EFFECTS OF FRUCTOSE ON LIPID METABOLISM IN HEPATOCYTES

A Thesis submitted by

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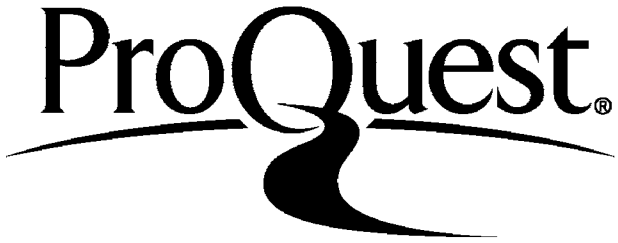

DEDICATION

To my parents.

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ABSTRACT

Dietary carbohydrate, especially fructose, causes hypertriglyceridaemia in humans and laboratory animals. The potency of fructose in this respect is attributed to the ease of its conversion in the liver to the precursors of triacylglycerol, i.e. sn-glycerol 3-phosphate and fatty acids. This thesis investigates the short-term effects of fructose on lipid metabolism in isolated hepatocytes.

Incorporation of D-[U-^{14}C] fructose into triacylglycerol was much greater than from D-[U-^{14}C] glucose. The majority of the radioactivity was associated with the glycerol moiety in each case, although there was a significant incorporation into fatty acids with fructose The extensive incorporation of D-[U-^{14}C] fructose into the glycerol moiety is the result of the ease of its conversion to triose phosphate and of the increase in sn-glycerol 3-phosphate content of the hepatocytes incubated with fructose. The latter effect occurs without a change in the cytoplasmic NADH/NAD$^+$ ratio. Thus the incorporation of D - [U-^{14}C] fructose into triacylglycerol is a good measure of triacylglycerol synthesis; it does not suffer from the disadvantage of the change in cytosolic redox potential observed with glycerol.

Increasing fructose concentration caused a stimulation of lipid synthesis from endogenous acyl CoA, an effect also observed when exogenous oleate was added to the hepatocyte which resulted in a much higher level of triacylglycerol synthesis. Oleate also stimulated glucose incorporation into total lipid but, in contrast to fructose, more phospholipid than triacylglycerol synthesis occurred. This suggested a specific stimulatory effect on triacylglycerol synthesis which was confirmed using [1-^{14}C] oleate as a precursor. The stimulation was attributed to the effect of the ketose on the cytoplasmic sn-glycerol 3-phosphate content of the cells. Glucagon was found to inhibit the fructose plus oleate stimulation of triacylglycerol synthesis.

Physiological concentrations of fructose also caused increased lipogenesis from L - [U-^{14}C] lactate and [1-^{14}C] acetate, but fatty acid synthesis was substantially inhibited at concentrations of the ketose above 2mM.

It is concluded that the hypertriglyceridaemic effect of fructose is the result of the specific stimulation of hepatic triacylglycerol synthesis and lipogenesis.

CONTENTS

ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
ACC	Acetyl CoA carboxylase
AMP	Adenosine 5'-monophosphate
BC	Biotin carboxylase
BCCP	Biotin carboxy carrier protein
Bt_2cAMP	Dibutyrylcyclic AMP
CAT I and II	Carnitine acyltransferase I and II
CoA	Coenzyme A
Cyclic AMP	Adenosine 3':5-phosphate
DHA	Dihydroxyacetone
DHAP	Dihydroxyacetone phosphate
DGAT	Diacylglycerol acyltransferase
ESR	External standard ratio
FAS	Fatty acid synthetase
FABP	Fatty acid binding protein
FFA	Free fatty acid
PFK I	Phosphofructokinase I
PFK II	Phosphofructokinase II
Fru 1,6-P_2	Fructose 1,6-bisphosphate
Fru 2,6-P_2	Fructose 2,6-bisphosphate
GPAT	sn-Glycerol 3-phosphate acyl transferase
GTP	Guanosine 5'-triphosphate
IMP	Inosine 5'-monophosphate
KRB	Krebs-Ringer bicarbonate
NAD^+	Oxidized β-nicotinamide-adenine dinucleotide
NADH	Reduced β-nicotinamide-adenine dinucleotide
$NADP^+$	Oxidized β-nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced β-nicotinamide-adenine dinucleotide phosphate
pO_2	Partial pressure of oxygen
PPH	Phosphatidate phosphohydrolase
SD	Standard deviation
SEM	Standard error of mean
TLC	Thin-layer chromatography
Tris	2-Amino-2-hydroxymethyl propane 1,3-diol
VLDL	Very low density lipoprotein

INTRODUCTION

There is a considerable body of literature which suggests that high carbohydrate diets cause hypertriglyceridaemia in humans (Macdonald and Braithwaite, 1964; Macdonald, 1966) and in laboratory animals (Nikkilä and Ojala, 1965; Macdonald, 1973). Sucrose feeding is more hypertriglyceridaemic than glucose or starch and this is thought to be due to the fructose moiety of the sugar (Macdonald, 1973; Macdonald, 1975). Sucrose and fructose are also thought to be lipogenic in the long term. They induce the synthesis of hepatic fatty acid synthetase (Bruckdorfer et al., 1971; Bruckdorfer et al., 1972a) and acetyl CoA carboxylase (Cohen et al., 1972) to a greater extent than glucose or starch.

This thesis is concerned with the short-term effects of the components of sucrose, i.e. glucose and fructose, on hepatic triacylglycerol and fatty acid synthesis. An attempt is made to show that, in addition to the long-term effects involving enzyme induction, fructose also has short-term effects on metabolism which can result in increased fatty acid and triacylglycerol synthesis by hepatocytes.

I. METABOLISM OF FRUCTOSE IN LIVER

A. Fructose uptake by the liver

In most animal species the liver is the principal site of D-fructose metabolism. Dietary fructose is transported to the liver from the small intestine via the hepatic portal vein. The concentration of the ketose in portal blood reaches a maximum of 2.2mM when rats are given a large fructose meal by intubation (Topping and Mayes, 1971). Under these conditions the concentration in the peripheral circulation reaches only 0.3mM and very little fructose is metabolised in extrahepatic tissues.

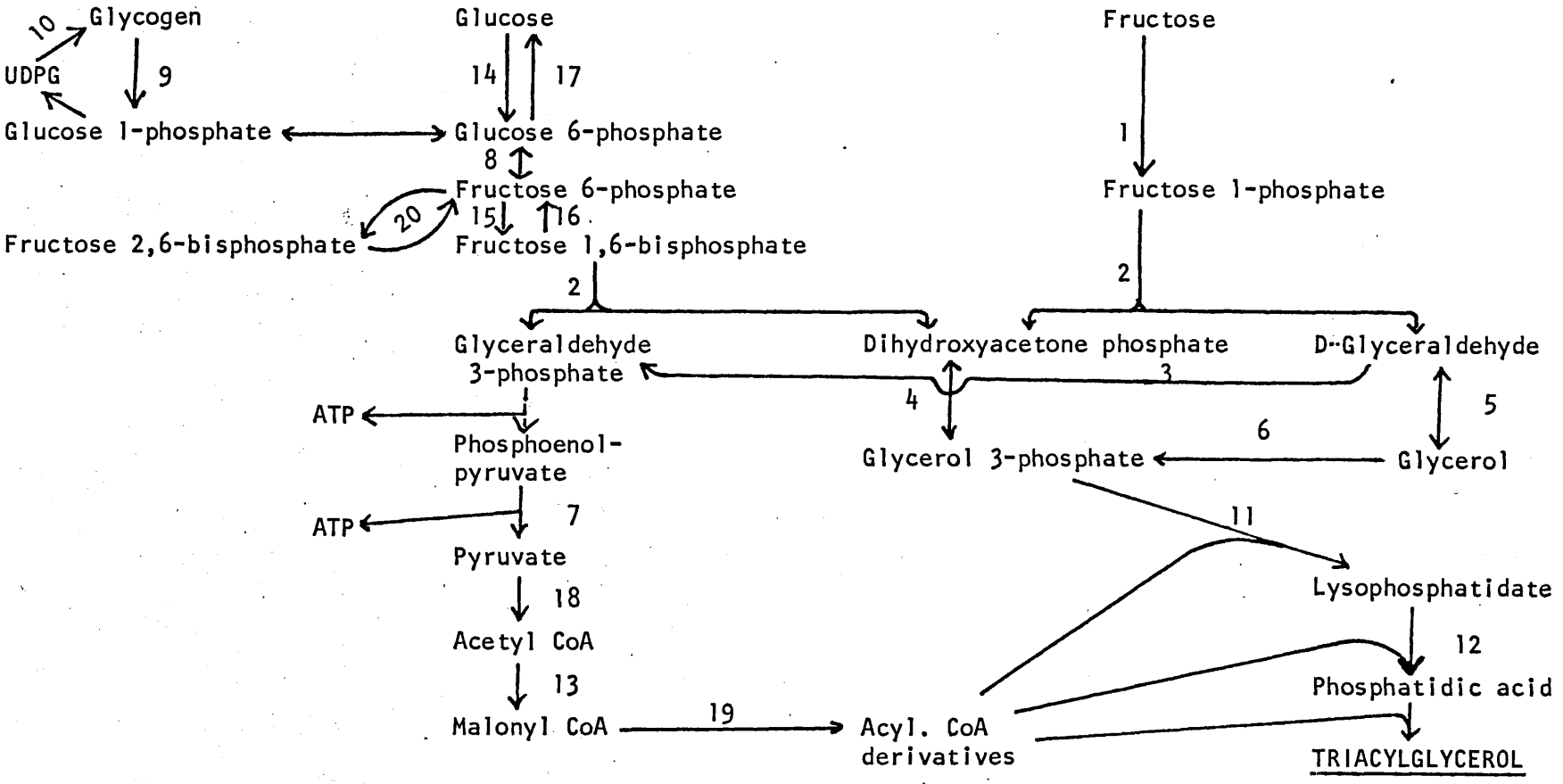
It has been reported that fructose does not enter the liver cells freely and that a carrier mechanism is involved, despite the existence of a steep gradient between the extra- and intra-cellular concentrations of the sugar (Woods et al., 1970; Sestoft and Fleron, 1974). The latter authors have found that the elimination of fructose from blood is a function of its concentration and follows Michaelis Menten kinetics. The Km of this system is 67mM and V_{max} is 30 µmole/min/g. Although Baur and Heldt (1977) were unable to show

