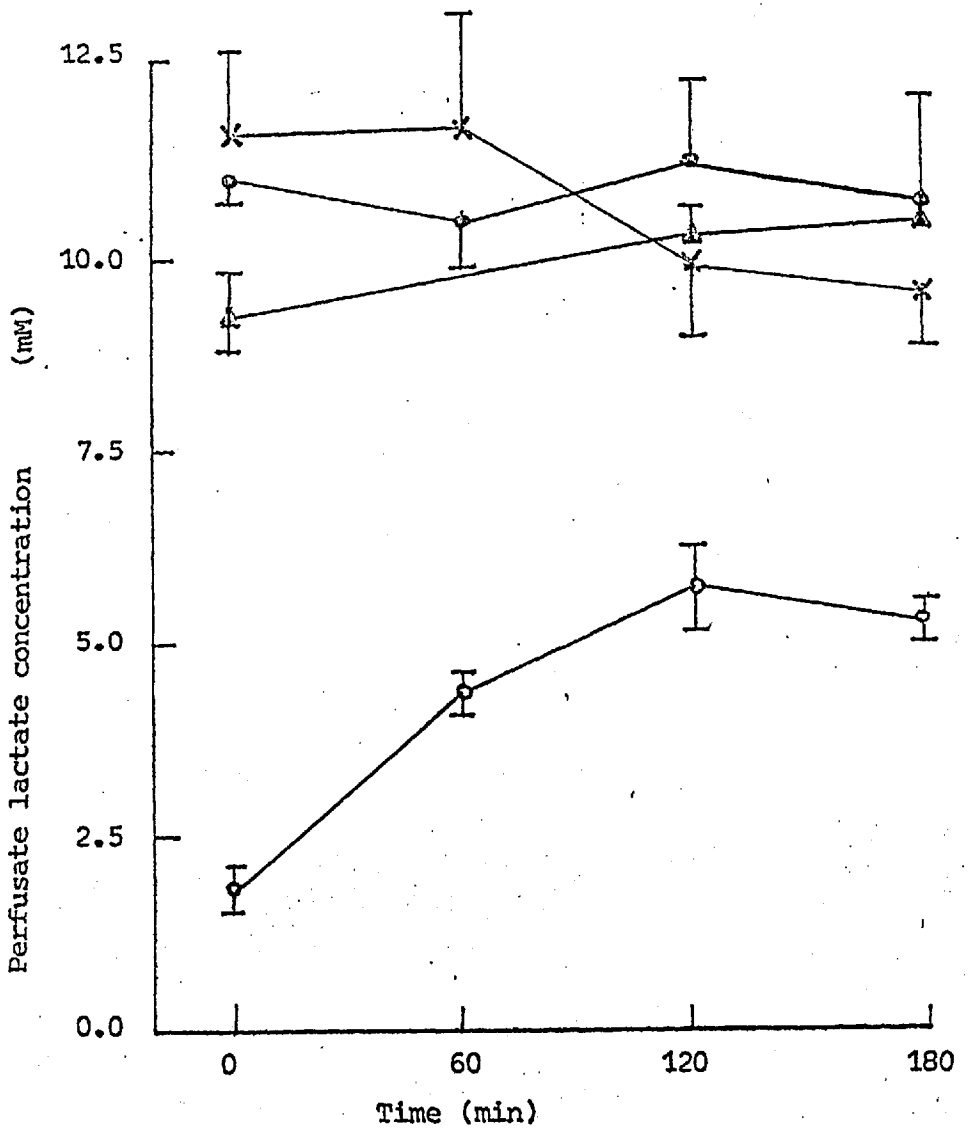


Fig.4.3. Time-course of changes in the medium glucose and lactate concentrations during perfusion of the mouse liver.

Livers of non-obese mice (female,fed ad lib.) were perfused with 60ml medium,consisting of 45ml bicarbonate-saline (Krebs & Henseleit,1932),washed human erythrocytes (25-30%) and bovine serum albumin (3.3%),as described in Section 2.11. Glucose only (open symbols),or glucose and lactate (closed symbols;initial lactate concentration 9-12mM) was added to produce a range of initial glucose concentrations.Each point was obtained from 3-7 perfusions (mean) and the bars represent the S.E.M.


Perfusate glucose concentration (mM)
25
20
15
10
5
0
0
60
120
180
Time (min)


Perfusate lactate concentration (mM)
12.5
10.0
7.5
5.0
2.5
0.0
0
60
120
180
Time (min)

present in the medium was likely to heve been derived through erythrocyte glycolysis since stored, human erythrocytes have a low capacity for this process (Rapoport, 1968).Rather, the initial medium contained lactate which had remained in erythrocytes during the preparative washing procedure, and slowly passed into the cell-free component of the medium during the course of the perfusions. This has previously been noted by Elliott et al (1972) and Woods & Krebs (1970), who also prepared erythrocytes from whole human blood stored in 30 mM glucose, in which lactate concentrations are high.

There were low rates of lactate release by livers perfused in the present experiments (Fig.4.3.).This is in contrast to the high initial rates of lactate release (in excess of 3 μmol $min^{-1}g^{-1}$, resulting in perfusate lactate concentrations of 8 mM after 120 min of perfusion) observed by investigators who perfused rat livers with erythrocyte-free perfusates (Glinsmann et al, 1969; Brunengraber et al, 1973).

When lactate (10 mM) was added initially to the perfusion medium, the perfusate glucose concentration rose by 2-3 mM.Net lactate uptake was observed only at initial perfusate glucose concentrations of 14 mM, conditions in which hepatic lactate output is minimal in the absence of added lactate (Woods & Krebs, 1970) (Fig.4.3.).

4.2. Relationships of carbohydrate metabolism to hepatic fatty acid synthesis in the the perfused mouse liver.

4.2.1. Calculation of total rates of de novo synthesis of fatty acids and cholesterol from the incorporation of isotopes.

The use of oxides of hydrogen isotopes to measure total FA synthesis was introduced by Schoenheimer & Rittenberg (1937) (1.1.1.). The basis of the technique resides in the stability of C-H bonds of FA to proton exchange (Van Heynigen et al, 1938) such that incorporation of hydrogen only occurs during synthesis. In experiments where the extent of incorporation of ^{3}H from $^{3}H_2O$ into FA was used to measure total rates of synthesis of LCFA, the incorporation has been expressed as μmol LCFA synthesized/g wet liver. This parameter has been calculated by correcting the "relative total activity" in liver FA for isotope discrimination against ^{3}H compared to ^{1}H during the hepatic synthesis of LCFA. The "relative total activity" (RTA) is defined as follows:

$$RTA = \frac{\text{d.p.m. } ^{3}H \text{ in liver LCFA/g wet liver}}{\text{d.p.m. } ^{3}H/\mu\text{g atom H in perfusate } H_2O}$$

Discrimination against ^{3}H relative to ^{1}H was corrected for in a similar manner to that used by Windmueller & Spaeth (1966). This yielded values within 95% of those calculated by the alternative method of Jungas (1968), which was based on data obtained from experiments with incubated adipose tissue. The discrimination factor used in the present work was derived from experiments in vivo (Bernhardt & Schoenheimer, 1940). The body water of mice on a fat-free diet was maintained at a steady deuterium content of 1.5% for 100 days by allowing them to drink $^{2}H_2O$. During this period, ^{2}H in

body LCFA had equilibrated at 0.43 of that in body water.A figure of 0.5 has been used in the present instance, since Jungas (1968) suggested that isotopic equilibrium might not quite have been obtained in the above experiments.A factor to correct for discrimination against ^{3}H relative to ^{2}H was obtained from experiments in which ^{3}H from $^{3}H_2O$, in the presence of excess $^{2}H_2O$, was found to be incorporated into LCFA with a preferential incorporation of deuterium over tritium of 1.19 (Eidinoff et al, 1953; Jungas, 1968).Thus it was assumed that ^{1}H from $^{1}H_2O$ was preferentially incorporated into LCFA relative to ^{3}H from $^{3}H_2O$ by a factor of (1.19/0.5)=2.38. Windmueller & Spaeth (1966) assumed that there was an average of 31.7 C-H bonds/μmol LCFA synthesized synthesized.This was validated by Foster & Bloom (1963) who showed that 16:0 was the major ^{3}H-FA synthesized from $^{3}H_2O$ in liver slices, and this was also supported by the present work using the perfused liver (Table 4.1.).Thus division of RTA in LCFA by 13.3 yields μmol LCFA synthesized.

Cholesterol synthesis may also be calculated in a similar manner. From similar considerations as those applied to determining a factor for converting RTA to μmol LCFA, Windmueller & Spaeth (1966) divided RTA in cholesterol by 18.9 to calculate μmol cholesterol synthesized. Brunengraber et al (1972) derived a comparable factor of 20.5 by showing a common precursor pool of acetyl CoA for both LCFA and cholesterol owing to the constancy of the ($^{14}C/^{3}H$) sterol ratio/ ($^{14}C/^{3}H$) LCFA ratio when ^{14}C-glucose and $^{3}H_2O$ were precursors.In the present work, a factor of 19 has been taken.

The rate of LCFA synthesis from glucose-C was calculated according to the following procedure.The initial specific radioactivity (d.p.m./μg-at glucose carbon) of perfusate glucose was determined by division of the total perfusate ^{14}C (d.p.m./ml) determined within 5 min of the start of the perfusion, by the

concentration of perfusate glucose (mg at/ml). It was shown that all perfusate ^{14}C in the initial medium sample was in glucose, and that the specific radioactivity of perfusate (^{14}C)-glucose was relatively constant for up to three hours of perfusion at glucose concentrations between 5 and 25 mM, by determination of the ^{14}C in glucose after t.l.c. (Sections 2.4.1. and 2.7.). The number of µg-at glucose carbon incorporated into each lipid fraction was obtained as the quotient of the expression (d.p.m. ^{14}C incorporated into lipid fraction/ initial specific radioactivity perfusate (^{14}C)-glucose). As shown by Foster & Bloom (1963) and suggested by the present experiments (Table 4.1.), the most highly labelled (^{14}C)-LCFA was 16:0. Thus the µg-atoms glucose-C incorporated into LCFA/g wet liver divided by 16 yielded umol LCFA synthesized from medium glucose.

LCFA synthesis from (U-^{14}C)-lactate was calculated in a similar manner as was that from (U-^{14}C)-glucose. The initial specific radioactivity of perfusate lactate, obtained by dividing the initial perfusate lactate concentration (µg at lactate carbon/ml) by initial perfusate ^{14}C (d.p.m./ml), has been used in these calculations.

The proportional contribution of (^{14}C)-labelled precursors to the total de novo synthesis of LCFA was calculated by division of total µmol LCFA synthesized (obtained from the incorporation of ^{3}H from $^{3}H_2O$ into LCFA) by the µmol LCFA synthesized from the (^{14}C)-labelled precursor.

Table 4.1. Degree of unsaturation of fatty acids synthesized from (U-^{14}C) glucose and $^{3}H_2O$ in the perfused mouse liver.

Livers were perfused with the standard medium (as described in 2.11) containing 3 mCi $^{3}H_2O$ and 5 uCi (U-^{14}C) glucose at the concentration indicated.After 3h of perfusion,total liver fatty acids were separated,as their methyl esters, according to their degree of unsaturation by argentation-t.l.c. (as described in 2.3.2).Each band was scraped ito vials and fatty acid methyl esters eluted from the silica into toluene-butyl P.B.D. scintillant before the simultaneous determination of ^{14}C and ^{3}H by liquid scintillation spectrometry (as described in 2.7.2.).Other details are in the text.Each set of results is from a single perfusion.All mice were non-obese.

Initial cooncentration of glucose in medium	% of radioactivity in total fatty acid methyl esters in:					
	saturated FAME		monoenoic FAME		polyenoic FAME	
mM	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
none	78.2	87.4	14.0	7.8	7.8	4.7
3	90.7	92.6	6.7	5.3	2.6	2.1
22	86.2	87.6	11.7	9.6	2.1	2.8
22	87.2	89.8	9.2	7.2	3.7	2.9

4.2.2. Dependence on medium glucose and lactate concentrations of the total rate of hepatic fatty acid synthesis.

The total rate of LCFA synthesis was measured in the perfused liver with ^3H_2O as described in Section 4.2.1. Fig.4.4(a) shows the time courses of the accumulation of LCFA synthesized de novo by the liver at different perfusate glucose concentrations.The incorporation at each of the three times was obtained by the method of liver biopsy described in Section 2.4.5.The possibility of differential incorporation between liver lobes was checked by simultaneous assay of ^3H in two different lobes at the end of twenty perfusions; the umol LCFA synthesized/g wet liver was found to be within ±5% of each other.The time course of incorporation was nearly linear in livers from non-obese mice (Fig.4.4(a).), except when glucose was not added to the medium initially when an inflexion was found after 60 min of perfusion approximately corresponding to the time of cessation of rapid glucose output (Fig.4.1) and equilibration of perfusate lactate levels.Two perfusions performed with non-recycling media and a constant glucose concentration of 2 mM gave similar low rates of LCFA synthesis (1.3 μmol LCFA synthesized/g wet liver/h) to that in the initial phase of recycling perfusions in which no glucose was added.Perfusions with non-recycling media gave a linear incorporation of ^3H from ^3H_2O into hepatic LCFA.When livers from ob/ob mice were perfused with a medium initially containing glucose at 6 mM, an inflexion in the rate of LCFA synthesis was found after 120 min of perfusion (Fig.4.4(a).).