that fructose transport is a saturable process, they found competition between the ketose and D-glucose or D-galactose for the transport system. Inhibition of transport of all three sugars by cytochalasin B, phloretin and phlorizin suggest that there is carrier mediated transport across the plasma membrane. Craik and Elliott (1980) have estimated a Km of 212mM for fructose for the transport system. The use of isolated hepatocytes has provided evidence for a common carrier mechanism for the hexoses.

The Km and V_{max} for fructokinase are reported to be 1mM and 10.3 µmol/min/g respectively (Sestoft et al., 1972; Sestoft, 1974a; Sestoft and Fleron, 1974). Thus the affinity of fructose for its carrier system is lower than the affinity of the ketose for fructokinase. It is therefore concluded that a carrier-mediated transport of fructose limits its metabolism under physiological conditions. Thus, although fructose is taken up more slowly than glucose by isolated hepatocytes, fructose is immediately metabolised within the cell (Heldt et al., 1974; Baur and Heldt, 1976). Therefore, unlike the uptake of glucose, the uptake of fructose does not come to a stop in isolated liver cells (Baur and Heldt, 1976). These authors have also shown that, if the phosphorylation of fructose within the cell is abolished by addition of an uncoupler, fructose uptake only proceeds until the external fructose concentration has been reached within the cells.

The metabolism of fructose by the liver is known to be much more rapid than that of glucose (Zakim et al., 1969; Macdonald, 1971; Walker et al., 1972). The major known differences in fructose and glucose metabolism occur in the initial phosphorylation and in the aldolase-catalysed cleavage of the phosphorylated hexoses (Cahill et al., 1958). Fructose is able to by-pass several enzymatic steps which are required for the metabolism of glucose. Thus, metabolism of fructose to triose phosphate does not involve any key regulatory enzymes. For this reason fructose is regarded as a much better precursor of both hepatic fatty acids and glyceride-glycerol (Zakim, 1973).

The major metabolic pathways for the conversion of glucose and fructose to triacylglycerol are summarised in Fig. 1.



1. Ketohexokinase
2. Fructose 1-phosphate and Fructose 1,6-bisphosphate aldolase
3. Triose kinase
4. Glycerol 3-phosphate dehydrogenase
5. Glycerol dehydrogenases (NAD or NADP)
6. Glycerol kinase
7. Pyruvate kinase
8. Phosphohexoisomerase
9. Phosphorylase a
10. Glycogen synthase
11. sn-glycerol 3-phosphate-O-acyltransferase
12. Acylglycerol phosphate acyltransferase
13. Acetyl CoA carboxylase
14. Hexokinase and glucokinase
15. Phosphofructokinase 1
16. Fructose bisphosphatase
17. Glucose 6-phosphatase
18. See Fig. 5
19. Fatty acid synthetase
20. Phosphofructokinase 2/ Fructose 2,6-bisphosphatase

Fig. 1 Metabolic pathways for the conversion of hexoses to triacylglycerol by the liver

The initial step in fructose metabolism by the liver is a phosphorylation by ketohexokinase yielding fructose 1-phosphate (Cori et al., 1951; Hers, 1952a). Cleavage of fructose 1-phosphate by liver fructose bisphosphate aldolase yields D-glyceraldehyde and dihydroxyacetone phosphate. The dihydroxyacetone phosphate may be converted to lactate by glycolytic enzymes, to glucose and glycogen by gluconeogenic enzymes or may be converted to sn-glycerol 3-phosphate and used for the synthesis of triacylglycerol. There are three possible enzymes which may be involved in the metabolism of D-glyceraldehyde; triokinase which phosphorylates D-glyceraldehyde to D-glyceraldehyde 3-phosphate, an NAD^+- specific aldehyde dehydrogenase that produces D-glyceric acid which can be further phosphorylated to 2-phosphoglycerate by glycerate kinase and NAD^+- and $NADP^+$- dependent alcohol dehydrogenases which can both reduce D-glyceraldehyde to glycerol which, in turn, can be phosphorylated to sn-glycerol 3-phosphate by glycerol kinase. The former route is thought to operate in vivo. Thus the metabolism of fructose by-passes the key regulatory enzymes of glucose metabolism, glucokinase and phosphofructokinase I (Heinz, 1973). The only regulatory enzyme which is common to the glycolysis of glucose and fructose is pyruvate kinase.

B. Key enzymes of fructose metabolism

1. Ketohexokinase

D-fructose is mainly phosphorylated to fructose 1-phosphate by an ATP-dependent ketohexokinase (ATP: D-fructose 1-phosphotransferase; EC 2.7.1.3) (Cori et al., 1951; Heinz, 1973). This enzyme is found in all mammalian livers (Heinz et al., 1968; Heinz and Weiner, 1969). Hepatic ketohexokinases have been purified by a number of investigators (Vestling et al., 1950; Parks et al., 1957; Adelman et al., 1967; Sánchez et al., 1971a). Purified rat liver ketohexokinase can be distinguished from the other hexokinase by an absolute requirement for K^+ ions (Sánchez et al., 1971b). The enzyme has a Km of 0.46mM for fructose and 1.56mM for the ATP-Mg^{2+} complex at a K^+ concentration of 0.4M (Sánchez et al., 1971a). The enzyme is strongly inhibited by ADP, one of its reaction products. The inhibition is non-competitive towards fructose and competitive with respect to the ATP-Mg^{2+} complex and is partially reversed by K^+ ions, (Sánchez et al., 1971b).

It has been reported that ketohexokinase is inhibited by fructose 1-phosphate (Froesch, 1959) but this inhibition was not observed by Parks et al. (1957). Ketohexokinase is not specific for D-fructose, it also catalyses the phosphorylation of other ketose such as L-sorbose, L-galactoheptulose, D-xylulose and D-tagatose and can therefore be regarded as a ketohexokinase. The affinity of ketohexokinase for these sugars also depends on the K^+ concentration (Adelman et al., 1967; Sanchez et al., 1971a).

Ketohexokinase activity differs in various species. In rat liver the activity of enzyme is 2-2.5 µmoles/min/g (Heinz et al., 1968) which equates well with the rates (3.0 µmole fructose/min/g tissue) of extraction of fructose from the medium during liver perfusion (Sestoft, 1974a). It has been shown that feeding rats on a diet enriched with fructose (65%) for 3 weeks causes an increase in hepatic ketohexokinase activity (Heinz, 1968).

2. Fructose 1-phosphate aldolase (E.C.4.1.2.13.).

The major fate of the fructose 1-phosphate is an aldolase-catalysed cleavage resulting in the formation of dihydroxyacetone phosphate and D-glyceraldehyde (Hers and Kusaka, 1953; Leuthardt et al., 1953). The same enzyme also catalyses the conversion of fructose 1,6-bisphosphate into D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, as well as the condensation of the triose phosphates to yield fructose 1,6-bisphosphate.

There are three isoenzymes of mammalian aldolase. Type A enzyme found in muscle, type B in the liver and type C in the brain (Rutter, 1964). Aldolase B also occurs in the intestine (Kawachi et al., 1973). Isoenzymes A and C catalyse mainly the cleavage of fructose 1,6-bisphosphate, but aldolase B utilises both fructose 1-phosphate and fructose 1,6-bisphosphate as substrates (Hers and Kusaka, 1953; Rutter et al., 1968). The ratios of fructose 1,6-bisphosphate: fructose 1-phosphate utilisation for type A, B and C are 50, 1 and 10 respectively (Penhoet et al., 1966). The hepatic enzyme is cytoplasmic (Hatzfeld et al., 1976). In the adult rat and rabbit livers both A and

B isoenzymes are found, but the latter is predominant. In foetal liver all isoenzymes are present but isoenzyme B becomes predominant just before birth (Rutter et al., 1968).

The Km values for fructose 1-phosphate and fructose 1,6-bisphosphate are 9 x 10^{-4} M and 1 x 10^{-5} M respectively (Heinz, 1973). The Vmax values reported for liver aldolase from male rat liver are 3.3 and 3.2 µmole/min/g (Hashemi, Dezfully 1979). A number of metabolites have an inhibitory effect on liver aldolase. It has been shown that aldolase B is competitively inhibited by AMP and ADP but not by ATP (Adelman, 1972). This inhibition could play a physiological role in in vivo controlling the activity of liver aldolase (Adelman, 1972). It has been suggested (Woods et al., 1970) that liver aldolase is inhibited after a fructose load because this treatment causes the accumulation of fructose 1-phosphate. This has been suggested to be due to an increase in IMP concentration which follows the hepatic depletion of the adenine nucleotides. However, Van den Berghe et al., (1977) haveshown that the FIP accumulation precedes that of IMP and thus the latter effect could not be considered to cause the accumulation of the ketose phosphate.