The dependence of hepatic LCFA synthesis on perfusate glucose concentrations between 5-30 mM was biphasic (Fig.4.5.).When rates of de novo synthesized LCFA accumulation in the liver were calculated between 120 and 180 min of perfusion, a rate of 1.5-2.0 μmol LCFA

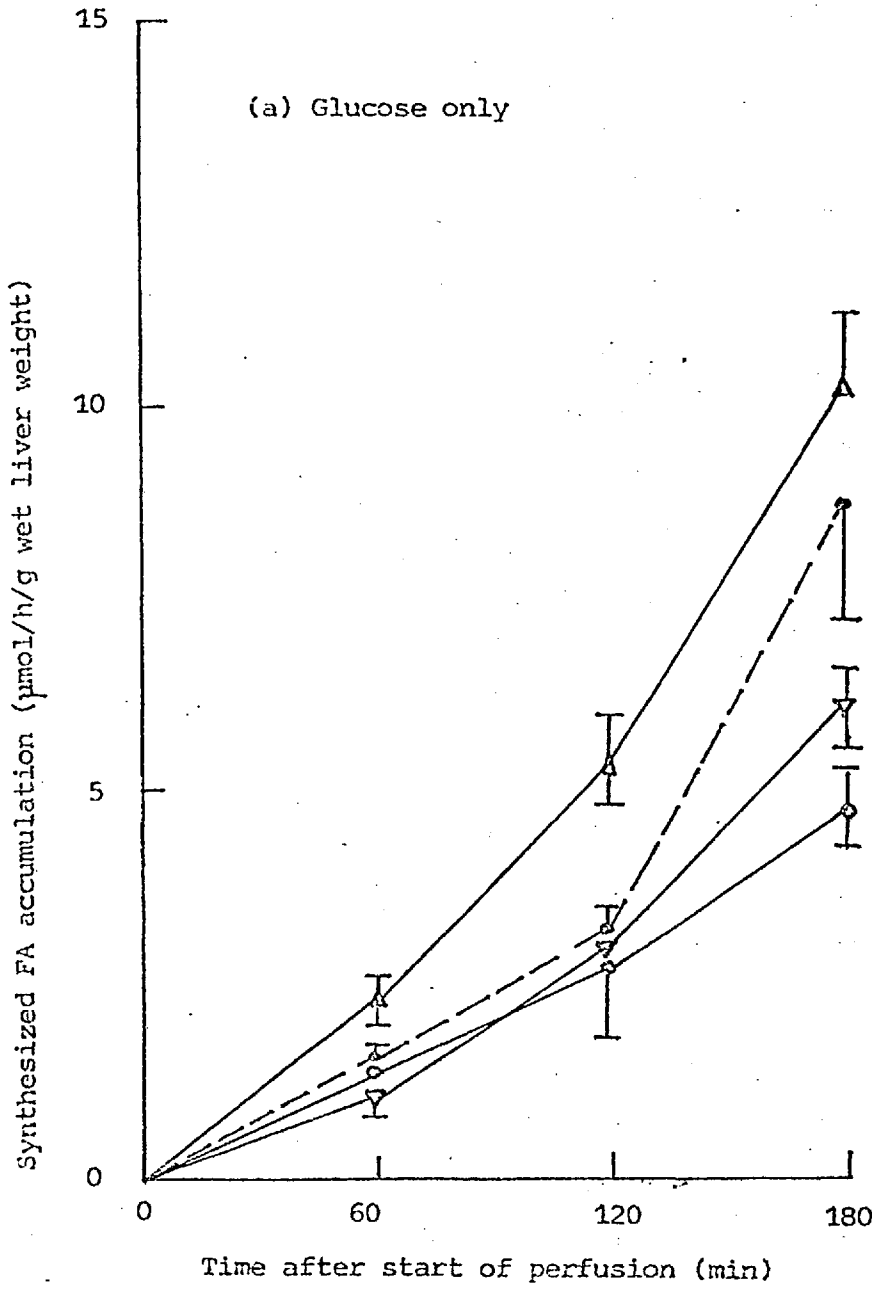


Fig. 4.4. Time-course of fatty acid synthesis in the perfused mouse liver.

Fatty acid synthesis was measured using $^{3}H_2O$ in the perfused mouse liver by methods described in Section 2.11. $^{3}H_2O$(3mCi) and various concentrations of glucose and lactate were present in the medium at the statr of perfusion. Successive halves of the median, bifurcated lobe were sampled after 60 and 120 min, and the left lateral lobe after 180 min of perfusion. The incorporation of ^{3}H into total liver FA was measured in each sample, and the umol FA synthesized calculated as described in the text. Results are expressed as 'synthesized FA accumulation' since the fraction of synthesized FA released into the perfusate (see Section 4.4) are not included in this measurement.
(a) Only glucose was added initially to the perfusion medium (see Fig. 4.1). Open and closed symbols represent perfusions of livers

(contd)

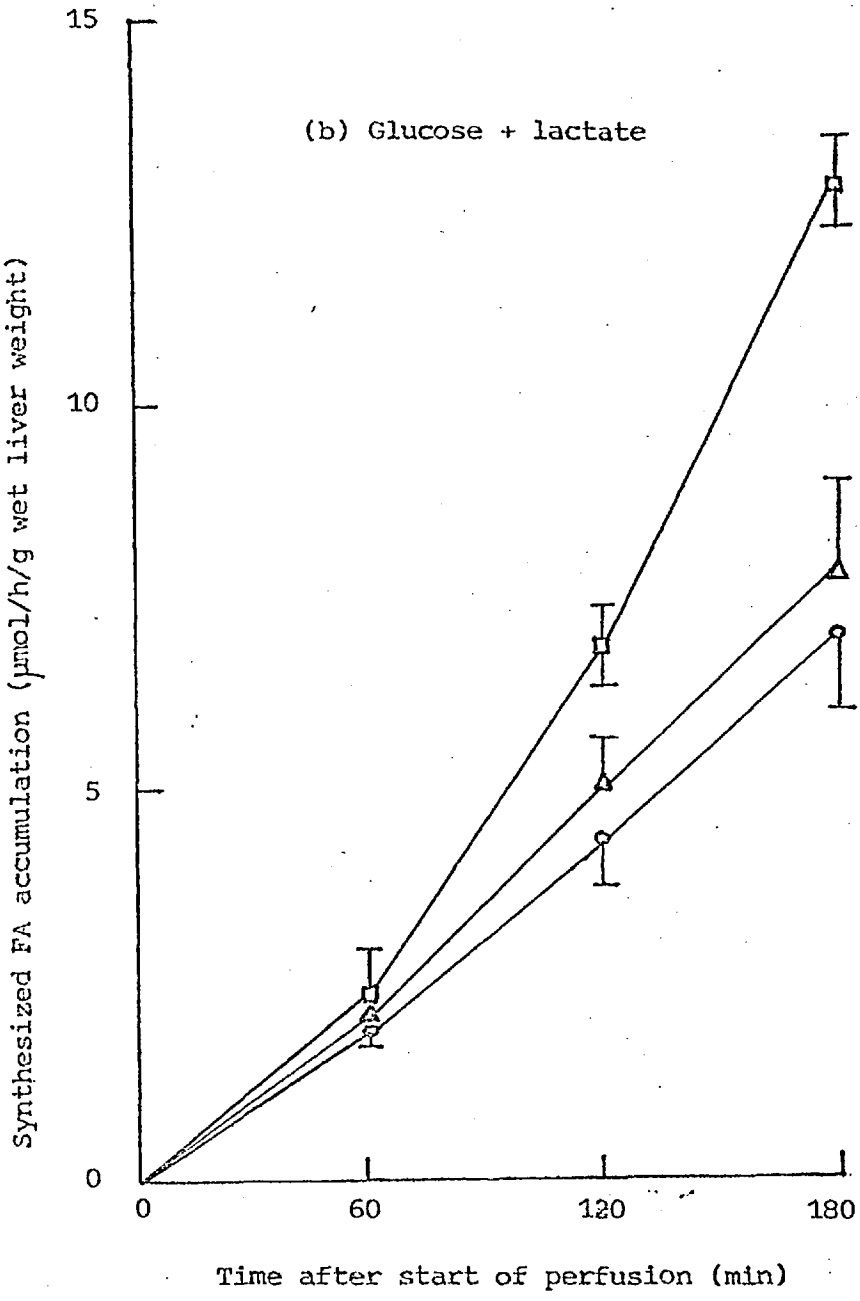


Fig.4.4. (contd. /caption)

of non-obese and ob/ob mice respectively.▽:no glucose added initially(4 perfusions);o,●:glucose 4-6 mM(3 perfusions); △:glucose 23-32 mM(7 perfusions).The bars represent the S.E.M.

(b)Glucose (at various concentrations) and lactate (10 mM) were added initially to the perfusion medium (see Fig. 4.3). o :glucose 4-6 mM(7 perfusions);□:glucose 12-17 mM(4 perfusions); △:glucose 20-25 mM(5 perfusions).The bars represent the S.E.M.

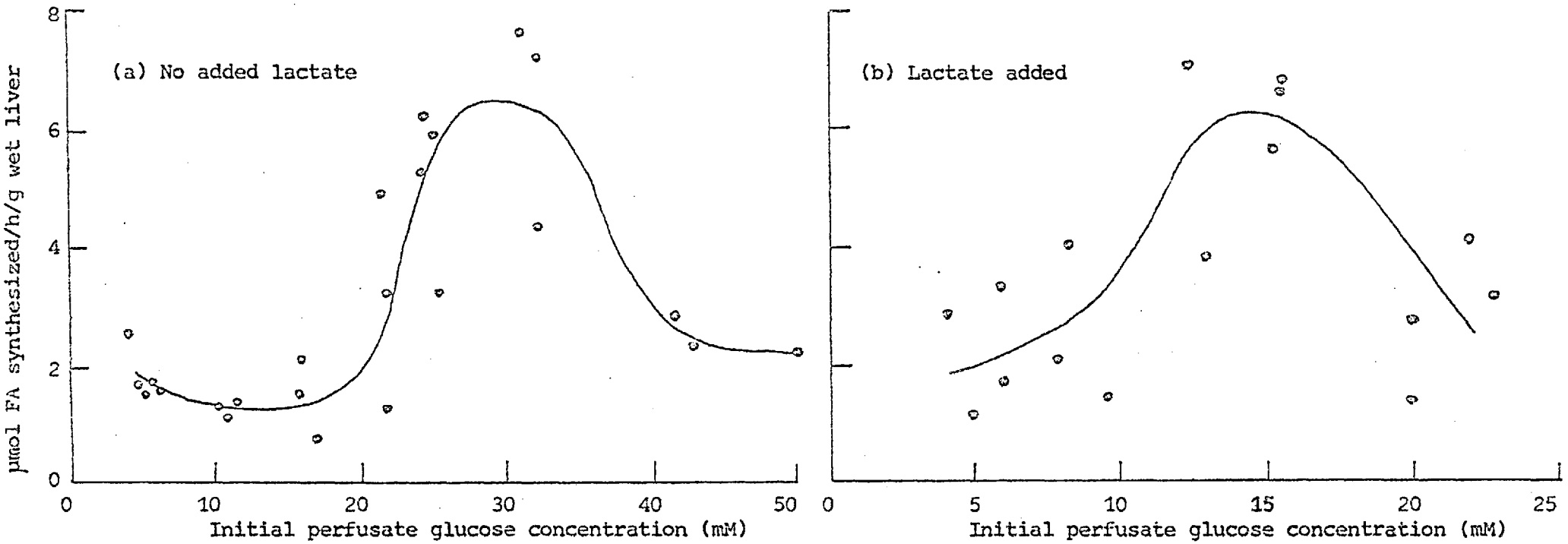


Table 4.5. The dependence on the medium glucose concentration of the total rate of fatty acid synthesis in the perfused mouse liver.

Livers of female, non-obese mice, fed ad lib., were perfused with 'standard medium containing ^3H_2O (3 mCi in 60 ml medium) and glucose at various concentrations (a) and in (b) with the addition of lactate at initial concentrations of 9-12 mM. Total rates of fatty acid synthesis were calculated from the incorporation of ^3H into total liver FA (as described in Section 4.2.1.) by subtracting the µmol newly-synthesized FA/g in one-half of the median, bifurcted lobe after 2 h of perfusion from that in the left lateral lobe after 3 h of perfusion. Each point represents the rate calculated from a single perfusion, and most of these perfusions are the same as those described in Table 4.2.

synthesized/g wet liver/h was obtained between 5-15 mM perfusate glucose. Above this concentration the rate of synthesis increased to a maximum between 25-30 mM glucose.Above 30 mM, a decrease in the rate of synthesis may have been due to hyperosmolarity of the medium relative to hepatocyte cytoplasm.The above concentration dependence of LCFA synthesis on perfusate glucose concentrations is different to that described by Brunengraber et al (1973) for the rat liver, in which the rate of synthesis rose from a low value at 4 mM glucose to a maximum at 8-15 mM glucose.However, the experimental conditions used by these authors vary considerably from those in the present study, including the use of rats fed a high-carbohydrate, fat-free diet, an erythrocyte-free medium and a shorter period of perfusion in the presence of isotopes.

The addition of 10 mM lactate to the perfusate altered the dependence of the total rate of LCFA synthesis on medium glucose concentrations such that a maximum rate was now observed at about 15 mM glucose (Table 4.2.).This now more closely resembled the glucose dependence of LCFA synthesis described by Brunengraber et al (1973).

Table 4.2. Synthesis of fatty acids from glucose and lactate in the perfused mouse liver.

Livers of non-obese mice (female, 12 weeks old and fed ad lib.) were perfused with standard medium containing $^{3}H_2O$ (3 mCi) and ^{14}C-labelled precursors at the initial concentration indicated. The incorporation of ^{3}H and ^{14}C into total liver FA were determined in sequential samples. The total quantity (μmol) of newly-synthesized FA was calculated from the ^{3}H values, and that formed from glucose or lactate from the ^{14}C results; the latter quantity is expressed as a percentage of the former. The rate of synthesis of FA de novo was calculated for the third hour of perfusion, and includes the fraction of newly-synthesized FA which was secreted into the perfusate (see Section 4.4.). Other details are in the text. Results are given as the means ±S.E.M. of the number of perfusions in parenthesis.

Additions to medium	initial concn. range (mM)	total newly-synthesized FA in liver (μmol/g wet liver)			calculated percentage of synthesized FA derived from (^{14}C)-precursor			calculated rate of FA synthesis μmol/h/g liv
		60 min	120 min	180 min	60 min	120 min	180 min	
(U-^{14}C) glucose	4 - 6	1.4±0.2(3)	2.8±1.0(3)	4.8±0.5(3)	6±1(3)	9±1(3)	11±1(3)	2.4
(U-^{14}C) glucose	11 - 17	1.2±0.2(3)	2.1±0.5(3)	4.0±0.7(4)	15±3(3)	14±2(3)	14±2(3)	2.3
(U-^{14}C) glucose	21 - 23	3.2±0.1(3)	6.4±0.5(3)	9.6±1.6(3)	20±1(3)	23±2(3)	22±1(3)	3.8
(U-^{14}C) glucose	24 - 32	2.3±0.3(6)	5.4±0.6(7)	10.3±0.9(7)	36±3(3)	38±4(3)	40±5(3)	5.8
(U-^{14}C) lactate + glucose	10 5 - 10	2.3±0.4(6)	4.5±0.7(7)	7.0±1.0(7)	34±7(5)	32±4(6)	31±7(6)	3.0
(U-^{14}C) lactate + glucose	10 12 - 17	2.4±0.5(4)	6.9±0.6(6)	12.8±0.5(4)	49±13(4)	36±4(6)	34±3(4)	7.0
(U-^{14}C) lactate + glucose	10 20 - 25	2.1±0.2(5)	5.1±0.7(5)	8.0±1.1(5)	30±4(5)	29±4(5)	26±(4)	3.5

4.2.3. The dependence on medium glucose concentrations of the contribution of glucose-C to total long-chain fatty acid synthesis.

In most of the experiments described in the previous sub-section, the rate of synthesis of LCFA was not only measured from the extent of incorporation of ^{3}H from $^{3}H_2O$, but also simultaneously from medium (U-^{14}C)-glucose.The results shown in Table 4.2 show that the contribution of glucose-C to LCFA synthesis was always less than 40%, and at normal blood glucose concentrations was less than 10%.The rate of synthesis from glucose-C was not linear, but tended to rise during three hours of perfusion.This indicated that there was a delay in the metabolism of glucose prior to its incorporation into LCFA.

The effect of perfusate glucose concentrations on the distribution of ^{14}C from (U-^{14}C)-glucose in hepatic lipid classes was investigated.In non-obese mice there was a propensity for ^{14}C accumulation in PL, whilst 15-30% was found in TG (Table 4.3).Other hepatic lipid classes contained less than 10% of the ^{14}C found in total lipids.At perfusate glucose concentrations of 5 and 25 mM, the proportion of ^{14}C in TG was greater and in PL less, than at 10 and 15 mM.

A different distribution of ^{14}C in hepatic lipid classes was observed in perfused livers from ob/ob mice.There were approximately equal amounts of radioactivity in PL and TG, but there was no clear change in this pattern with changes in glucose concentration.The incorporation of ^{14}C into other lipid classes was less than 10% of that in total lipids, (Table 4.3).

Table 4.3. Distribution of ^{14}C from (U-^{14}C) glucose in lipid classes during perfusion of the perfused mouse liver.

Livers were perfused with standard medium containing 2.5-5.0 μCi (U-^{14}C) glucose as described in 2.11. Liver samples were obtained after 2h (median, bifurcated lobe) and 3h (left lateral lobe. Lipids were extracted and lipid classes separated by adsorption-t.l.c. (as described in 2.3.2.). The content of ^{14}C in each band was determined. The sum of the radioactivity recovered in all bands was more than 90% of that which was applied. Other details are in the text. Results are expressed as the % of the total radioactivity recovered from the plate, and are the values obtained from one or the mean of two perfusions ± range/2. All sets of values given at 2 and 3h are from the same perfusion(s).

mouse type	initial medium glucose concn.	time of sampling (h)	number of perfusions	percentage distribution of ^{14}C in lipid classes							^{14}C in *TL (μg-at/g)
				PL	MG	FFA	FC	DG	TG	CE	
non-obese	5	1	1	54.9	1.4	1.8	5.6	6.2	28.3	1.8	8.7
		2	2	60.8±6.3	0.5±0.1	0.9±0.1	5.1±2.0	3.1±1.2	28.9±2.8	0.8±0.3	14.1±0.8
	10	1	1	59.6	0.9	0.6	2.0	4.3	27.1	5.5	10.9
		2	1	69.5	0.8	0.9	2.4	1.8	23.5	0.9	
	15	1	2	67.4±2.1	2.6±0.1	2.3±0.2	3.8±1.1	3.8±0.4	17.8±3.8	2.6±0.3	8.1±1.4
		2	2	68.8±9.1	0.8±0.1	0.7±0.1	2.9±0.4	3.7±0.7	20.1±7.1	3.2±2.3	17.3±3.5
	25	1	1	54.4	2.2	0.9	2.6	0.1	37.8	2.0	64.9
		2	1	59.2	2.7	3.6	2.8	0.1	31.1	0.4	88.9
ob/ob	4	1	2	52.5±1.9	0.8±0.4	1.1±0.2	2.3±0.5	4.1±0.5	36.8±2.5	0.8±0.1	
		2	2	46.8±2.2	0.7±0.1	0.9±0.2	2.2±0.9	4.6±0.4	44.3±1.7	0.7±0.1	31.5±12.4
	12	1	2	74.1±4.7	0.3±0.1	0.7±0.1	1.4±0.5	1.9±0.5	21.5±3.6	0.5±0.1	16.8±4.7
		2	2	53.2±2.5	0.3±0.1	0.5±0.1	4.7±1.9	2.2±0.1	39.6±1.5	0.8±0.2	30.6±5.7
	25	1	1	25.8	1.4	2.0	2.6	4.1	31.7	0.5	35.9
		2	1	41.6	1.2	1.0	2.6	3.4	49.1	1.0	69.2

*TL total lipid

4.2.4. The dependence on medium glucose concentrations of the contribution of lactate carbon to total long-chain fatty acid synthesis.

In view of the finding that glucose provided little carbon for de novo FA synthesis, the contribution of lactate carbon to this process was investigated, since this represented another group of metabolites which is both present in blood and a potential precursor of LCFA. In the liver, compounds such as lactate and some amino acids (eg. alanine) are rapidly converted to pyruvate, the major fate of which is gluconeogenesis or oxidation to CO_2 (Exton et al, 1972). It was therefore possible that acetyl CoA formed as an intermediate in the latter process contributed a major proportion of the total carbon of acetyl CoA utilized in hepatic FA synthesis.

Table 4.2 shows the results of a series of experiments in which livers were perfused with a medium containing lactate (5 or 10 mM) and glucose at concentrations ranging from 4-23 mM. The percentage of total LCFA synthesized during the first hour rose from 33.8% at 5 mM glucose to 49.3% at 15 mM glucose, but decreased to 31.0% and 33.7% respectively by the third hour of perfusion. Above an initial perfusate glucose concentration of 20 mM, a decrease in both the total rate of LCFA synthesis and incorporation of lactate carbon into LCFA occurred.

4.2.5. The relationship of the initial hepatic glycogen content to the total rate of long-chain fatty acid synthesis, and the contribution of glucose carbon to this process.

In previous experiments it was shown that the contribution of carbon from glucose and lactate was unable to account fully for the total carbon utilized in LCFA synthesis in livers perfused with normoglycaemic media.

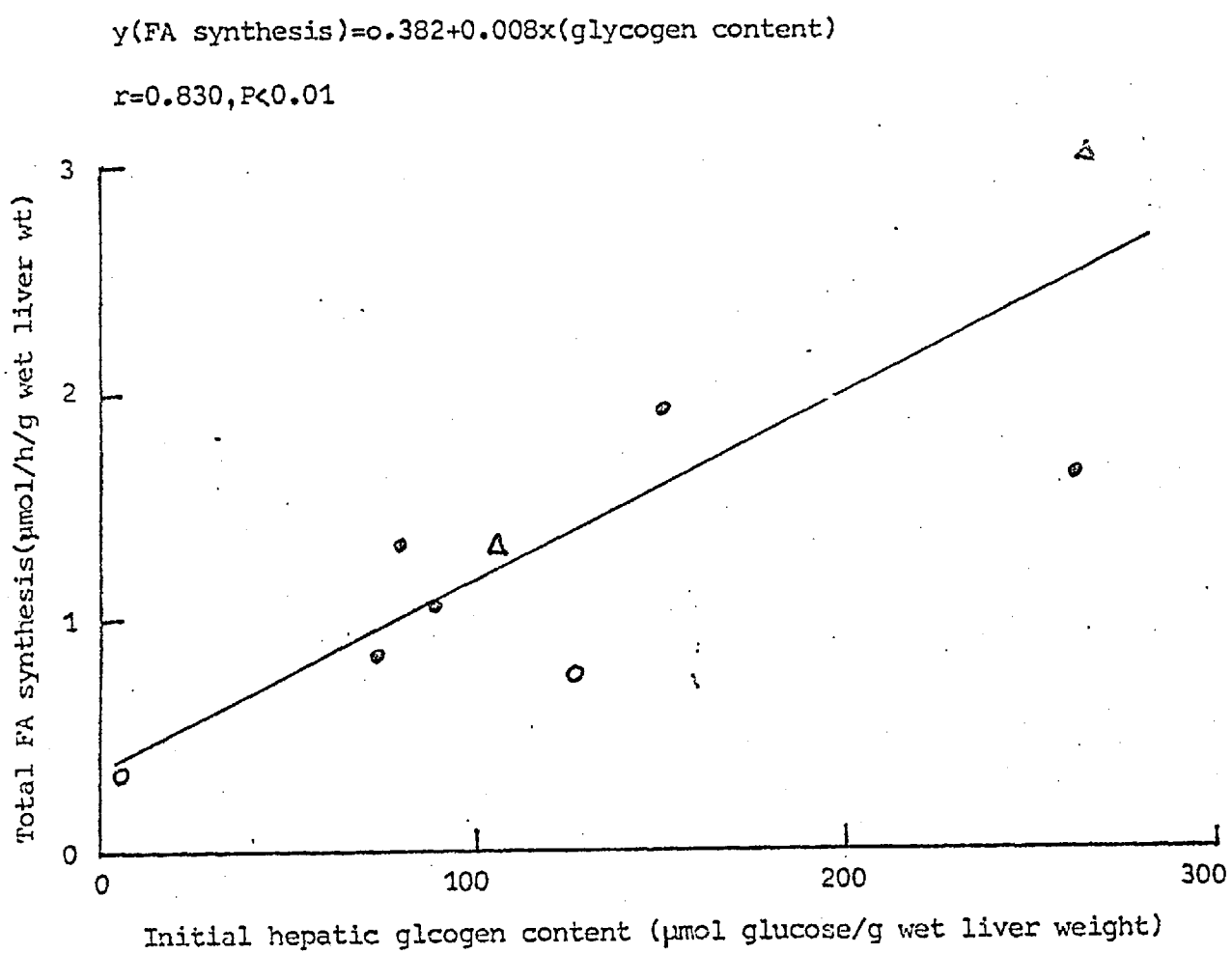


Fig. 4.6. Relationship between the total rate of fatty acid synthesis and the initial glycogen content in the perfused mouse liver.

Livers of non-obese mice (female, fed ad lib.) were perfused with standard medium (as described in Section 2.11) containing ^3H_2O (3 mCi) and 5 mM glucose. After 5 min, the glycogen content in the median, bifurcated lobe was measured, and after 60 min the incorporation of ^3H into total liver FA was determined in the left lateral lobe, from which the rate of FA synthesis was calculated.
Δ: livers pre-perfused during cannulation procedures using bicarbonate-saline containing 20 mM glucose.
●: 'standard' perfusions.
○: pre-perfusion for 30 or 60 min with medium without added glucose.
Results are from individual perfusions, and the line was fitted by linear regression analysis.

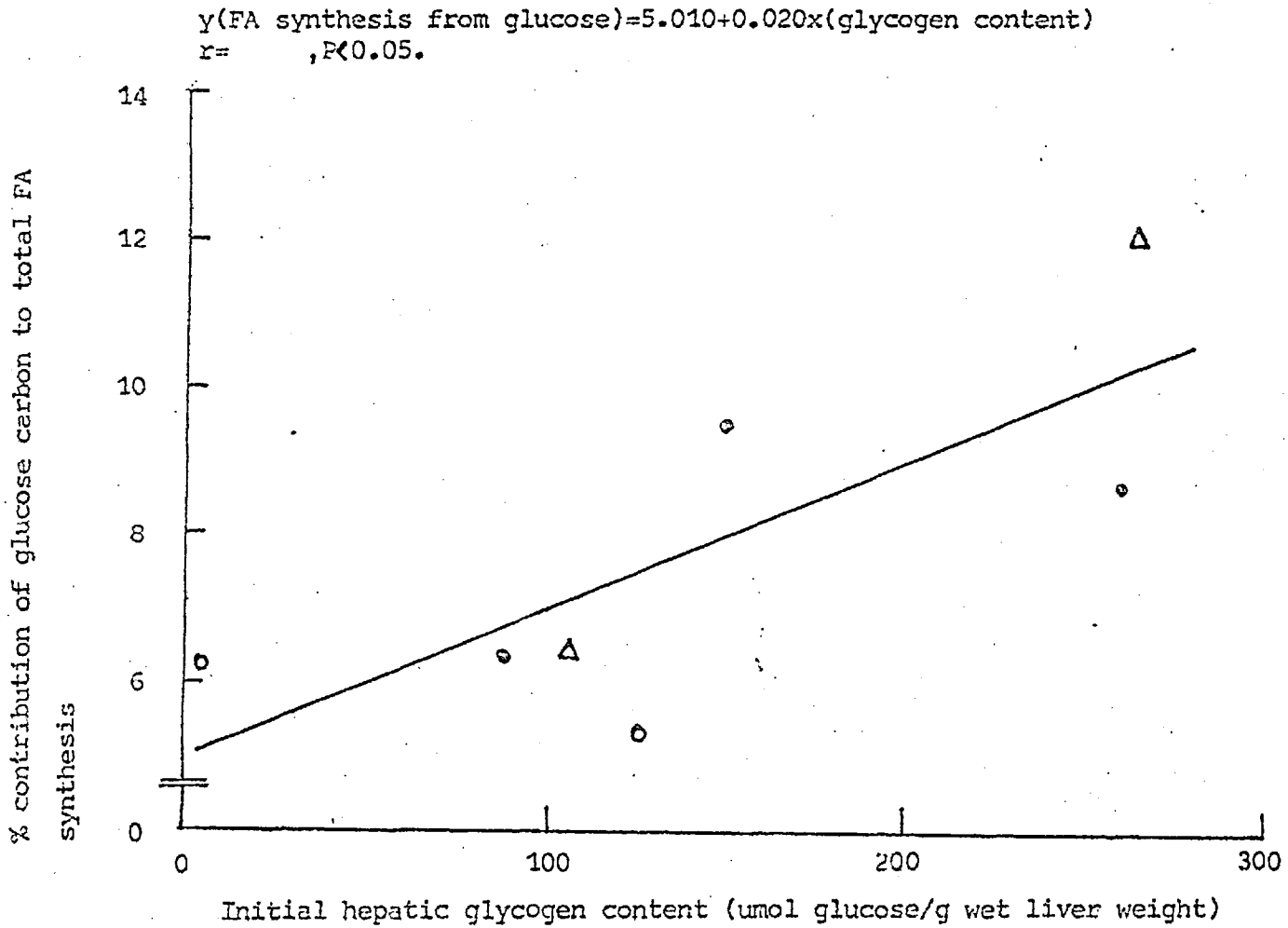


Fig. 4.7. Relationship between the % of total fatty acid synthesized derived from medium glucose carbon and the initial glycogen content in the perfused mouse liver.