Liver aldolase is regulated by dietary carbohydrate (Adelman and Spolter, 1966). The total activity of the enzyme decreases to about one-third of the normal activity if the animals are fasted for 48-72 h, but activity can be restored to normal by re-feeding with glucose, fructose or sucrose when the fasted rats are given diets rich in glucose or fructose; the activity of the enzyme is restored to normal when diet is administered for three weeks (Adelman and Spolter, 1966).

3. Triokinase (ATP: D-glyceraldehyde 3-phosphotransferase; E.C.2.7.1.28)

The ATP-dependent phosphorylation of D-glyceraldehyde into D-glyceraldehyde 3-phosphate occurs by the action of triokinase (Hers and Kusaka, 1953). In vivo D-glyceraldehyde is normally derived from C-4, C-5 and C-6 of fructose. The high activity of triokinase in both human and rat livers and the low K_m of this enzyme for D-glyceraldehyde strongly suggests that triokinase is the most important enzyme in D-glyceraldehyde metabolism (Sillero et al., 1969). The same conclusion was reached following a study of the conversion of [4-^{3}H, 6-^{14}C]

fructose to liver glycogen in mice (Hue and Hers, 1972). These authors have shown that ^{3}H is not lost in the conversion of fructose into glycogen. If the pathway proceeds via glyceric acid there should be a loss of ^{3}H during the oxidation of D-glyceraldehyde (Fig. 2). These authors have found that the $^{3}H/^{14}C$ ratio in glycogen was 36.6% of that in the injected fructose. The loss of tritium through fructose metabolism was equal to 29.2% and the C-6 to C-1 randomization to fructose metabolism was 27.4%. These results are in agreement with the hypothesis that the D-glyceraldehyde formed in the liver upon aldolytic cleavage of fructose 1-phosphate is metabolised by phosphorylation to D-glyceraldehyde 3-phosphate under the action of triokinase. The kinetic properties of triokinase also reflect the central role of the enzyme in fructose metabolism (Frandsen and Grunnet, 1971). These authors have shown that the amount of fructose converted to D-glucose in rat liver slices corresponds to the activity of triokinase and also that the enzyme displays a K_m of 0.77mM for Mg-ATP. It is possible that D-glyceraldehyde phosphorylation is regulated to a significant degree by ATP levels *in vivo*. Triokinase, therefore, is effective when the concentration of ATP is high which would favour other gluconeogenic processes and inhibit glycolysis. These authors have also suggested that at low ATP levels the glycerate and/or glycerol pathways may operate.

4. Pyruvate Kinase (ATP: pyruvate phosphotransferase, E.C.2.7.1.40)

Pyruvate kinase catalyses the final step in the formation of pyruvate from glucose via the glycolytic pathway. This enzyme catalyses the transfer of the 'energy rich' phosphate group from phosphoenolpyruvate to ADP producing enolpyruvate and ATP (Heinz, 1973).

Hepatic pyruvate kinase (L-type) is localised in the cytoplasm. In the absence of any effectors the liver pyruvate kinase shows markedly sigmoidal kinetics with respect to phosphoenolpyruvate with a $[S]_{0.5}$ of about 10^{-3} M (Seubert and Schoner, 1971). Fructose 1,6-bisphosphate is a potent stimulator of the enzyme (Carminatti *et al.*, 1968). The V_{max} is not altered but the K_m is markedly decreased to about 10^{-4} M.

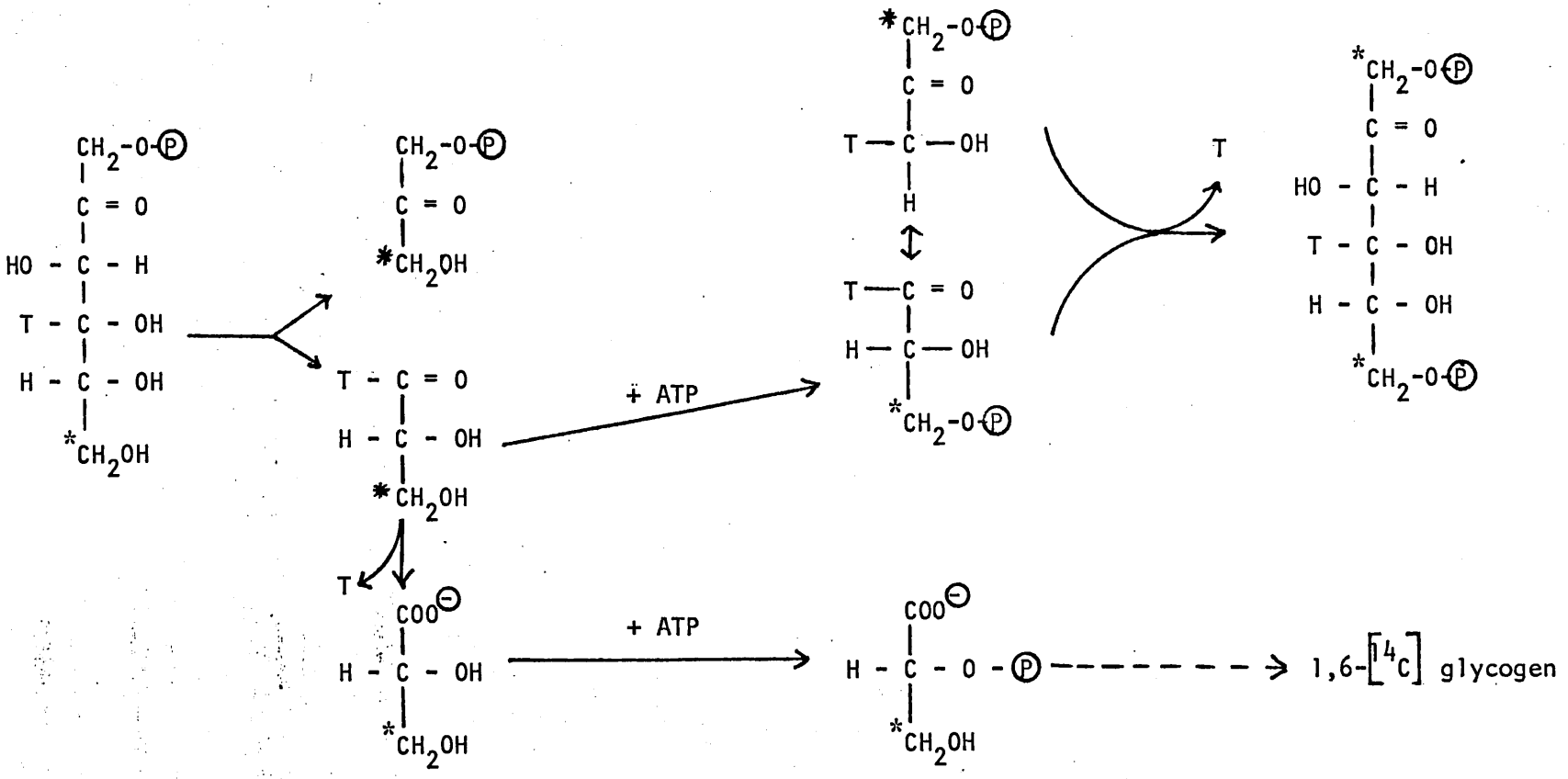


Fig. 2 Labilization of tritium (T) and randomization of carbon in the conversion of [4-^{3}H,6-^{14}C] fructose into fructose bisphosphate (Hue and Hers, 1972).

L-type pyruvate kinase is allosterically inhibited by ATP and alanine (Seubert and Schoner, 1971; Imamura and Tanaka, 1972). The presence of physiological concentrations of these effectors can have a profound effect on the degree of co-operativity that the enzyme shows, as well as on the $[S]_{0.5}$ for phosphoenolpyruvate. Physiological concentrations of fructose 1,6-bisphosphate, however, can completely reverse the inhibition by these effectors. It has also been reported that pyruvate kinase activity assayed at sub-optimal phosphoenol-pyruvate concentration is increased by fructose 1-phosphate after perfusion of rat liver with 10mM fructose (Eggleston and Woods, 1970). Acetyl-CoA has also been shown to inhibit rat liver pyruvate kinase activity (Weber et al., 1967).

The activity of the L-type isoenzyme is markedly altered by phosphorylation catalysed by cyclic AMP-dependent protein kinase (Ljungström et al., 1974; Engström, 1978). Moreover, phosphorylation has been shown to occur in intact liver preparations in which the cyclic AMP levels have been increased by glucagon (Ben-Bassat et al., 1977; Gurtler and Emmerich, 1978). Phosphorylation converts the enzyme into a less active form. Dephosphorylation has been demonstrated in the presence of a protein phosphatase extracted from liver (Engström, 1978). The phosphorylated and dephosphorylated forms of the enzyme are characterised by high (800 μM) and low (300 μM) values for the $[S]_{0.5}$ for phosphoenol-pyruvate respectively. Recently, it has been shown that L-type pyruvate kinase can also undergo a proteolytic cleavage which produces a less active enzyme molecule (Hall et al., 1979). These authors speculated that the cleavage is a secondary response to phosphorylation and inactivates the enzyme irreversibly.