In most of the perfusions described in Fig. 4.6., (U-^{14}C) glucose (2.5 µCi) was also present in the perfusion medium. The incorporation of ^{14}C and ^{3}H (from $^{3}H_2O$) into total liver FA was determined simultaneously, and the % of the toal umol FA synthesized derived from glucose ca bon calculated as described in Section 4.2.1. The symbols denote the type of pre-perfusion in each case, and are identicl to those described in Fig.4.6. Results are from individual perfusions, and the line was fitted by linear regression analysis.

It was thought that glycogen might be a potential source of such carbon especially under conditions in which hepatic glycogen shows a net decrease during the course of perfusion (see Section 4.1.2).

A series of perfusions were therefore carried out in which the initial median (notched) lobe glycogen content varied over a range of 5-300 μmol glycogen glucose/g wet liver; the total rate of LCFA synthesis was measured in the left lateral lobe during the first hour. Fig.4.6 shows that a linear relationship existed between these two parameters.

Fig.4.7 shows the relationship of the initial hepatic glycogen content of the proportion of total LCFA synthesized derived from glucose. A positive linear correlation was found between these two parameters.

4.2.6. The incorporation of radioactivity from isotopically-labelled precursors into 'lipid glycerol'.

Radioactivity in the water-soluble fraction obtained after extraction with light petroleum (b. pt. 40°-60°C) of saponified, acidified total lipids presumably resided almost entirely in lipid glycerol. Extraction and washing of the total lipid fraction removed more than 99% of the radioactivity associated with (^{14}C)- and (^{3}H)-labelled precursors as shown by the absence of detectable radioactivity in total lipid extracts of medium samples taken within 5min. of the start of perfusion. However, in view of the fact that glycerol was not isolated from other solutes in the extracted aqueous phase after saponification, radioactivity found in this fraction is referred to as being incorporated into 'lipid glycerol'.

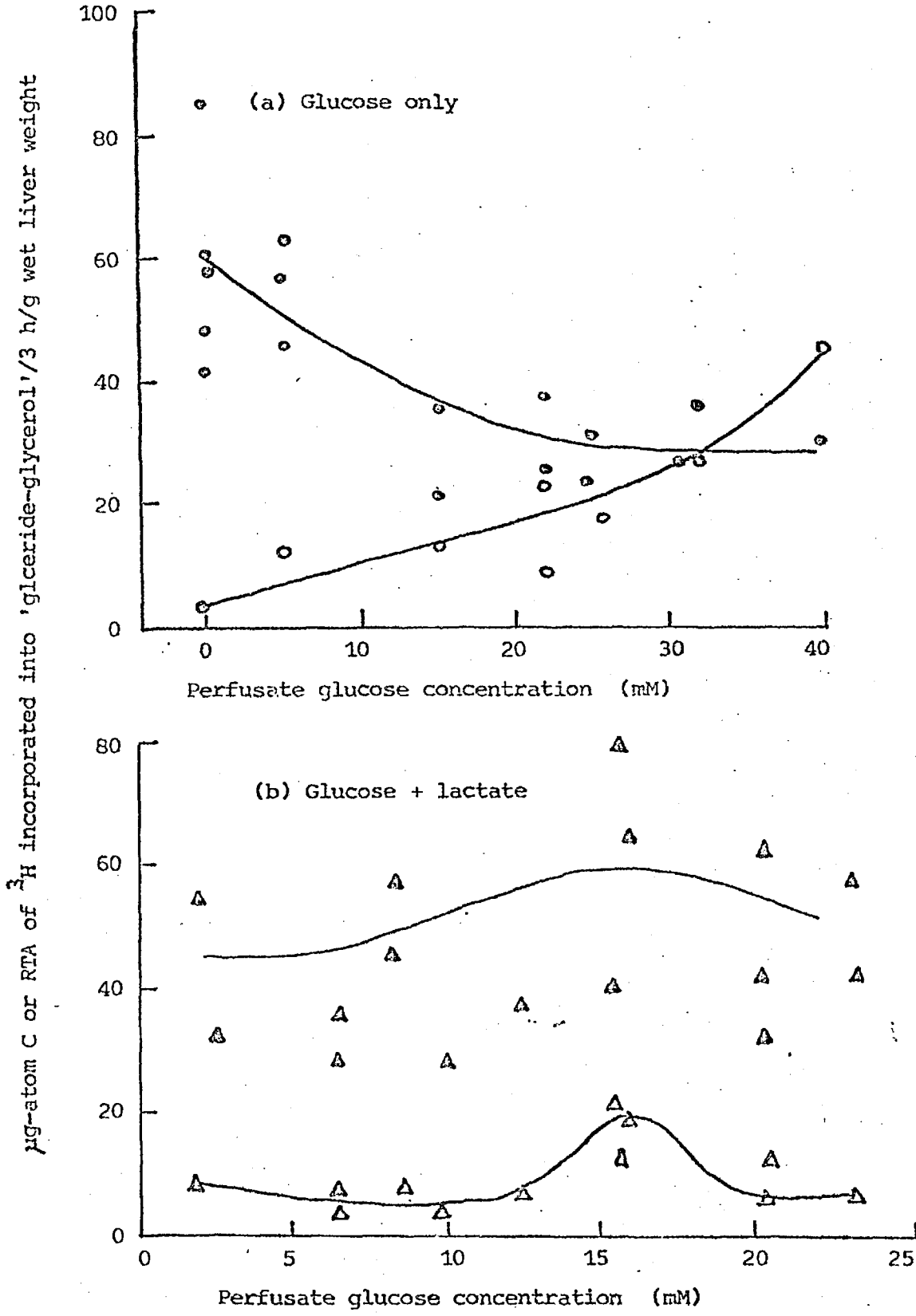


Fig.4.8. Dependence on the medium glucose concentration of the incorporation of radioactivity from $^{3}H_2O$, (U-^{14}C) glucose and (U-^{14}C) lactate into 'glyceride-glycerol' in the perfused mouse liver.

Fig. 4.8 shows the dependence of the μg-at C, from (U-^{14}C)-glucose or (U-^{14}C)-lactate, and the RTA of ^{3}H in 'lipid glycerol' on the medium glucose concentration.An increase in the extent of incorporation of ^{14}C from (^{14}C)-labelled precursors was found under conditions when an increased rate of FA synthesis from these precursors was observed (cf. Table 4.3).The incorporation of ^{3}H from $^{3}H_2O$ was complex.An increased RTA of ^{3}H in lipid glycerol was not associated with high rates of FA synthesis, in perfusions with or without added lactate.

4.2.7. The effect of oleate on the rate of fatty acid synthesis in the perfused liver.

Hepatic LCFA synthesis has been shown to be subject to end-product feedback inhibition; thus palmityl-CoA inhibits both FA synthesis in cell free systems and purified acetyl CoA carboxylase (Bortz & Lynen, 1963; Robinson et al , 1963; Tubbs & Garland, 1964).Oleate markedly inhibits synthesis of LCFA in the perfused rat liver (Mayes & Topping, 1974) when perfusate concentrations exceed 0.3-0.4mM.This effect was investigated in the perfused mouse liver with respect to the contribution of glucose carbon to LCFA synthesis.

In one series of experiments, oleic acid, bound to bovine serum albumin, was added initially to the perfusate to raise the concentration to about 0.3mM, and was infused during perfusion to maintain a relatively steady concentration (Fig. 4.9).The rate of uptake of oleate was then equal to its rate of infusion.Uptake by the perfused non-obese mouse liver was similar to those reported by Assimacopoulos et al (1973), but that by the ob/ob mouse liver was greater on account of the greater weight of livers from these mice (Table 4.4).

Table 4.4. Rates of uptake of oleic acid from the medium by perfused livers from ob/ob and non-obese mice.

Livers were perfused with standardmedium containing glucose at the concentration indicated.Oleic acid,bound to bovine serum albumin,was added to the medium at the start of the perfusion,and then infused at a constant rate to maintain a relatively steady concentration (see Fig. 4.8.).The rate of infusion was then equal to its rate of uptake.The hepatic uptake of oleate was assumed to be a first order process,and was therefore compared between perfusions of livers of ob/ob and non-obese mice by calculating the uptake rate (R_u) at a standard concentration (0.5mM) per g wet liver using the relationship: $R_u=(\ln 2/t_{\frac{1}{2}}).v.c$,
where $t_{\frac{1}{2}}$ is the half-time for the uptake of oleate (min.),v is the volume of distribution of oleate(ml) and c is the concentration of oleate in the medium (mM). **The data refer to single perfusions.**

Mouse type	perfusate glucose concentration (mM) range	perfusate oleate concentration (mM) range	$t_{\frac{1}{2}}$ (min.)	v (ml)	observed R_u (μmol/min/liver)	calculated R_u at c=0.5mM (μmol/min/g wet liver
non-obese	10.8-11.4	0.31-0.48	76.6	64.5	0.25	0.19
non-obese	2.9-5.2	0.33-0.54	86.2	85.7	0.30	0.23
non-obese	26.2-36.0	0.35-0.43	98.5	92.3	0.30	0.22
ob/ob	9.5-17.5	0.33-0.73	49.3	61.5	0.50	0.19

Fig. 4.9. next page

Fig. 4.9. Influence of perfusate glucose and oleate concentrations on fatty acid synthesis in the perfused mouse liver.

Livers were perfused as described in Fig. 4.1.,except that oleic acid bound to bovine serum albumin was added at the start of perfusion to produce an initial concentration of about 0.35 mM and subsequently infused to maintain this concentration (as described in Section 2.11.).3 mCi of $^{3}H_{2}O$ was also present in the medium.After 60.120 and 180 min of perfusion,samples of liver and medium were taken and the incorporation of ^{3}H into total liver FA determined. •,denotes perfusions to which no oleate was added,glucose concentrations 5-10 mM (same perfusions as shown in Table 4.2.) and are the means of three perfusions and the bars represent the S.E.M.The open symbols denote perfusions to which oleate was added and are the results of one perfusion in each case.

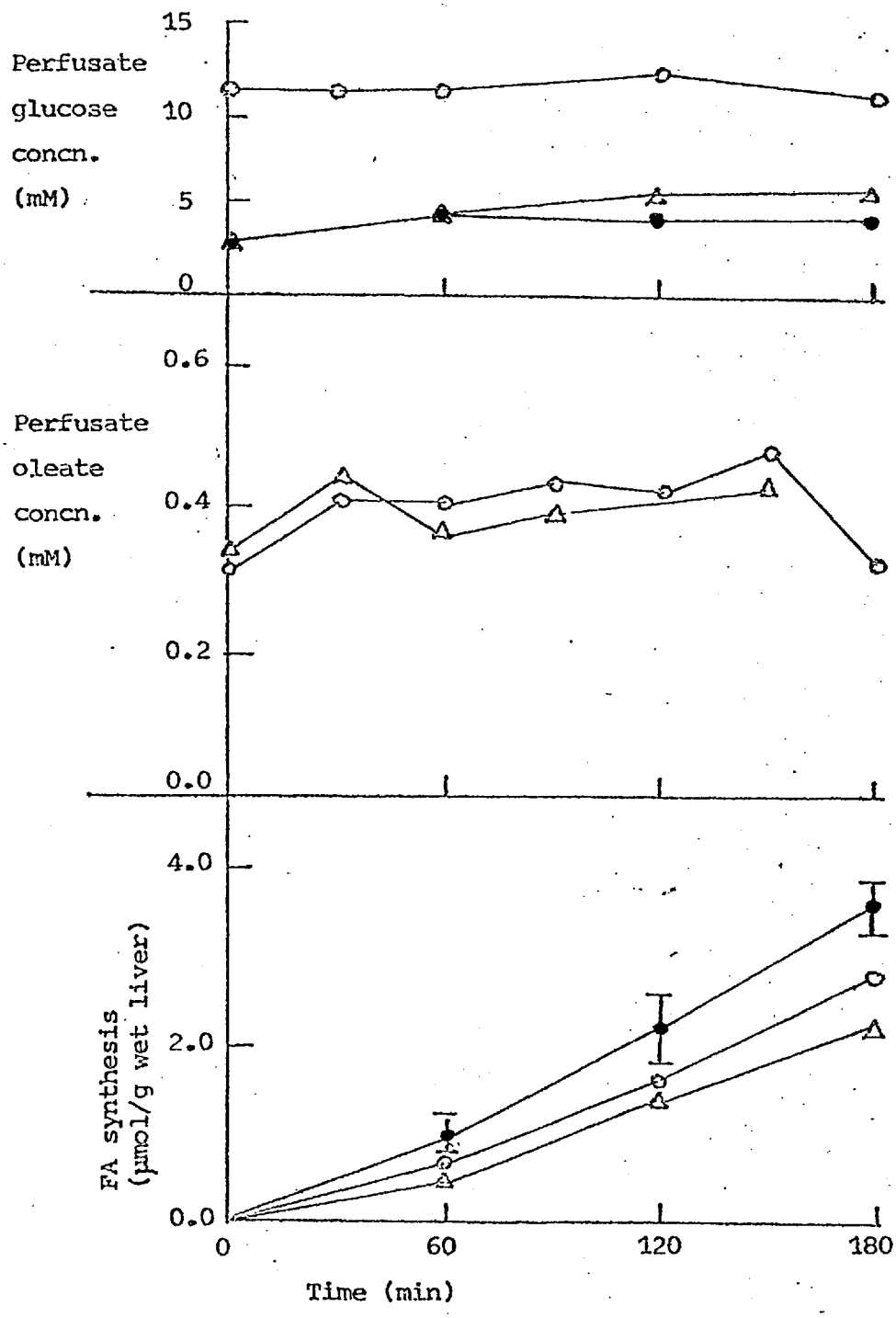


Fig. 4.9. Influence of perfusate glucose and oleate concentrations on fatty acid synthesis in the perfused mouse liver.

Fig.4.9 shows that when the perfusate oleate was 0.3-0.4mM, about 25% inhibition of total LCFA synthesis occurred. This was less than that reported recently in isolated rat liver parenchymal cells (Nilsson et al, 1973), but of the same order as obtained by Mayes & Topping (1974) in the perfused rat liver. The contribution of glucose carbon to total LCFA synthesis was decreased in the presence of oleate in livers from both types of mice. No increase was observed in hepatic glucose output above that when oleate was omitted from the medium (Fig.4.9) in contrast to the report of Kohout et al (1971) in the perfused rat liver, although this effect may have been masked by the larger medium/liver ratio used in the present perfusion system.

4.3. Synthesis of cholesterol in perfused liver.

In some experiments, free and esterified cholesterol were isolated by t.l.c. (2.2.4.) from total lipids extracted from livers after 180 min of perfusion. It was found that not less than 75% of both ^{3}H and ^{14}C in (FC and CE) was in free cholesterol. In addition, Exton et al (1972) have shown that 95% of ^{14}C from ^{14}C-lactate incorporated into these two sterol fractions is associated with the unesterified fraction. Thus it was likely that most of the radioactivity in CE was in the FA moiety. Table 4.6 shows the extent of free cholesterol synthesis in livers of non-obese and ob/ob mice perfused with $^{3}H_2O$ and various concentrations of (U-^{14}C)-glucose for 3 hours. Assuming a linear time course, rates of synthesis are of the same order as reported by Windmueller & Spaeth (1966, 1967) and Mayes & Topping (1974) in perfused livers of rats fed ad lib. From the limited numbers of observations, it appears that cholesterol synthesis may be slower in the ob/ob mouse liver.

Table 4.5. Cholesterol synthesis in the perfused mouse liver.

Livers were perfused with standard medium containing 3mCi $^{3}H_2O$ and 2.5-5.0uCi (U-^{14}C) glucose for 3h.After this time,lipids were extracted from the left lateral lobe and separated by adsorption t.l.c. The content of ^{14}C and ^{3}H in the chlesterol (free sterol) fraction was determined by liquid scintillation spectrometry.The perfusions are the same as those described in Table 4.3.The methods of calculation of rates of cholesterol synthesis and other experimental details are described in the text.Values are those obtained from one or the mean of two perfusions.

Type of mouse	initial glucose concentration (mM)	number of perfusions	cholesterol synthesis (nmol / g wet liver)	calculated average % of synthesized FC derived from glucose-carbon
non-obese	5	2	302.5	8.3
non-obese	10	1	163.2	8.6
non-obese	15	2	147.4	12.6
non-obese	25	1	294.7	31.5
ob/ob	5	2	216.1	11.9
ob/ob	25	1	225.3	29.6

4.4. Secretion of lipids by the perfused mouse liver.

In the absence of added FFA, the precursors of lipids secreted by the perfused liver are endogenous lipids and TG synthesized de novo.

The rate of secretion of lipids synthesized de novo by perfused livers from non-obese mice increased during the first two hours of perfusion but was almost constant during the third hour (Fig.4.9), in agreement with Windmueller & Spaeth (1966, 1967), although the rate of accumulation of ^{3}H in hepatic lipids was nearly linear throughout these perfusions (Fig.4.4).The rate of secretion of lipids by livers from ob/ob mice increased throughout three hours of perfusion such that the fraction of newly-synthesized lipid that was secreted was greater after three hours of perfusion (25%) than by livers from non-obese mice (18%).

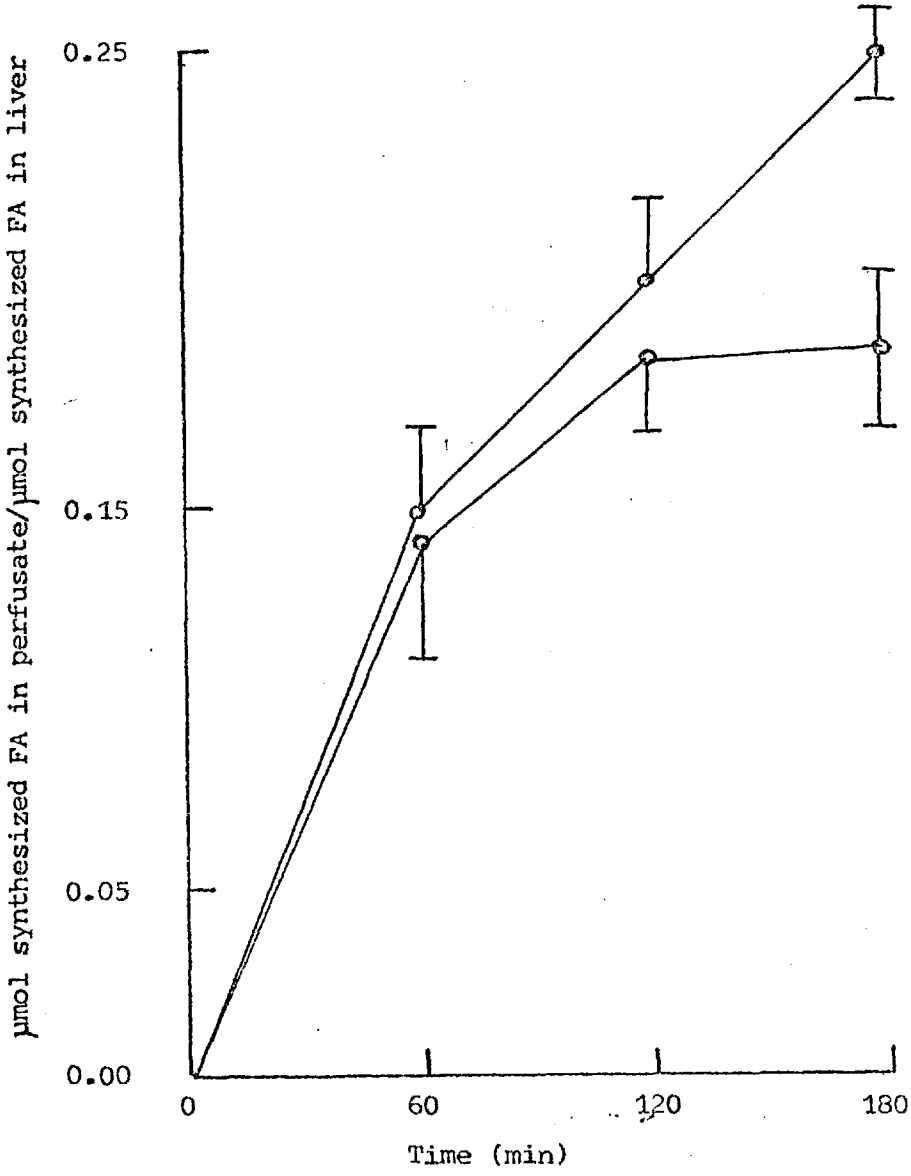


Fig. 4.10. Time-course of the ratio of the ^{3}H-labelled lipid secreted into the perfusate to that accumulated in the liver.

Livers of mice were perfused with 'standard medium to which $^{3}H_2O$ (3 mCi) and glucose (initial concentration 5-10 mM) was added. After the times indicated, The liver and perfusate was sampled and the incorporation of ^{3}H into total lipids was determined. The results are from four prefusions each of livers from non-obese (○), or ob/ob (●) mice. The bars represent the S.E.M.

CHAPTER 5.

DISCUSSION

5.1 Hyperlipoproteinaemia in ob/ob mice.

5.2 Turnover of plasma triglycerides in mice.

5.2.1 Rate of influx of triglycerides into plasma.

5.2.2 Rate of removal of triglycerides from plasma.

5.2.3 Overall estimate of the rate of turnover of plasma triglycerides.

5.2.4 Significance of increased turnover rates of plasma triglycerides in ob/ob mice.

5.2.5 Possible endocrine abnormalities implicated in the increased plasma triglyceride turnover in ob/ob mice.

5.3 Precursors of plasma triglycerides in ob/ob mice.

5.4 Control of fatty acid synthesis by substrate supply in the perfused mouse liver.

5.4.1 Suitability of the perfused mouse liver preparation for investigation of fatty acid synthesis.

5.4.2 Utilization of glucose carbon in fatty acid synthesis.

5.4.3 Role of lactate in fatty acid synthesis.

5.4.4 Role of glycogen in the de novo synthesis of fatty acids in the liver.

5.5 Roles of the liver in the altered lipid metabolism in ob/ob mice.

CHAPTER 5.

DISCUSSION.

5.1. Hyperlipoproteinaemia in ob/ob mice.

The present experiments confirmed the occurrence of hyperlipoproteinaemia in ob/ob mice (Tuller & Mayer,1959),due largely to increased quantities of cholesterol (Mayer & Jones,1953) as both FC and CE (Enser,1972)in HDL (d. 1.075-1.21) and possibly HDL_1 (identified on polyacrylamide gel disc electrophoresis).Genetically obese (fa/fa) rats also show hypercholesterolaemia,but in contrast,rats or mice rendered obese through goldthioglucose injection do not (Barry & Bray,1969; Zucker,1965;Schonfeld & Pfleger,1971;Mayer & Jones,1953).In man,plasma cholesterol is largely associated with LDL (Havel et al,1955;Lewis,1971), whereas the three techniques which were employed in the present experiments for the separation of plasma LP classes showed that plasma from both types of mice,like plasma from rats and a number of other species (Havel et al, 1955;Lewis et al,1952) contains relatively more HDL and less LDL than in man. This difference could explain why hypercholesterolaemia is associated with the deposition of atheromatous arterial deposits in man (Schettler,1972; Carlson & Bottinger,1972),but such pathological consequences have not been observed in ob/ob mice (Russell & Meir,1966).

Neither plasma TG or FFA levels were found to be raised in ob/ob mice relative to non-obese mice.In contrast,fa/fa rats have marked increases in the concentrations of these two plasma lipid classes (Zucker,1965; Barry & Bray,1969;Schonfeld & Pfleger,1971),and in the obese sand rat increased plasma TG levels are associated with increased TG deposition

(Robertson et al,1973).In common with most other species (Nichols et al, 1969),plasma TG in ob/ob mice was predominantly associated with VLDL (d.<1.006).Most of this plasma TG appeared to be of endogenous origin, since at the time when the murine plasma was obtained for analyses (12.00 ±1.00 h) a negligible yield of chylomicra was obtained (measured by nephelometry after centrifugation of plasma at 10,000 g for 1 h).

5.2. Turnover of plasma triglycerides in mice.

Since measurements of plasma TG levels do not distinguish between rates of TG secretion and removal,but merely represent a resultant steady state,measurements of TG turnover rates were carried out in order to define more accurately differences in plasma TG metabolism in ob/ob mice. The present experiments showed that the turnover of VLDL-TG was faster in ob/ob than in non-obese mice,due both to an increase in TG secretion and its rate of uptake from blood.These two processes are accelerated to about the same extent,resulting in similar plasma TG concentrations in the two types of mice.This follows from calculations both of the rates of secretion of endogenous TG and of the uptake of (^{14}C)-VLDL-TG and the capacity for uptake of exogenous,emulsified (Intralipid) TG (see Chapter 3),the bases of which are dicussed in the following two sub-sections.

5.2.1. Rates of influx of triglycerides into plasma.

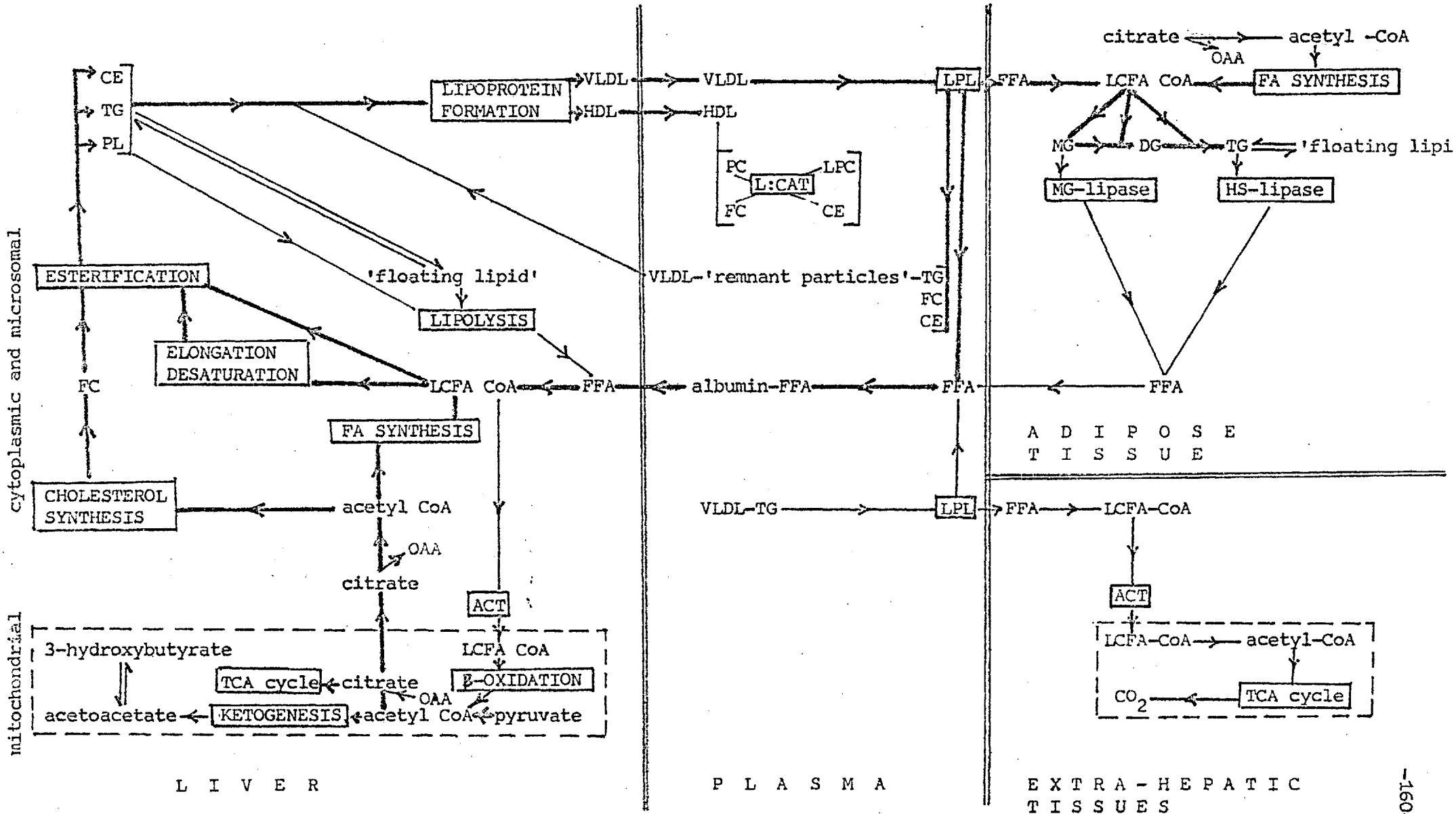
The present approach to estimating rates of TG entry possessesthe inherent advantage that blood metabolite levels are maintained in the normal physiological range during the experimental period.The calculation of the rate of influx of TG into plasma involves consideration of a number of processes,illustrated in Fig. 5.1.The rate of secretion of plasma TG was calculated from the time course of incorporation of ^{14}C from a trace intravenous dose of (U-^{14}C) palmitate into plasma TG.The presumption was made that pools of extravascular TG (likely to be located mainly in the liver) became labelled with ^{14}C over an initial period during which release of (^{14}C)-labelled TG into plasma was negligible,and that the time course of appearance of ^{14}C in plasma TG depended on these extravascular pools