Pyruvate kinase activity has been studied in meal-trained animals fed on either carbohydrate or Chow diets (Hopkirk and Bloxham, 1979). In both groups of rats the total activity of enzyme increases during feeding and is maximum 1-5 h postprandially. Thus the period of maximum enzyme activity coincides closely with the period of maximum fatty acid synthesis. The total activity of pyruvate kinase was about three times as great in homogenates from high-carbohydrate fed animals compared with chow-fed animals. This increase parallels the enhanced fatty acid synthesis in high carbohydrate fed rats (Hopkirk and Bloxham, 1979).

C. Consequences of hepatic fructose loading

The levels of fructose 1-phosphate in the liver are increased following fructose feeding (Hue and Hers, 1972). Oral and especially parenteral administration of fructose cause a rapid accumulation of fructose 1-phosphate and a depletion of ATP and P_i in the tissues that possess ketohexokinase. The accumulation of fructose 1-phosphate has been demonstrated in the livers of rats and mice (Burch et al., 1969; Van den Berghe et al., 1973; 1979; Iles et al., 1980) and normal humans (Woods et al., 1970; Sestoft et al, 1972). It is postulated that the breakdown of fructose 1-phosphate is much slower than its formation by fructokinase, as a result there is an increase in the concentration of the fructose ester. It has been concluded that the accumulation of IMP (an inhibitor of aldolase), which is also observed when liver is perfused with fructose, is the reason for the accumulation of ketose phosphate (Woods et al., 1970). The finding that the accumulation of the fructose ester precedes the increase in IMP in vivo contradicted the above explanation (Van den Berghe et al., 1977). Another explanation, the inhibition of fructokinase by fructose 1-phosphate, has also been disputed (Sestoft, 1974a) and the fact that the concentration of ADP is barely changed after fructose load rules out the possibility of inhibition by the other product of the reaction. The decrease in ATP caused by a fructose load (Mäenpää et al., 1968) could decrease the velocity of the fructokinase mechanism, although not sufficiently to stop the accumulation of fructose 1-phosphate. The availability of P_i also plays a role since, in isolated liver, the rate of fructose uptake (Sestoft, 1974b), as well as the accumulation of fructose 1-phosphate (Woods, 1972), can be enhanced by increasing the concentration of P_i in the perfusion medium.

Besides the accumulation of fructose 1-phosphate and IMP, several other metabolites are increased after the administration of a fructose load (Van den Berghe, 1978). It has been reported that the concentration of glyceraldehyde, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and especially sn-glycerol 3-phosphate are increased following fructose load (Heinz and Junghänel, 1969; Burch et al., 1969; Woods et al., 1970). It has been shown that the concentration of sn-glycerol 3-phosphate is increased approximately eightfold in rat liver perfused with 10mM fructose (Weiland and Matschinsky, 1962; Woods et al., 1970).

Davies and Mapungwana (unpublished observations) have also shown that fructose can elevate sn-glycerol 3-phosphate levels in hepatocytes incubated with fructose, even at physiological concentrations of the ketose, but the increase is only of the order of 1.5-2.0 fold (from approximately 200 nmoles/gcells). The dihydroxyacetone phosphate concentration, on the other hand, was unaffected by the ketose.

Recently, fructose metabolism has been studied with ^{31}P n.m.r. in perfused livers from rats starved for 48 h (Iles et al., 1980). These authors have shown that a rapid fall in the concentration of both ATP and P_i and a decrease in intracellular pH occurs after infusion of fructose. These changes are accompanied by a rapid rise in fructose 1-phosphate. The uptake of lactate by the liver indicated that the fall in intracellular pH was caused primarily by production of protons accompanying the formation of lactate from fructose, as shown below.

$$\text{Fructose} + \text{Mg ATP}^{2-} \longrightarrow \text{Fructose 1 - P}^{2-} + \text{Mg ADP}^{1-} + \text{H}^{+}$$

$$2\ \text{Lactate}^{-} + 2\text{H}^{+} \longrightarrow \text{glucose}$$

$$\text{Fructose} \longrightarrow 2\text{H}^{+} + 2\ \text{Lactate}^{-}$$

However, this proton release is transient since it is limited by the magnitude of the initial increase in fructose 1-phosphate concentration and the fall in ATP concentration. Further metabolism of fructose 1-phosphate to glucose and re-conversion of ADP into ATP, to maintain the new steady state concentrations, will result in a net balance of zero protons released. However, at the same time it is apparent that a change from lactate uptake to lactate output occurs by this mechanism, which presumably accounts for the lowering of intracellular pH (Iles et al, 1980). The concentration of other nucleotides has also been reported to be decreased by fructose loading. These are UTP, UDP-glucose (Burch et al., 1969; 1970) and GTP (Van den Berghe et al., 1977). The decrease in the GTP and UTP occurs more slowly than the decrease of ATP. The reason for the decrease in GTP is thought to be its utilisation for the phosphorylation of D-glyceraldehyde by triokinase (Frandsen and Grunnet, 1971).

The mechanism of D-fructose-induced hyperuricemia in humans is probably by way of the rapid phosphorylation of fructose which causes depletion of ATP and inorganic phosphate in the liver (Mäenpää et al., 1968; Woods et al., 1970; Sestoft, 1974a, Van den Berghe et al., 1977; Van den Berghe, 1978). Both are essential for the stabilisation of AMP level and, therefore, the total adenine nucleotide content of the tissue. When the inhibition of AMP deaminase becomes less effective the hepatic IMP concentration increases and both AMP and IMP undergo dephosphorylation with the formation of adenine and inosine (Wood et al., 1970; Van den Berghe et al., 1980) and eventually hypoxanthine, uric acid and allantoin, as shown in Fig. 3 (Woods, 1972; Fox and Kelly, 1972).

The most likely route for the degradation of AMP appears to be via AMP deaminase, since the enzyme is stimulated by ATP and inhibited by physiological levels of GTP and P_i. The depletion of the latter metabolic intermediates appear to result in the loss of an important regulatory control of AMP deaminase, i.e. the inhibition of the enzyme under physiological substrate and regulator levels (Van den Berghe et al., 1977). Therefore, fructose infusion results in an increase in the serum level of uric acid (Heukenkamp and Zöllner, 1971). In experimental animals such as rats that possess uricase, the administration of a fructose load causes an increase in the plasma level of allantoin in addition to the increase in uric acid (Mäenpää et al., 1968; Kekomäki et al., 1972). The mechanism of fructose induced nucleotide catabolism was studied using isolated rat hepatocytes in which the adenine nucleotide pool was pre-labelled with [^{14}C] adenine (Smith et al., 1977). Incubation of isolated rat hepatocytes with fructose (28 mM) caused a rapid depletion of the [^{14}C] adenine nucleotides and a corresponding increase in [^{14}C] allantoin (Van den Berghe et al., 1980). The production of allantoin by isolated hepatocytes is not influenced by the addition of low concentrations of coformycin (0.1 µM), but is decreased by concentrations of coformycin (50 µM) that are inhibitory for AMP deaminase. With 50 µM - coformycin the production of allantoin is decreased by 85% and the formation of radioactive allantoin from [^{14}C] adenine nucleotides is completely suppressed. In the presence of 0.1 µM coformycin, or in its absence, the addition of fructose to the incubation medium causes a rapid degradation of ATP, without an equivalent increase in ADP and AMP, followed by transient increases in IMP and in the rate of production of allantoin. In the