Fig. 5.1. next page

Fig. 5.1. Major processes involved in inter- and intra-tissue fatty acid metabolism.

The diagram outlines some of the major movements and interconversions of fatty acids, fatty acyl esters, their precursors and their catabolites. Evidence for many of the pathways shown is cited in the text. The most heavily drawn lines denotes those processes thought to predominate in an animal fed a low fat diet *ad lib.* The processes shown to occur within mitochondria (enclosed by dotted line) are only those thought to occur exclusively in this compartment. However, some other processes (*e.g.* elongation and desaturation of LCFA-CoA) may occur both intra- and extra-mitochondrially. Most abbreviations are explained in the Preface, but those peculiar to the diagram are as follows:

ACT	:	acylcarnitine transfer
HS-lipase	:	hormone-sensitive lipase
LPL	:	lipo-protein lipase
MG-lipase	:	monoglyceride lipase
OAA	:	oxaloacetate
LPC	:	lysophosphatidyl choline
L:CAT	:	lecithin:cholesterol acyl transferase
PC	:	phosphatidyl choline


CE
TG
PL
LIPOPROTEIN FORMATION
VLDL
HDL
VLDL
HDL
PC
L:CAT
LPC
FC
CE
LPL
FFA
LCFA CoA
citrate
acetyl -CoA
OAA
FA SYNTHESIS
MG
DG
TG
'floating lipi
MG-lipase
HS-lipase
ESTERIFICATION
'floating lipid'
VLDL-'remnant particles'-TG
FC
CE
LIPOLYSIS
ELONGATION DESATURATION
FC
LCFA CoA
FFA
albumin-FFA
FFA
FFA
FA SYNTHESIS
A D I P O S E
T I S S U E
CHOLESTEROL SYNTHESIS
acetyl CoA
OAA
citrate
ACT
VLDL-TG
LPL
FFA
LCFA-CoA
ACT
3-hydroxybutyrate
LCFA CoA
TCA cycle
citrate
β-OXIDATION
OAA
acetoacetate
KETOGENESIS
acetyl CoA
pyruvate
LCFA-CoA
acetyl-CoA
CO_2
TCA cycle
cytoplasmic and microsomal
mitochondrial
L I V E R
P L A S M A
E X T R A - H E P A T I C
T I S S U E S

subsequently behaving as if there was one pool whichretained fairly constant specific radioactivity (for 5-60 min).This analysis of events after an intravenous,trace dose of (^{14}C) palmitate agrees in general with that of Laurell (1959),Baker & Schotz (1964,1967),Havel et al (1962),Farquhar et al (1965) and Gross et al (1967).These authors have discussed evidence for several different ways in which (^{14}C) palmitate may recycle between plasma FFA,liver fatty acyl esters,plasma TG and extrahepatic TG during the course of such experiments.These possibilities have been included in Fig. 5.1.Whilst there is abundant evidence that endogenous plasma TG are almost solely of hepatic origin (Harper et al, 1953;Stein & Shapiro,1959;Byers & Friedman,1960;Borgstrom& Olivecrona, 1961;Havel & Goldfien,1961),plasma FFA may assimilated by muscle,adipose tissue and other organs,in addition to the liver (Dole,1956;Fredrickson & Gordon,1958;Friedberg et al,1960;Havel et al,1962;Bragdon & Gordon,1958), and may be recycled as FFA from these sites,either directly or following temporary storage as glycerides (Fredrickson & Gordon,1958;Laurell,1959). In addition,TG released into plasma from the liver may be reassimilated by the liver intact,whence the majority returns to the circulation without undergoing hydrolysis (Havel et al,1962;Baker & Schotz,1964; Farquhar et al,1965;Olivecrona,1965;Bergman et al,1971).The present assumption concerning the behaviour of extravascular pool(s) of (^{14}C)-labelled TG was reasonable as shown by further consideration of the rising phase of the time course (Fig. 3.4),which reflected turnover of secreted (^{14}C)-labelled TG (during its gradual dilution by the relatively unlabelled plasma pool) and whose time scale resembled that of VLDL-TG removal from blood.If the above presumption were not correct, the rising phase of the curve of incorporation of ^{14}C would have had a slower scale than that of TG removal.

As this calculation concerned the rate of TG secretion from a hypothetical TG pool (prelabelled with (^{14}C) palmitate),it yielded values for the total rate of entry of TG into plasma (Table 3.8),but gave no measure of the contribution of individual precursors to this process.

The rates of TG secretion,calculated as described above,may be compared to those in other experiments in which a trace dose of (^{14}C)-labelled FFA was administered.The rate in non-obese mice (0.65 µmol TGFA/min/100g body weight) is similar to those in rats (Baker & Schotz,1964). If the present approach to the calculation of TG secretion rates is applied to the time courses of incorporation of ^{14}C into plasma TG in rats (Baker & Schotz,1964),dogs (Gross et al,1967) and rabbits (Havel et al, 1962),the calculated rate of secretion is of the order reported (which was not obtained by the present approach);this lends additional credibility to the present method of calculation of the rate of entry of TG into plasma.

5.2.2. Rate of removal of triglycerides from plasma.

The removal of both endogenous and exogenous TG from the plasma of mice was investigated in the present experiments - respectively as TG associated with native VLDL or as a stable TG-PL emulsion (Intralipid). The former resemble endogenous TG of hepatic origin (Havel et al,1962) and the latter chylomicra (Gries et al,1966).

The calculation of rates of TG efflux from plasma from measurements of the time course of disappearance of injected VLDL-TG depended on the presumption that the assimilation of injected VLDL exhibited first order kinetics,and that the VLDL mixed with and resembled that in the plasma of mice.The first presumption is supported by studies of the

uptake of plasma TG in other species (reviewed by Bierman,1965).The second presumption was reasonable because the (^{14}C)-VLDL-TG was prepared from donor mice which resembled the recipient mice,including the presence,or not,of genetic obesity.The delay of 30 min before collecting VLDL presumably allowed formation from (^{14}C)-palmitate of a variety of (^{14}C)-labelled TG entities,so that the preparations may be regarded as resembling the VLDL pool of plasma TG in the recipients.

The usefulness of measurements of clearance of Intralipid-TG may be questioned since the reticulo-endothelial system may participate to a greater extent in the uptake of emulsified TG than in that of chylomicra. However,Waddell et al (1954) concluded that this was unlikely,since saturation of the reticulo-endothelial system with "blockers" (e.g. lithium carmine,trypan blue) had little effect on the rate of elimination of emulsified TG from plasma.Furthermore,changes in the rate of disappearance of TG from plasma and the tissue distribution of assimilated TG are similar in different nutritional and hormonal states for both chylomicra and emulsified TG (Gries et al,1966;Kessler,1962a,b).

Similar $t_{\frac{1}{2}}$ values were obtained in the present work for the disappearance of TG associated with VLDL and Intralipid from plasma. This is in agreement with work in rats (Schotz et al,1966 ;Harris & Felts, 1973),although this is not the case in man,in whom the rate of uptake of plasma TG is inversely correlated with the size of the lipoprotein substrate (Bierman,1965;Quarfordt & Goodman,1966).

5.2.3. Overall estimate of the rate of turnover of plasma triglycerides in mice.

In a steady state, the calculated rates of secretion and assimilation of endogenous TG should theoretically be identical. The calculated value for the secretion was almost a third of that for the disappearance of plasma TG, but the former may have been subject to the greatest error due to the method of calculation. The rate of secretion of plasma TG was calculated from the fastest rising phase of the time course of the appearance of injected (U-^{14}C)-palmitate into plasma TG. Since this occurred between 5-10 and 10-20 min after injection in non-obese and ob/ob mice respectively, there was sufficient time (equivalent to about two half-times of plasma TG turnover) for significant uptake of newly-secreted TG to occur such that the observed rate was less than the true rate of secretion.

An estimate of the actual rate of plasma TG turnover in mice (female, aged 8-12 weeks, concious and fed ad lib.) may be made by combining the results of the calculations for both types of experiment, and giving greater weight to the values for uptake. These values are about 0.3 and 1.3 μmol TGFA/min/mouse in non-obese and ob/ob respectively. These are similar to the turnover rates of plasma TG found before and after the induction of obesity in sand rats (Robertson et al, 1973). It is also in agreement with the occurrence of an increased plasma TG turnover in obese humans (Nestel & Whyte, 1968; Birkenhager & Tjabbes, 1969).

Both the present measurements of plasma TG turnover, and those of Robertson et al (1973) mentioned above, include a component due to recycling of TG between liver and plasma (see Fig. 5.1). This has been estimated as 20-30% of the total flux in rats (Baker & Sehotz, 1964; Olivecrona, 1966), 25% in rabbits (Havel et al, 1962) and 40% in the case of

chylomicron TG in dogs (Bergman et al,1971).Thus the net flow of TG to extrahepatic tissues in ob/ob mice is likely to be about 1 μmol TGFA/min/mouse.

5.2.4. Significance of an increased turnover rate of plasma triglyceride in ob/ob mice.

The experiments in which Intralipid was injected into mice provided a measure of the capacity for removal of plasma TG by tissues (i.e."TG tolerance").This process is enhanced in ob/ob mice,which explains the absence of hyperglyceridaemia in the presence of an increased plasma TG turnover rate.This is in agreement with increases in the activity of lipoprotein lipase in heart and adipose tissue (deGasquet & Pequignot, 1972;Enser,1972) and skeletal muscle (Rath et al,1974) in ob/ob mice, especially when calculated per whole tissue.Hence these tissues are likely to be implicated in the extrahepatic assimilation of plasma TG in ob/ob mice,which was previously calculated to occur at a rate of 1 μmol/min/mouse.This process may be an important source of FA in adipose tissue,since the estimated rate of extrahepatic TG assimilation is in excess of the rate of TG accretion by ob/ob mice (calculated previously to be an average of 0.7 μmol/min in mice of the same age,sex,and nutritional status).

The hyperglycaemia and "insulin resistance" (see 1.3) may be exacerbated by an increased extrahepatic assimilation of plasma TG. Increased metabolism of FFA by tissues can cause impairment of glucose uptake (as if there were competition between substrates) at least in muscle;this process is part of the "glucose-FA" cycle (Randle et al,1963;

Ruderman et al,1969).Since plasma TG is hydrolyzed to release FFA and glycerol by the enzyme lipoprotein lipase,a similar relationship could pertain with respect to TG and glucose.The product FFA of the lipoprotein lipase process may enter tissues immediately or may recirculate bound to plasma albumin;from experiments using isolated perfused adipose tissue, it has been estimated that about one-third of the product FFA may be returned to the circulation (Scow et al,1972).If this were the case in ob/ob mice,it could be the origin of part of the increased FFA (and glycerol) flux in these animals (Dade & Hems,1972;Elliott et al,1974).

5.2.5. Possible endocrine abnormalities implicated in the increased plasma triglyceride turnover in ob/ob mice.

In several species a number of hormonal alterations result in accelerated rates of plasma TG turnover;some of these have been identified in ob/ob mice.Insulin increases the activity of lipoprotein lipase in adipose tissue,both in vivo and in vitro (reviewed by Robinson, 1970),and has also been shown to increase the net rate of secretion of VLDL-TG by the perfused rat liver (Topping & Mayes;1972).The effects of insulin in vivo,however are subject to various interpretations (as indicated by Nikkila (1969),who suggested that the ob/ob mouse represented a valuable model for the study of this problem,owing to its chronic hyperinsulinaemia).For example,a correlation of plasma insulin levels with TG turnover rates has not been obtained in obese human subjects, neither does the presence of an insulinoma in hypoglycaemic human patients result in an increased plasma TG turnover rate (Farquhar et al,1966). As a result of such observations,Farquhar et al (1966) proposed that both hyperglycaemia and hyperinsulinaemia(implying some degree of insulin

resistance) are pre-requisites for a marked increase in plasma TG turnover. Such a state is characteristic of obesity, which is associated with increased plasma corticosteroid levels and abnormal adrenocortical function (Cope & Black, 1959; O'Connell et al, 1973; Solomon & Mayer, 1973). Furthermore, Klausner et al (1967) have shown that corticosterone increases net TG output by the perfused rat liver, and Reaven et al (1974) showed that administration of this steroid to rats increases hepatic VLDL assembly and release. Thus a component of the actions of adrenal glucocorticoids on tissues in vivo probably involves direct effects on hepatic lipoprotein metabolism.

A further speculation in relation to steroid interactions with plasma TG metabolism implicates plasma oestrogen levels. There are indications that some obese humans (irrespective of sex) have an excessive oestriol secretion rate (Brown & Strong, 1965), and it is possible that a disturbance of oestrogen metabolism in ob/ob mice may be related to the infertility of these mice when fed ad lib. Perfused livers from female rats show twice the TG secretion rate that is found in perfused livers from male rats (Heimberg et al, 1973). Kekki & Nikkila (1971) showed a marked increase in plasma TG turnover rate, associated with both increased secretion and uptake in women receiving prolonged oestrogen treatment, in combination with progesteron as a contraceptive. This situation represents, in the human, a rare state of accelerated TG turnover not associated with proportionately increased plasma TG concentrations, and bears a resemblance to the plasma TG kinetics in ob/ob mice.

5.3. Precursors of plasma triglycerides in ob/ob mice.

Since there is abundant evidence that the major source of plasma TG is the liver (see Section 5.2.1.), the nature of circulating precursors of plasma TG in ob/ob mice may be reasonably be inferred from measurements of the incorporation of ^{14}C from (^{14}C)-labelled precursors into hepatic FA. The results suggest that both plasma FFA and FA synthesized de novo within the liver contribute to the accelerated TG secretion in ob/ob mice. This follows from the more rapid formation in ob/ob mice ofhepatic TG and PL from (^{14}C)-palmitate and (U-^{14}C)-glucose.

The rate of synthesis of FA from glucose in the liver of ob/ob mice in vivo was calculated to be 0.7 μg-atoms glucose carbon converted to FA/min/g fresh liver, or 6 μmol FA ($C_{16:0}$)/h/liver. This represents a maximal potential rate of hepatic secretion of FA synthesized from glucose into plasma. However, not all the (^{14}C)-labelled FA may have been synthesized directly from (U-^{14}C)-glucose entirely in the liver, since extrahepatic tissues metabolize glucose very rapidly in comparison to the liver, which may have led to the release of (^{14}C)-lactate into the blood, to be removed largely by the liver. In addition, some newly-formed FA may reach the liver via the blood, having been synthesized in other tissues. Finally, some newly-formed FA may not be released from the liver. Despite these reservations, even the calculated rate of synthesis is low when compared to the estimated total rate of synthesis from $^{3}H_2O$ in vivo (see Table 3.12) and the toal rate of secretion (non-recycled) of plasma TGFA in ob/ob (60 μmol TGFA/h/mouse). Furthermore, the total rate of de novo FA synthesis in all tissues in ob/ob mice used in the present experiments is likely to be of the order of 40 μmol/h on average, considering that the difference

in the rate of weight gain between ob/ob and non-obese mice is about 0.5 g/day.Half of this may be accounted for by synthesis of TG in view of their decreased total body FA turnover (Bates et al, 1955b).Hence,it appears that hepatic FA synthesis from blood glucose makes only a minor contribution (one quarter or less) to either the total rate of TGFA secretion of the liver or to total body FA synthesis.Although it is possible that hepatic FA synthesis was not investigated under the optimal conditions (e.g. the possible significance of diurnal variations in FA synthesis),this conclusion is in accord with the small contribution (about 10% at normal physiological blood glucose concentrations) of glucose carbon to FA synthesis found on perfusion experiments.

A major proportion of newly-formed TGFA which is secreted by the liver in the post-absorptive mouse appears to be formed from plasma FFA.This is suggested by the ease with which ^{14}C from (^{14}C)-palmitate was detected in plasma TG,and by the faster rate of hepatic esterification of FFA to form liver TG and PL (viz:0.39 and 0.12 umol FFA/min in ob/ob and non-obese mice respectively. The increase in hepatic esterification in ob/ob mice,compared to non-obese mice,may be interpreted as a preferential direction of hepatic FA-CoA into esterification reactions rather than into oxidative pathways.This is in accord with similar observations in liver slices (Winand et al,1968),their increased microsomal esterification activity (Howard & Lowenstein,1962) and the decreased rate of hepatic ketogenesis in the perfused mouse liver (Stein et al,1970;Elliott et al,1974).

The question arises as to likely causes for the alterations in hepatic FA synthesis and esterification dicussed above. As in the case of increased plasma TG turnover rates in ob/ob mice, both insulin and corticosterone may be involved. The potential of insulin to stimulate hepatic FA synthesis has been considered previously (see section 4.1.) and the most positive effects have been obtained in the perfused rat liver (Haft & Miller, 1956, 1958; Haft, 1967), but in vivo the effects have not been conclusive. This aspect requires further clarification. However, insulin increases the rate of hepatic esterification of oleate in perfusion experiments (Topping & Mayes, 1972). Corticosterone also exerts effects on the activity of microsomal esterification enzymes when rats were treated with the steroid (Hayes & Hill, 1965).

5.4. The control of fatty acid synthesis by substrate supply in the perfused mouse liver.

The perfused mouse liver proved to be eminently suitable for the further investigation of several problems arising from work discussed in previous sections. Experiments carried out *in vivo* showed that the major precursors of plasma TG in *ob/ob* mice are hepatic FA synthesis and plasma FFA. The control of the proportional contribution of these two precursors has been the subject of a number of investigations. Topping & Mayes (1972) demonstrated that the addition of insulin and fructose to the medium perfusing the rat liver decreased the contribution of medium FFA to to VLDL-TG secretion, although the net rate of the latter increased. Barter & Nestel (1973,1974) observed a similar phenomenon in humans after a glucose infusion, which was more marked in obese subjects. However, both these authors were unable to unequivocally ascribe an increased contribution of hepatic FA synthesis to TG secretion owing to the absence of a method for the quantitative measurement of total rates of FA synthesis. This was overcome by Windmueller & Spaeth (1966,1967) by the use of ^3H_2O (see section 4.2.1.), who reported a correlation between the total rate of FA synthesis and the net FA secretion by the perfused rat liver. Similar experiments were carried out in mice, both in the light of the above discussion and the evidence implicating the livers of *ob/ob* mice as a major site of increased FA synthesis.

A further point was raised from the results of the experiments *in vivo*, which had the implication that blood glucose carbon may be only a minor precursor of newly-synthesized FA in the liver. Further investigation

of this phenomenon in the normal animal was carried out before attempting to elucidate the situation in the ob/ob mouse. Although the results of some perfusions of ob/ob mice are reported, most of the perfusions in the present work were carried out using non-obese mouse livers, and yielded results which have interesting implications concerning hepatic FA synthesis in obesity. These experiments are an initial step in describing precursor contributions to FA synthesis and the control of the total rate of this process by substrate supply under the well-defined conditions permitted by the use of liver perfusion.

5.4.1. Suitability of the perfused mouse liver preparation for investigation of fatty acid synthesis.

The rates of FA synthesis in the non-obese mouse liver perfused in the present conditions resemble those in the liver of similar mice measured *in vivo* using $^{3}H_2O$ (1-4 μmol/h/g wet liver: Hems *et al*, 1974, unpublished data), in the rat liver *in vivo* (Lowenstein, 1971) and in the perfused rat liver (Windmueller & Spaeth, 1966, 1967; Brunengraber *et al*, 1972, 1973; Mayes & Topping, 1974). They are about ten times faster than those which can be obtained in other liver preparations (e.g. slices or isolated parenchymal cells: see Nilsson *et al*, 1973), presumably as a result of the greater functional viability of the perfused preparation.

In the reports cited above, FA synthesis was measured by the incorporation of ^{3}H from $^{3}H_2O$ into FA. The rates obtained are higher than in most experiments with (^{14}C)-labelled substrates. The question arises of the validity of measurements made with $^{3}H_2O$. This has been discussed in Section 4.2.1., but further credibility was shown by results of other experiments. ^{3}H incorporation into polyunsaturated FA accounted for

less than 15% of that in (^{3}H)-labelled FA(Foster & Bloom,1963;present work),indicating that ^{3}H in FA was largely in those FA which are the products of avidin-sensitive FA biosynthesis,which is chiefly located in the high-speed supernatant fraction (Harlan & Wakil,1963).In addition, rates of FA synthesis *in vivo* show expected alterations in response to changes in hormonal or nutritional states of the animal (Fain *et al*,1962; Fain& Scow,1965;Lowenstein,1971) or administration of inhibitors of enzymes associated with *de novo* FA synthesis (e.g. (-)-hydroxycitrate: Lowenstein,1971;c-AMP: Allred & Roerig,1973).Particular validation of the use of $^{3}H_2O$ in the present work is derived from the fact that in liver, as in adipose tissue (Jungas,1968),a substantial proportion of the carbon of newly-synthesized FA can be identified from experiments with (^{14}C)-labelled precursors (up to 60%,if the (U-^{14}C)-lactate and (U-^{14}C)-glucose data are taken as being additive).McGarry & Foster (1971) also found similar rates of FA synthesis in the perfused rat liver to those which have been found using $^{3}H_2O$ by dominating the hepatic acetyl-CoA pool(s) with (^{14}C)-acetyl-CoA derived from (1-^{14}C)-octanoate.

The fastest rates of FA synthesis in the present experiments, expressed as umol of acetate consumed,were about 1.0μmol/min/g wet liver.This is of the same order as the rate of degradation of acetate in the tricarboxylic acid cycle (see also Brunengraber *et al*,1973;McGarry & Foster, 1971).Hence the process of FA synthesis can represent a major fate of acetyl-CoA in the liver,at least in rodents ingesting a carbohydrate-based,low-fat diet.In contrast,hepatic cholesterol synthesis is not fast enough to be of significance in the control of rapid metabolic events; this was reflected in its lack of dependence on the circulating glucose concentration.

The perfused liver releases a proportion of newly-synthesized lipid to blood, 95% of which is as TG and the rest as PL and cholesterol. (Windmueller & Spaeth, 1966). This process must be considered in assessing the functional state of the perfused liver preparation. A comparison of data obtained from perfusion experiments with estimations of the rate of this process *in vivo* must take into account the many presumptions made in such calculations. However, in both the present *in vivo* experiments, and those of Haft (1973), only about 2% of secreted TG was estimated to be derived from newly-synthesized FA (representing a rate of about 0.04 umol TGFA/ h/g wet liver derived from (U-^{14}C)-glucose. This was similar to the rate found during perfusions. Furthermore, only 20% of newly-synthesized FA was secreted by the mouse liver during 3h of perfusion, indicating that, as *in vivo*, synthesized FA is either utilized mainly within the liver or is subject to a delay of several hours before it is secreted. This would allow sufficient time for elongation and desaturation to occur, such that the FA composition of newly synthesized FA might more nearly resemble that of plasma TG. However, there is no evidence to suggest any impairment of the secretory process in the present preparation.

The above considerations suggest that the charateristics of FA synthesis observed in the liver perfused in the present conditions may reasonably be presumed to obtain in the liver of mice.

5.4.2. Utilization of glucose carbon in fatty acid synthesis.

The present experiments with (U-^{14}C)-glucose suggest that glucose, in the concentration range usually present in blood (4-15mM), is not a significant carbon source for FA (or cholesterol) synthesized *de novo*

in the liver.However,glucose at higher concentrations contributes more significantly to FA synthesis,and also increases the absolute rate by more than could be accounted for in terms of increased glucose carbon availability.These observations were probably a reflection of the extent of glucose entry into the liver.This appears to be a finely controlled process,dependant on mechanisms which provide homeostasis of blood glucose concentrations by the liver,a major function of this organ *in vivo*. From experiments using the dog liver *in situ*,Soskin *et al* (1938) first described the capacity of the liver to maintain the glucose concentration of the perfusing blood within a narrow concentration range.Cahill *et al*, (1959) confirmed this observation and proposed that rapid alteration in glucose output or uptake in response to the circulating glucose concentration was mediated by a hormone-sensitive glucostatic system, which may be controlled by the relative activities of glucokinase and glucose-6-phosphatase.In experiments with the isolated,perfused liver,the equilibration glucose concentration is inversely proportional to the medium erythrocyte content (Burton & Ishida,1965;Glinsmann *et al*, 1969),and resembles blood levels *in vivo* between 18-36% haematocrit.The endogenous control of hepatic glycogen content also has a role in the direction of the glucose flux across the hepatocyte membrane,since this operates parallel to the above glucostatic system.Glycogenolysis occurs occurs below perfusate glucose concentrations of 15-20mM (Glinsmann *et al*, 1969;present work) and only above this concentration range does net glycogen deposition occur.This is brought about by the regulation of glycogen synthesizing and degrading enzymes by glucose,both in perfusion (Glinsmann *et al*,1970;Buschiazzo *et al*,1970) and in the intact mouse (deWulf & Hers,1968).Thus rapid glucose uptake by the liver,perfused under conditions in which these mechanisms survive,is only allowed at glucose concentrations

above 20mM;this coincided with a rapid increase in both the total rate of FA synthesis,and its rate from glucose, at glucose concentrations between 20 and 30mM in the present work.

The total rate of de novo FA synthesis in the perfused mouse liver was influenced by the perfusate glucose concentration to a greater extent than can be explained by its carbon contribution to FA synthesis both in the presence and absence of added lactate.In the latter conditions,net glucose output occurred in association with high rates of FA synthesis,and yet glucose was required to achieve these rates (as shown by the lower rates at lower glucose concentrations).This effect of glucose resembles that on glycogen synthesis in the perfused liver of starved rats, in which glucose was again not the sole net carbon source of the synthetic product (Hems et al,1972).Glucose appears to exert a regulatory effect on the biosynthesis of glycogen (see Hers et al,1970), which may now be said to affect FA synthesis.