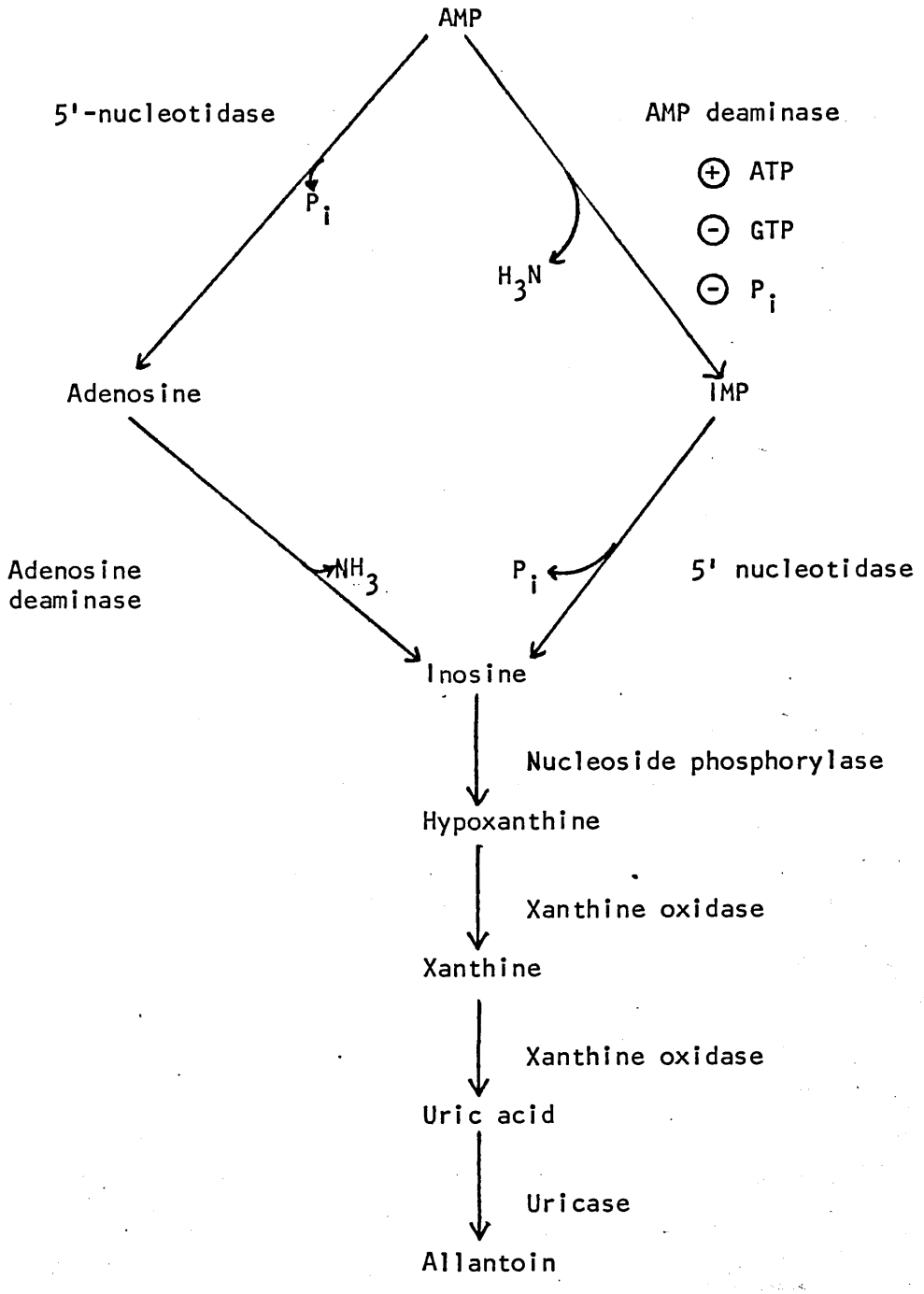


Fig. 3 Pathway of AMP degradation(After Woods et al., 1970)

presence of 50 µM-coformycin, the fructose-induced breakdown of ATP is not modified, but the depletion of the adenine nucleotide pool proceeds much more slowly and the rate of production of allantoin increases only slightly. A rise in IMP concentration is not detectable but AMP level is increased. Inhibition of adenosine deaminase requires only 0.1 µM-coformycin (Van den Berghe et al., 1980). This is evidence for a major role of AMP deaminase in the regulation of the adenine nucleotide breakdown.

D. End products of fructose metabolism

The major end products of fructose metabolism are glucose, lactate and glycogen. It has been shown that when livers from fasted rats are perfused with 20mM fructose for 1h, 52% of the ketose is recovered as glucose, 18% as lactate plus pyruvate and 8% as glycogen; the remaining 22% is assumed to be metabolised to triacylglycerol, CO_2 and ketone bodies and to glycerol and sorbitol (Exton and Park, 1967). From studies performed on perfused liver, it is well known that fructose is one of the best gluconeogenic substrates. The maximum rate of glucose formation from fructose by perfused liver is about twice that from lactate (Exton and Park, 1967; Ross et al., 1967). It has been shown that fructose is utilised very effectively by isolated liver cells from both fed (Seglen, 1974; Arinze and Rowley, 1975) and fasted (Veneziale and Lohmar, 1973; Seglen, 1974) rats. At 20mM fructose the rate of gluconeogenesis is approximately 3 µmoles/min/g and glycogen has been shown to accumulate (Seglen, 1974). Gluconeogenesis from fructose (5-20mM) is not subject to end-product inhibition by glucose at physiological concentrations in cells from fasted rats (Seglen, 1974). In vivo studies with [1-^{14}C] fructose in experimental animals (Friedmann et al., 1970) and in human (Atwell and Waterhouse, 1971; Kellen and Froesch, 1972) have demonstrated a rapid conversion of the ketose into blood glucose which results in hyperglycemia. Parenchymal cells isolated from the livers of 40h starved, but not from 16h-fasted rats, show an increase in the rate of gluconeogenesis from fructose compared to fed rats (Seglen, 1974).

Glucagon, adrenaline or cyclic nucleotides stimulate glucose

synthesis from fructose in isolated rat liver cells (Garrison and Haynes, 1973). Glucagon enhances glucose formation from low concentrations of fructose in isolated hepatocytes from 24 h fasted rats but does not enhance the initial rate of gluconeogenesis from 10mM fructose (Zahlten et al., 1973). Other authors have also found that at high concentrations of substrate glucagon has no significant effect on glucose production (Ross et al., 1967; Exton and Park, 1968; 1969). The effect of glucagon on gluconeogenesis is mediated through changes in the concentration of cyclic AMP leading to a decrease in phosphofructokinase (Van Schaftingen et al., 1981) and an increase in fructose 1,6-bisphosphatase (Van Schaftingen and Hers, 1981) activity as the result of a lowering of the fructose 2,6-bisphosphate concentration in the liver (Van Schaftingen et al., 1980). Fructose at low concentration elevates hepatocyte Fru 2,6-P_2 but the level of the effector is depleted at high concentrations of the sugar (D.R. Davies, unpublished). This could explain the apparent discrepancy between the effect of glucagon at different concentrations of fructose.

Perfusion of rat liver from fed animals with physiological concentrations of fructose leads to a significant lowering of glucose in the perfusate and a concomitant increase in liver glycogen (Topping and Mayes, 1976). A reduction in glucose concentration has also been reported in the hepatic venous blood of rats absorbing a fructose meal (Topping and Mayes, 1971). Fructose is known to cause an increase in hepatic glucose 6-phosphate concentration (Walli et al., 1975; Miller, 1978). This may activate glycogen synthetase and inhibit phosphorylase (Hers et al., 1974) which may account for the increase in glycogen deposition observed by Topping and Mayes (1976).

Perfusion of rat liver by fructose has been reported to result in a transient increase in phosphorylase a activity followed by an inactivation of the enzyme (Walli et al.,1975; Jakob, 1976). Van den Berghe et al., (1973) have also reported an inactivation of phosphorylase in mouse liver when fructose is administered intraperitoneally. The activation of phosphorylase by fructose is in contrast with the widely held belief that the ketose is a good precursor of glycogen. Miller (1978) has provided evidence that the activation of phosphorylase by

fructose involves a cyclic AMP-dependent mechanism, since perfusion of liver with fructose increases cyclic AMP concentration within the cells and also increases cyclic AMP-dependent protein kinase. However, very high levels of the ketose were used by this author. Van der Werve and Hers (1979), however, were unable to demonstrate any change in phosphorylase kinase or histone kinase activity in isolated hepatocytes in response to the sugar. They correlated the increase in phosphorylase a to the decreased concentration of ATP, since the activation of phosphorylase was also observed with other ATP depleting agents such as glycerol, tagatose and glucose. The latter effect was only observed when hexokinase was added. They suggest a mechanism for the activation of phosphorylase involving a decrease in the level of ATP-Mg, resulting in a change in the ratio of Mg^{2+}/ ATP-Mg which would, in turn, stimulate phosphorylase kinase and thus increase the conversion of phosphorylase b to phosphorylase a. The inactivation of phosphorylase observed at longer time intervals is reported to be related to the lowering of cyclic AMP by fructose (Van den Berghe et al., 1973).

Fructose 1-phosphate has also been reported to inhibit purified phosphorylase a so that at concentrations of 10mM fructose 1-phosphate and 1.5mM P_i, conditions which are likely to occur in hepatocytes subjected to a fructose load, the enzyme may be inhibited by approximately 70% (Van den Berghe et al., 1973).

It has been reported that glycogen is synthesised from fructose (20mM) at high rates by hepatocytes from fed or 16h-fasted rats, but not in cells from 40h-starved animals (Seglen, 1974). There is a strong synergism between the effects of fructose and of glucose on glycogen synthesis. Glycogen synthase activity has been reported to be increased following fructose administration in vivo (Van den Berghe et al., 1973; Hue et al., 1973; Thurston et al., 1974) and in perfused liver (Walli et al., 1975; Whitton and Hems, 1975). The effect of fructose on glycogen synthase is thought to be secondary to the effect on phosphorylase, since it has been shown that increasing glucose concentration from 5.5mM to 55mM causes a sequential inactivation of glycogen phosphorylase and activation of glycogen synthase in perfused liver (Hue et al, 1975). The explanation for this is that glucose binds to phosphorylase a and, as a result, there is a rapid

conversion to phosphorylase b by phosphorylase phosphatase (Hers et al., 1977). Synthetase phosphatase, which activates glycogen synthase, is strongly inhibited by phosphorylase a; thus, the conversion of phosphorylase a to phosphorylase b results in an activation of glycogen synthase. However, Walli et al., (1975) found that fructose increased phosphorylase a without a concomitant decrease in glycogen synthase, although the decrease in phosphorylase a observed after a longer perfusion period resulted in an increase in glycogen synthase.

It has been shown that lactate formation from fructose is several-fold faster than from glucose in liver slices (Renold et al., 1956; Thieden and Lundquist, 1967) in the perfused liver (Exton and Park, 1967) and in isolated liver cells (Seglen, 1974; Davies and Mapungwana, unpublished). This difference is explained by the higher activity of fructokinase in comparison with the glucose phosphorylating capacity of hepatic glucokinase (Adelman et al., 1967; Aminoff, 1974) and the fact that fructolysis bypasses the regulatory enzymes, glucokinase and phosphofructokinase (Arinze and Rowley, 1975). In addition pyruvate kinase is stimulated by fructose 1-phosphate (Eggleston and Woods, 1970) and by the depletion of ATP. Fasting has been shown to decrease the formation of lactate from fructose (Seglen, 1974; Sestoft, 1974a). It has also been shown that, under anaerobic conditions glycolysis from fructose is stimulated in hepatocytes (the Pasteur effect) and gluconeogenesis and glycogen synthesis is is strongly inhibited (Seglen, 1974). Administration of fructose in humans has been shown to cause considerable increase in blood lactic acid, accompanied by a fall in pH (Bergström et al., 1968). It has been shown that perfusion of liver with 8.9mM fructose resulted in a net lactate production at low and high concentrations of non-esterified fatty acid but a net uptake occurred at physiological fructose levels (Laker and Mayes, 1979).

E. Antiketogenic effect of fructose

The metabolism of long-chain fatty acid by the liver is a complex process which is dependent on the hormonal and dietary condition of the animal. The blood-borne fatty acids are converted to the

acyl CoA derivative by the cytoplasmic acyl CoA synthase (Williamson, 1979). The fate of the long-chain acyl CoA is either esterification with sn-glycerol 3-phosphate to form triacylglycerol (McGarry and Foster, 1980a) or β-oxidation to acetyl CoA. The latter product may be used for the generation of ketone bodies or for the synthesis of citrate which, in turn, can either be exported from the mitochondria and used for fatty acid synthesis or oxidised to CO_2 via the tricarboxylic acid cycle (Fig. 4).

β-Oxidation involves the successive oxidative removal of acetyl unit as acetyl CoA from long-chain fatty acids. The carnitine-dependent transfer of fatty acid across the mitochondrial membrane is established as an obligatory step in the β-oxidation of long-chain fatty acid. The formation of acylcarnitine and the subsequent oxidation may be regulated in the intact cell (Bremer et al., 1978) but present evidence indicates that it is the mitochondrial uptake of fatty acid which is the most important factor (Williamson, 1979).

The regulation of the fate of acyl CoA appears to be under hormonal control. In the fed animal esterification predominates and oxidation is shut down. In this case the major source of mitochondrial acetyl CoA may be pyruvate. In the fasted state lipogenesis and triacylglycerol synthesis are suppressed and ketone body formation is increased. The uptake and utilisation of citrate for oxidation is virtually unaffected by dietary status. These changes have been ascribed to alterations in the plasma glucagon/insulin ratio (McGarry and Foster, 1980a; Zammit, 1981).

The reciprocal relationship between oxidation and esterification of free fatty acid taken up by the liver has been well demonstrated (McGarry and Foster, 1971a; Topping and Mayes, 1972; McGarry et al., 1975). The channelling of fatty acids through this metabolic branch point may be governed by the esterifying capacity of the liver which, in turn, is thought to be dependent upon the availability of sn-glycerol 3-phosphate (Fritz, 1961; Wieland and Matschinsky, 1962; Tzur et al., 1964; Mayes and Felts, 1967). Accordingly, the enhanced rate of hapatic fatty acid oxidation seen in insulin-deficient states is postulated to be secondary to a diminished esterification capacity

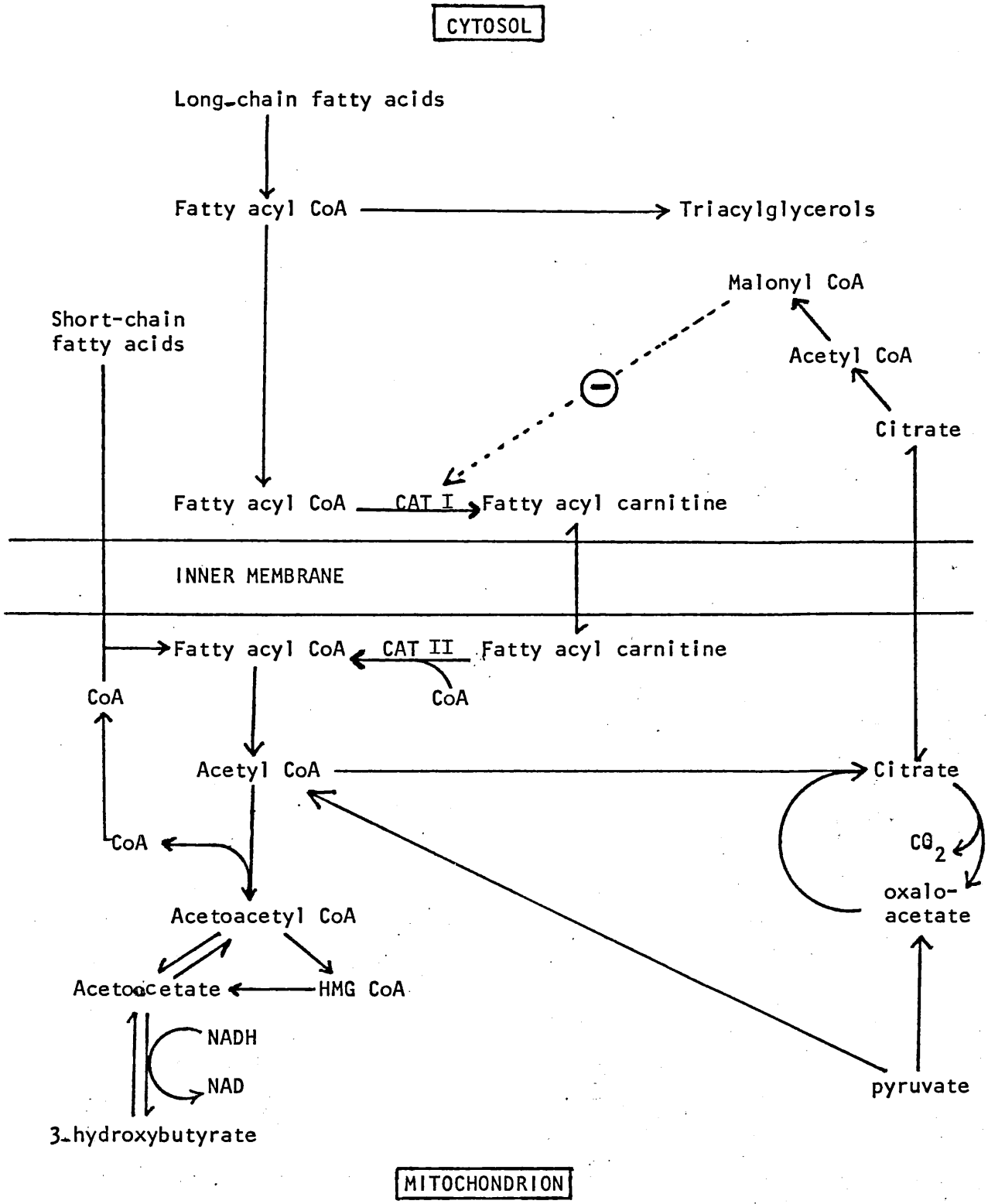


FIG 4. Major disposal routes of long-chain fatty acids in liver. CAT I and CAT II refer to carnitine acyltransferase I and II respectively. Scheme as defined by (Zammit, 1981).

resulting from a fall in sn-glycerol 3-phosphate levels. However, it is not established that fluctuations in the sn-glycerol 3-phosphate concentration can influence the rate of fatty acid oxidation (Williamson et al., 1969; McGarry and Foster, 1971a, Christiansen, 1979), but recent evidence by Zammit (1981) shows that sn-glycerol 3-phosphate levels increase in parallel to total ketone body concentration. In addition, Debeer et al., (1981) have found that hepatocyte sn-glycerol 3-phosphate levels may regulate palmitate oxidation via changes in esterification rates but there is no claim that sn-glycerol 3-phosphate affects oxidation directly. It is suggested (Zammit, 1981) that the raised sn-glycerol 3-phosphate concentration is the result of increased delivery of glycerol to the liver from the adipose tissue under ketogenic conditions and that sn-glycerol 3-phosphate availability is unlikely to limit esterification.

The addition of fructose to isolated liver preparation from fasted animals decreases the formation of ketone bodies (McGarry and Foster, 1971a; Ontko, 1972; Sestoft, 1974c). Such an antiketogenic effect has also been described in man, following intravenous infusion of fructose (Dietze et al., 1978). The antiketogenic effect of fructose is most probably due to the inhibition of β-oxidation of fatty acids and an enhanced utilisation of acetyl CoA for lipogenesis. From in vitro experiments it has been concluded that the effect of fructose can be attributed to the accumulation of sn-glycerol 3-phosphate resulting in increased esterification and thus a decreased amount of free fatty acid available for β-oxidation. On the other hand, an increased activity of the citric acid cycle has been postulated to be responsible (McGarry and Foster, 1971b). Ontko (1972) found that fructose elevated CO_2 production from palmitate by hepatocytes at low fructose concentration but Topping and Mayes (1972), using physiological levels of the ketose, found inhibition of β-oxidation to CO_2. The increased activity of the citric acid cycle in response to fructose may be associated with the fall in ATP concentration in the liver. A more recent study of Christiansen (1979) using hepatocytes treated with glucagon revealed that fructose, dihydroxyacetone, glycerol, sorbitol and lactate were capable of a similar antiketogenic effect despite large differences in the accumulationof sn-glycerol 3-phosphate in response to each of these substrates. It is suggested that the

antiketogenic effects of these substrates may be exerted via changes in the levels of long-chain acyl CoA and malonyl CoA.