5.4.3. Role of lactate in FA synthesis.

In contrast to glucose, lactate can serve as a major carbon source of FA in the perfused liver, irrespective of glucose concentration.This has recently been confirmed by Clarke et al (1974),using an isolated rat hepatocyte preparation,although a detailed description of the dependence of FA synthesis from lactate carbon on the medium glucose concentration was not reported by these authors.The highest rates of FA synthesis in the perfused mouse liver (7 μmol/h/g wet liver) were obtained in the presence of intermediate glucose concentrations and 10mM lactate.In this situation, lactate contributed up to 50% of the carbon utilized in FA synthesis.The general implication of this observation is that circulating precursors which can rapidly form pyruvate may, under some circumstances

be major precursors of FA synthesized in the liver *in vivo*.At times when there are intermediate blood glucose concentrations in the hepatic portal vein (*e.g.* after starch containing meals),blood glucose is assimilated by muscle, and partly released as lactic acid (see review by Exton,1971). Part of this excess circulating C_3-material may be converted to FA in the liver,although its major fate appears to be glcogen, glucose and CO_2 (Exton *et al*,1972).A similar situation could obtain after excercise, when blood lactate levels are high.In other states such precursors probably contribute rather less carbon to FA synthesis since their concentrations in blood are relatively low (1mM or less).This inference is supported by experiments with trace injections of (U-^{14}C)-labelled lactate or alanine in mice (Elliott *et al*, 1974).

Although lactate (1-2mM) was present during perfusions in which glucose alone was added,the addition of lactate (10mM) produced a several fold acceleration of the rate of FA synthesis when livers were perfused in the presence of 12-17mM glucose which was not entirely due to the provision of carbon.Lactate appears to control FA synthesis through substrate supply, in the range 1-10mM, as is true for gluconeogenesis (Exton & Park,1967)and antiketogenesis (McGarry *et al*,1973).This effect of lactate on FA synthesis is similar to that described by McGarry & Foster (1971), who showed that the addition of lactate redirected ^{14}C from (1-^{14}C)-octanoate from ketogenesis to long-chain FA in perfused livers of both fed and starved rats.The enhancement of the rate of FA synthesis under conditions when lactate (10mM) and glucose (12-17mM) were present in the perfusion medium could be due to an unknown direct effect of blood glucose on FA synthesis (which would be solely catalytic,since net hepatic glucose output occurred under these conditions) or to an indirect effect of blood glucose concentrations

on hepatic lactate utilization.Whilst there is little direct evidence to support the former suggestion, the existence of the latter effect would be in accordance with the following considerations.In view of the reversibility of the lactate dehydrogenase reaction in the liver, net lactate uptake is presumably determined by the difference between rates of uptake and output.Glinsmann et al (1969) and Woods & Krebs (1971) have discussed the control of hepatic lactate output by the liver of fed rats by the perfusate glucose concentration, in the absence of added lactate.Glycogenolysis is rapid at low glucose concentrations and results in lactate output.As the perfusate glucose concentration is increased, net glycogenolysis is reduced until it ceases at 15-20mM glucose.This coincides with low rates of lactate output.At higher glucose concentrations, rapid rates of glucose output result from glycolsis of medium glucose.This suggests that the fastest rates of lactate uptake should occur at glucose concentrations in the range 10-20mM when lactate is added to the medium.In the present work, this was the only glucose concentration range in which lactate uptake was significantly detected.

Uptake of lactate may be an important factor in controlling hepatic FA synthesis in ob/ob mice.There is more rapid accumulation of (^{14}C)-FA in livers of ob/ob mice after injection of (^{14}C)-lactate in vivo (Shreeve et al,1967; Elliott et al,1974).Although the estimated rates of FA synthesis from lactate were relatively low in these experiments, this process may be more rapid shortly after ingestion of a carbohydrate-based meal.It is noteworthy that Clarke et al (1974) have shown that about five times as much of the lactate utilized by hepatocytes from meal fed rats is directed into FA synthesis (the total

rate of which is also increased to a similar extent) compared to rats fed ad lib. In this context, it is relevant that higher activities of lactate dehydrogenase isoenzyme 5 have been found in ob/ob mice, relative to non-obese mice, in skeletal muscle, adipose tissue and pancreas (Prochazka et al, 1970). Ob/ob mice also have higher total activities of this enzyme in the liver (Seidman et al, 1970).

5.4.4. Role of glycogen in the synthesis de novo of fatty acids in the liver.

The use of circulating (^{14}C)-labelled precursors during perfusion identified the carbon source of up to 50% of the FA which was synthesized at low glucose concentrations (4-17mM). The remainder of the carbon was probably derived from an endogenous source, such as glycogen, protein or lipid. Of these sources, glycogen is the most likely as it is known to exhibit rapid turnover in relation to metabolic events.

The possibility that glycogen contributes extensively to the carbon requirement for lipid synthesis is not open to direct testing as glycogen cannot be exclusively pre-labelled with ^{14}C (e.g. prior to liver perfusion). However, circumstantial evidence supports the above suggestion that glycogen can provide significant carbon for hepatic FA synthesis. It has long been known that glycogen accumulation tends to be associated with rapid rates of FA synthesis (e.g. in liver slices prepared from cold stressed rats, Masoro et al, 1950; and in rats refed after a period of starvation, Tepperman & Tepperman, 1958). This generalization was refined in the present experiments by the demonstration that FA synthesis is proportional to the initial glycogen content. Glycogen can provide substantial carbon to the glycolytic pathway in aerobic conditions, especially at low glucose concentrations when lactate and glucose output occur simultaneously (Glinsmann et al, 1969; Woods & Krebs, 1971). Presumably glycogen is then a major source of carbon dioxide since glycogen breakdown may not be totally accounted for as lactate or glucose. Thus Glinsmann et al (1969) calculated a deficit (between glycogen breakdown, and glucose and lactate release) of about 32μmol glycogen-glucose/g liver/h when no

glucose was added initially to the medium.If glycogen can provide acetyl CoA for oxidadtion, it is reasonable to suggest that it is also a potential source of acetyl CoA for FA synthesis (as discussed by Woods & Krebs, 1971).The extent of glycogen breakdown minus glucose and lactate production, in the present experiments, at glucose concentrations less than 15mM, was sufficient to account for FA synthesis.

In the intact animal, when the blood glucose concentration is in the normal range (4-15mM), glycogen is likely to be carbon source of FA synthesized de novo in the liver.This follows because a major role for glucose is precluded by the present results,(as discussed previously), and because other precursors in blood are not present in sufficient amounts (vide supra).In post-absorptive or late post-prandial conditions such a role for glycogen, in providing acetyl residues for lipogenesis would permit simultaneous release of glucose from the liver (i.e.zero contribution of glucose as a net carbon source of fat).Such a conjunction of hepatic glucose release (see Elliott et al, 1971) and FA synthesis, which may be quite common in vivo, is exaggerated in ob/ob mice.One role of the increased hepatic glycogen turnover in ob/ob mice (Shull & Mayer, 1959) may be in supplying carbon for FA synthesis in the post-absorptive condition.

At higher glucose concentrations (15mM or more), the breakdown of hepatic glycogen is suppressed (Glinsmann et al, 1969).This was confirmed in the present experiments.In such a situation, glycogen would be less likely to contribute significant net carbon for FA synthesis.If glycogen is the major carbon source of FA synthesized in

post-absorptive animals, this raises the question of which circulating precursors form glycogen (and subsequently, FA) or serve as precursors of FA during net glycogen accumulation. It is probable that both gluconeogenic precursors (see Hems et al, 1972) and glucose are implicated, depending on the state of the animal.

Glycogen appears to exert a controlling influence on FA synthesis, apart from being a major carbon source. This follows because in perfusions with high initial glycogen levels, the extent of conversion of (^{14}C)-glucose to FA was increased, as well as the total rate of synthesis. A similar result was obtained in liver slices, with (^{14}C)-acetate (Hauggard & Stadie, 1949). This effect of glycogen could have a variety of origins and its mechanism remains to be elucidated.

5.5. Roles of the liver in the altered lipid metabolism in ob/ob mice.

In the preceding sections, several changes in hepatic lipid metabolism in ob/ob mice have been discussed.These are summarized in Table 5.1.It was established that these mice have an increased plasma TG turnover, and under appropriate conditions of substrate supply FA synthesis could contribute much of the additional FA required for this process.However, the turnover of plasma FFA and the rate of hepatic esterification of plasma FFA is also faster.It was also found that there is an increased desaturation and elongation of liver esterified FA, both relative to corresponding lipid classes in non-obese mice, and to their major precursor in the post-absorptive animal, plasma FFA. Several interpretations are possible for these data concerning the relationship of liver to adipose tissue with respect to FA metabolism in obesity.

In the light of the above observations, it is appropriate to review the possible role of the liver as a site of increased FA synthesis de novo in ob/ob mice.Studies on adipose tissue of the animals using isotopically-labelled precursors have all failed to find an increased accumulation of isotope in adipose FA.Such experiments do not conclusively indicate low rates of FA synthesis in this tissue, since in vivo newly-synthesized FA could be rapidly mobilized as plasma FFA, or in vitro conditions may not accurately reflect those in vivo. Rapid mobilization of newly-synthesized FA from adipose tissue could occur in order that they may be transported to the liver and desaturated whence they could be returned to adipose tissue as esterified FA in VLDL.It is pertinent that significant desaturase activity has not been

Table 5.1. Estimated rates of some major processes involved in fatty acid metabolism in liver and plasma in mice.

The data refer to female mice, aged 12 weeks and fed ad lib. The turnover rate of plasma FFA is taken from data of Elliott et al (1974), and is derived from the means obtained from two types of experiments in which (U-^{14}C)-palmitate was either injected as a single trace dose or continuously infused into the tail vein of mice. Total rates of hepatic FA synthesis were measured using $^{3}H_2O$, as described in Table 3.12. The rate of hepatic esterification of plasma FFA was calculated from the incorporation of ^{14}C from intravenously injected (1-^{14}C)-palmitate-albumin complex 5 min after injection (see Section 3.3.1.). The turnover rate of plasma TG was calculated from the results of two types of experiments, in which plasma TG were either endogenously labelled (see Section 3.3.2.) or injected as VLDL-TG, pre-labelled with (U-^{14}C)-palmitate prepared from donor mice (see Section 3.3.3.). The values are those estimated for conscious mice, and the method of calculation is described in the relevant section or reference in each case.

Process	Estimated rate of process (μmol FA/min/mouse)		Ratio
	ob/ob(A)	non-obese(B)	(A/B)
Turnover of plasma FFA	1.8	0.9	2.0
Turnover of plasma TG	1.0	0.24	4.0
Esterification of plasma FFA by liver	0.39	0.12	3.3
Total hepatic FA synthesis	0.22	0.08	2.6

observed in adipose tissue of mice or rats (Hollenberg, 1966; Winand et al, 1971,1974; Wahle, 1974) or of genetically-obese (New Zealand) mice (Winand et al, 1968a).Thus selective mobilization of the products of de novo FA synthesis in adipose tissue would be required in order to produce the FA profile characteristic of adipose TGFA.Several observations support this hypothesis.At least two pools of TGFA exist in adipose tissue, one of which is small and has a rapid turnover rate (Kerpel et al, 1961; Stein & Stein, 1962; Angel & Farkas, 1970).In addition, plasma FFA contain a high proportion of saturated FA (Miller et al, 1962; Redding & Schally, 1967; present work).Such selectivity in the release of the products of lipolysis is not, however, found in experiments using isolated adipose tissue (Haessler & Crawford, 1965; Winand et al, 1968).Alternative support may be sought from the occurrence of an increased FFA turnover in the plasma of ob/ob mice (Elliott et al, 1974).It is possible that this represents an increased flux of relatively saturated FA from adipose tissue to the liver.However, this is unlikely in view of the hyperinsulinaemia of ob/ob mice, which probably reduces the activity of hormone-sensitive lipase in adipose tissue (reviewed by Froesch, 1967).It is known that enlarged adipocytes in both ob/ob mice and old rats have increased activity of the high K_m phosphodiesterase enzyme (Kaplan et al, 1973; De Sanitas et al,1974) which may account for the lack of sensitivity of isolated adipose tissue from ob/ob mice to agents which normally stimulate lipolysis through increased levels of adipocyte c-AMP(Lovell-Smith & Sneyd,1973).

In order for the preferential release of newly-synthesized FA from fat to be of significance with respect to the increased FA accretion in adipose tissue of ob/ob mice, it is necessary to demonstrate an

increased capacity for FA synthesis in this tissue.Among enzymes thought to be rate-controlling in FA synthesis, ATP-citrate lyase was found to have a decreased activity (u/mg high speed supernatant protein) in [redacted] adipose tissue of ob/ob mice in the present work.The activity of this enzyme increases during long-term changes in FA synthetic capacity in order to facilitate an increased supply of cytoplasmic acetyl-CoA (see Section 1.4).In contrast, Martin et al (1973) found a small increase in the activity of malate dehydrogenase in ob/ob adipose tissue (relative to non-obese mice), which is known to show large changes in activity parallel with that of ATP-citrate lyase (Ball, 1966; Shrago et al, 1969; McLean et al, 1972).It is notable that the changes in the activities of these enzymes are consistently greater in the livers of ob/ob mice (present work; Spencer & Lowenstein, 1966; Martin et al, 1973; Kaplan & Fried, 1973) than in adipose tissue.Thus it does not seem likely at present that adipose tissue from ob/ob mice (aged 3 months) possesses an increased capacity for FA synthesis.

In conclusion, it is suggested that much of the TGFA accumulated by ob/ob mice originates in the liver.A number of processes appear to contribute to this altered, hepatic function: de novo synthesis of FA, elongation and desaturation of LCFA, LCFA esterification and secretion of VLDL-TG.The rates of all these processes are increased in ob/ob mice.Conditions found to be conducive to enhanced rates of de novo synthesis of FA in non-obese mice in liver perfusion experiments may also pertain in the livers of ob/ob mice.Increased availability of glycogen and lactate increasesthe rate of FA synthesis by more than may be solely accounted for by increased carbon supply, and it is likely that the supply of these substrates is a major factor limiting

the synthesis of FA over short periods in the liver of the fed animal. It is relevant that both the turnover of glycogen and the capacity of uptake of lactate are increased in the livers of ob/ob mice. The present experiments suggest that the hyperglycaemia (which is probably maintained by hepatic glucose output) frequently found in ob/ob mice also exerts significant control over the rate of FA synthesis by a mechanism unrelated to the role of glucose as a carbon precursor of FA.

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