Prager and Ontko (1976) have investigated the effect of fructose with a cell-free system utilising intact mitochondria and they have found that fructose inhibits a step in the β-oxidation sequence since palmitoyl carnitine oxidation is inhibited substantially, an effect which is independent of changes in esterification since the microsomes were removed in the procedures.

II. HEPATIC LIPOGENESIS

The de novo synthesis of fatty acids in the liver involves the conversion of acetyl CoA to acyl CoA in the cytosol of the hepatocytes. The initial step catalysed by acetyl CoA carboxylase is the carboxylation of acetyl CoA to malonyl CoA (Volpe and Vagelos, 1976). The synthesis of the acyl CoA is catalysed by the fatty acid synthetase complex which utilises acetyl CoA, malonyl CoA and NADPH as substrates. The cytoplasmic acetyl CoA required for lipogenesis is thought to be derived from citrate by the action of ATP citrate lyase. The conversion of pyruvate to fatty acid involves the mitochondrial uptake of the former and its conversion to acetyl CoA in the mitochondria by pyruvate dehydrogenase. Acetyl CoA combines with oxaloacetate in the mitochondria by action of citrate synthase and the citrate which leaves the mitochondria is utilised for the resynthesis of acetyl CoA in the cytoplasm (Bhaduri and Srere, 1963).

A. Key enzymes of fatty acid synthesis

1. Acetyl CoA Carboxylase (E.C.6.4.1:2) or : Acetyl CoA - CO_2 Ligase (ADP forming)

The initial step in the biosynthesis of long-chain fatty acids is catalysed by acetyl CoA carboxylase. Malonyl CoA is formed by carboxylation of acetyl CoA. This is generally regarded as a rate limiting step in de novo fatty acid biosynthesis. Acetyl CoA

carboxylase catalyses the biotin-dependent carboxylation of acetyl CoA to form malonyl CoA in a two-stage reaction:

$$ATP + HCO_3^- + \text{biotin} - E \xrightarrow[BC]{Mg^{2+}} CO_2^- - \text{biotin} - E + ADP + Pi$$

$$CO_2^- - \text{biotin} - E + CH_3CO - SCoA \xrightarrow[TC]{} {}^-OOCCH_2OO - SCoA + \text{biotin} - E$$

Acetyl CoA carboxylase is an allosteric enzyme with a biotin prosthetic group and consists of three sub-units; biotin carboxylase (BC), biotin carboxy carrier protein (BCCP) and transcarboxylase (TC). Biotin carboxylase catalyses the first half-reaction, the carboxylation of biotinyl moiety of the BCCP (Volpe and Vagelos, 1976). The trans-carboxylase component of acetyl CoA carboxylase catalyses the reversible transfer of carboxyl group from CO_2 - BCCP to acetyl CoA to form malonyl CoA (Alberts *et al.*, 1971; Guchhalt *et al.*, 1971; Polakis *et al.*, 1974).

The enzyme exists in two forms, an inactive monomeric form and an active polymeric form (Gregolin *et al.*, 1968). Evidence from *in vitro* studies suggest that rat liver acetyl CoA carboxylase is also regulated by a phosphorylation - dephosphorylation mechanism and it has been postulated that the phosphorylation of the enzyme protomer leads to a decrease in enzyme activity Carlsonand Kim, 1973; Lee and Kim, 1977).

Thus the activity of purified acetyl CoA carboxylase depends on its state of aggregation. The enzyme from animal tissues requires a pre-incubation in the presence of a tricarboxylic acid, notably citrate, to reveal the carboxylase activity (Lane *et al.*, 1974; Witters *et al.*, 1979a). The citrate activation of the purified enzyme has been shown to be the result of the polymerisation of the protomer to give a catalytically active filamentous structure (Gregolin *et al.*, 1966; Hashimoto and Numa, 1971; Moss and Lane, 1972; Moss *et al.*, 1972; Lane *et al.*, 1974; Ashcraft *et al.*, 1980). However, the physiological significance of effect of citrate preincubation has been somewhat doubtful since the Ka for citrate is 3-4mM which is about 10-fold the level which occurs in the liver cell. It is thought that the activation

induces a conformational change in the protomer which results in polymerisation (Lane et al., 1974). Polymerisation is also enhanced by the presence of P_i, albumin, a pH between 6.5 - 7.0 and a high enzyme concentration, whereas depolymerisation is induced by ATP - Mg^{2+} and HCO_3^-, malonyl CoA, fatty acyl CoA and alkaline pH (Lane et al., 1974). It is generally accepted that the major effect of the activation of citrate is to increase the V_{max} of the enzyme rather than to change the Km values for the substrate (Volpe and Vagelos, 1976).

The Km of the carboxylase for acetyl CoA is 3×10^{-5}M, the Km for bicarbonate is 6×10^{-3}M and for ATP 2×10^{-4}M under optimum assay conditions (Alberts and Vagelos, 1972; Soler-Argilaga et al., 1978a).

It has been reported that physiological concentrations of CoA can activate a partially purified acetyl CoA carboxylase from rat liver but this effect was lost on further purification of the enzyme. This activation of the partially purified enzyme is accompanied by a lowering of the Km for acetyl CoA from 200 μM to 4 μM, the latter value is closer to the physiological concentration of cytosolic acetyl CoA. The activation of acetyl CoA carboxylase by physiological concentrations of CoA has been shown to be sigmoidal. Below 60 μM CoA there is little or no activation of the enzyme, suggesting that small changes in the concentration of CoA can cause significant changes in carboxylase activity. These authors have suggested that the binding site for CoA is different from that for citrate (Yeh and Kim, 1980).

Treatment of the purified enzyme with Dowex 1 - X8 to remove tightly-bound citrate results in the restoration of the CoA activation of the enzyme, in this case there is an increase in the V_{max} apparently without a change in the Km. The activation by CoA is accompanied by a polymerisation of the enzyme. The phosphorylated form of acetyl CoA carboxylase does not respond to CoA activation at all at physiological concentrations. There appears to be one high affinity binding site for CoA per sub-unit, binding at this site is unaffected by citrate but is inhibited by palmityl CoA. Bovine serum albumin and CoA together can activate the palmityl CoA inhibited enzyme (Yeh and Kim, 1980).

It has been claimed that acetyl CoA carboxylase in liver

homogenates is activated by incubation at 37° *in vitro* (Swanson *et al.*, 1968; Allred and Roehrig, 1978). The latter workers found that a liver preparation obtained by homogenising the tissue in isotonic mannitol and centrifugation at 27,000 x g contained an enzyme which was capable of the acetyl CoA dependent fixation of HCO_3^- into an acid-stable product. However, more recent work Davies *et al.*, (1982) has identified the major product of such an incubation as $[^{14}C]$ aspartate, presumably formed as the result of the action of pyruvate carboxylase and a transaminase. The former enzyme has almost identical substrate and effector requirements as acetyl CoA carboxylase except that pyruvate is the substrate for pyruvate carboxylase whereas acetyl CoA is an allosteric stimulator. Davies *et al.*, (1982) have also shown that sufficient pyruvate accumulation occurs during homogenisation and incubation to account for the enhanced rates of carboxylation observed by Allred and Roehrig (1978). The observations of Davies *et al.*, (1982) also cast doubt on some of the recent evidence that acetyl CoA carboxylase activity in isolated hepatocytes is influenced by hormones. Geelen *et al.*, (1978b) reported a covariance between acetyl CoA carboxylase level and lipogenesis in hepatocytes treated with insulin and glucagon. Witters *et al.*, (1979a) reported an inhibition of acetyl CoA carboxylase in isolated hepatocytes by glucagon and a stimulation by insulin. Demonstration of these hormonal effects required for isolation of the enzyme extract at room temperature using a vigorous homogenisation technique. Similarly, Assimacopolous-Jeannet *et al.*, (1981) have also found that insulin and vasopressin increase lipogenesis from ^3H_2O and the latter hormone increases acetyl CoA carboxylase activity. All these observations can be simply explained by the fact that these hormones all regulate glycolysis and thus the level of pyruvate in the crude extracts which are used for enzyme assay. It is also possible that changes in pyruvate concentration could also account for the differences in the rate of lipogenesis observed by these authors. In fact, the latter group have shown that the stimulatory effect of insulin is abolished at a high concentration of pyruvate.

Some early reports (Swanson *et al.*, 1967) indicated that acetyl CoA carboxylase present in an inactive form in a high-speed supernatant from rat liver could be stimulated by incubation with trypsin. In this context Abdel-Halim and Porter (1980) have found a non-dialysable factor in such a preparation which inhibits acetyl CoA carboxylase.

This inhibition is destroyed by heating and by trypsin treatment, suggesting that a regulatory protein is present in the high-speed supernatant which modulates the activity of the protein. This factor has recently been purified (Shiao et al., 1981). These authors have shown that this factor behaves as a cAMP-independent protein kinase that inactivates acetyl CoA carboxylase by phosphorylation; this kinase has a molecular weight of 16,000 and it requires ATP and Mg^{2+} for activity (Shiao et al., 1981).

Hardie and Guy (1980) have also described an effect of trypsin on the enzyme which results in partial hydrolysis of the protomer; this treatment, however, appeared to have no effect on the activity of the carboxylase despite the apparent loss of one of the sites phosphorylated by cAMP-dependent protein kinase.

A number of different candidates for the inhibition of the enzyme in vivo have been postulated. Long-chain fatty acyl CoA thioesters at micromolar concentrations inhibit the carboxylase from rat liver; the inhibition is competitive with respect to citrate (Bortz and Lynen, 1963; Numa et al., 1965a; 1965b; Volpe and Vagelos; 1976). The most potent thioesters are those of the C_{16} - C_{18} fatty acids, such as palmityl, stearyl and oleyl CoA (Goodridge, 1973b; Goodridge et al., 1974; Lunzer et al., 1977). This inhibition is thought to be of physiological importance since half-maximal inhibition occurs at concentrationsof the thioesters between 3 and 8×10^{-7} M and the concentration of acyl CoAs in vivo is thought to be 15-140 μM (Greenbaum et al., 1971; Yeh and Leveille, 1971).

It has been demonstrated that palmitoyl CoA binds tightly and reversibly to mammalian acetyl CoA carboxylase in an equimolar ratio to completely inhibit the enzyme (Ogiwara et al., 1978). The inhibition constant (Ki) is as low as 6nM, about three orders of magnitude smaller than the critical micellar concentration of palmitoyl CoA. Comparison of the Ki values for various structural analogues of palmitoyl CoA indicate that 3'-phosphate of the CoA moiety and the long-chain acyl residue are essential for the inhibition of the enzyme (Nikawa et al., 1979). The inhibition is competitive with respect to citrate but non-competitive with respect to acetyl CoA, bicarbonate, or ATP (Numa et al., 1965a, 1965b). Regulation of acetyl CoA carboxylase activity by

palmityl CoA has also been studied in chicken liver (Goodridge, 1972). In the presence of bovine serum albumin (25 mg/ml), palmityl CoA (100 μM) inhibited incorporation of [^{14}C] citrate into fatty acids by a 100,000 x g supernatant of chicken liver and inhibited the activity of acetyl CoA carboxylase purified from chicken liver (Goodridge, 1972). This "specific" inhibition of acetyl CoA carboxylase can be reversed by one of three ways: (1) increase in concentration of albumin, (2) increase in concentration of citrate or (3) addition of (+) - palmityl carnitine. Palmityl carnitine may act as a structural analogue that binds to the enzyme at the same site as palmityl CoA but does not inhibit enzymatic activity (Goodridge, 1972).

Malonyl CoA is a potent inhibitor of the avian liver acetyl CoA carboxylase (Gregolin et al., 1966; Chang et al., 1967) exhibiting a Ki of about 10^{-5}M. It has been established that the inhibition by malonyl CoA is competitive with respect to both acetyl CoA and the tricarboxylic acid activator (Gregolin et al., 1966). While competitive inhibition by malonyl CoA with respect to acetyl CoA is of the "classical" type, the competitive relationship between malonyl CoA and isocitrate affects the protomer/polymer equilibrium (Lane et al., 1974). Citrate and isocitrate cause a shift in the equilibrium towards the catalytically active polymeric form and malonyl CoA is known to promote depolymerisation (Gregolin et al., 1966; Gregolin et al., 1968) thereby shifting the equilibrium towards the catalytically inactive form (Lane et al., 1974). The capacity of the acetyl CoA carboxylase to generate malonyl CoA approximately equals the capacity of the fatty acid synthetase to incorporate malonyl CoA into long-chain fatty acids, suggesting that circumstances may arise in vivo when the malonyl CoA concentration would be sufficient to inhibit acetyl CoA carboxylase activity (Chang et al., 1967). The tissue malonyl CoA level has been shown to fluctuate in the range 0.7 - 3.5 nmol/g wet weight (McGarry and Foster, 1980a). Inhibition of acetyl CoA carboxylase by malonyl CoA may provide a safeguard against excessive acetyl CoA utilisation for malonyl CoA production, thereby providing a fine control over the fatty acid synthesis (Lane et al., 1974). It has been shown that the cellular content of malonyl CoA in hepatocytes from fed rats is lowered as the concentration of oleate in the medium is raised and this is accompanied by a proportional fall in the rate of endogenous fatty acid

synthesis (McGarry and Foster, 1980a). This is most likely due to the inhibition of acetyl CoA carboxylase by an increase in the intracellular concentration of long-chain acyl CoA as described above.

Thus there are a number of regulatory metabolites which have been postulated to control the activity of acetyl CoA carboxylase *in vivo*. The malonyl CoA content of liver has been reported to change under different dietary regimes. Values of about 4nmol/g in fasted or diabetic animals, and a level of 15 nmol/g in rats fed on a high carbohydrate lipid-free diet have been found (McGarry *et al.*, 1978). These authors suggest a central role for malonyl CoA in the co-ordination of fatty acid synthesis and β-oxidation in isolated hepatocytes. Glucagon has an acute effect on hepatocyte fatty acid metabolism causing a switch from synthesis to oxidation. The high level of malonyl CoA inhibits mitochondrial carnitine acyltransferase, thus inhibiting fatty acid oxidation. The effect of glucagon is, therefore, thought to be exerted at two levels; firstly, the regulation of glycolysis and, secondly, by partial inhibition of acetyl CoA carboxylase. The rationale behind this conclusion is that the inhibition of lipogenesis by the hormone could be largely, but not completely, offset by addition of pyruvate and lactate. Additionally, glucagon inhibits the generation of citrate and malonyl CoA in cells from fed animals. However, addition of lactate and pyruvate in the presence of glucagon restores the cellular citrate level, but only partially restores malonyl CoA concentration. This is interpreted as a direct effect of the hormone on acetyl CoA carboxylase or, possibly, on citrate cleavage enzyme (McGarry *et al.*, 1978).

Watkins *et al.*, (1977) found that glucagon inhibited fatty acid synthesis by isolated chick hepatocytes without an apparent change in acetyl CoA carboxylase activity. This inhibition was ascribed to the decrease in cytosolic citrate after hormone treatment. Cook *et al.*, (1977) were also unable to show any effect of the glucagon administered *in vivo* on acetyl CoA carboxylase activity, although the hepatic malonyl CoA concentration was reduced by 70% and the long-chain acyl CoA content was only slightly elevated.

On the other hand, Witters *et al.*,(1979a) and Geelen *et al.*,

(1978b) have claimed that insulin stimulates and glucagon inhibits acetyl CoA carboxylase in isolated hepatocytes, and Assimacopolous-Jeannet et al., (1981) have reported a positive effect of vasopressin on the enzyme. It is postulated that the effect of the hormones on acetyl CoA carboxylase is mediated via a protein kinase and that the enzyme is controlled by a phosphorylation/dephosphorylation mechanism (Carlson and Kim, 1973; 1974). The phosphorylation and inactivation of the carboxylase stimulated by physiological concentrations of AMP and the degree of enzyme phosphorylation is related to the energy change. Maximum phosphorylation and inactivation occurred in the presence of 1.6 mM ATP and 2.4 mM AMP (Yeh et al., 1980)

Pekala et al., (1978) have reported a cAMP independent incorporation of $^{32}P_i$ into acetyl CoA carboxylase by chick liver cells. The fully phosphorylated enzyme appears to be fully active and they suggest that it is the dephosphorylated form which is inactive. This is in contradiction to the more recent reports by Yeh et al., (1980) and Shiao et al., (1981). The latter group have provided evidence for the presence in rat liver of a cAMP-independent kinase that inactivates acetyl CoA carboxylase by phosphorylation and also a phosphoprotein-phosphatase which dephosphorylates the enzyme and regenerates the carboxylase activity. All three enzymes are separable.

Acetyl CoA carboxylase in isolated hepatocytes is known to be phosphorylated in response to glucagon (Witters et al., 1979a; Witters, 1981) but there is no convincing evidence that this leads to a decrease in enzyme activity, although a number of reports have appeared which claim to observe changes in response to the hormone (Witters et al., 1979b; Geelen et al., 1978b; Assimacopolous-Jeannet et al., 1981)

Insulin causes a small increase in phosphorylation of acetyl CoA carboxylase, an effect which is additive to that of glucagon and independent of cAMP. The effect of insulin on enzyme activity was not, however, examined (Witters, 1981).

Thus, most of the evidence so far points to a regulation of acetyl CoA carboxylase by various phosphorylation/dephosphorylation mechanisms which, allied to changes in effectors, could change rates of lipogenesis in response to hormones. There is no direct conclusive

evidence for the short-term regulation of the liver enzyme activity by hormones. However, the enzyme from mammary gland does appear to be inactivated by a cAMP-dependent protein kinase (Hardie and Guy, 1980) and Brownsey et al., (1981) have reported evidence for the phosphorylation and activation of adipose acetyl CoA carboxylase by a plasma membrane-associated, cAMP-independent kinase. It is postulated that insulin may regulate this kinase.

The activity of acetyl CoA carboxylase is enhanced by a fatty acid and acyl CoA binding protein (FABP) from rat liver cytosol, which overcomes the inhibition of the enzyme by palmityl CoA. This discovery led to the theory that FABP may participate in the short-term regulation of lipogenesis (Lunzer et al., 1977).

The hypotriglyceridaemic agent clofibrate, when given to rats increases the concentration of FABP in the liver cytosol by two-fold. This can be correlated to an increase in the uptake of free fatty acid by perfused livers from clofibrate-treated rats. However, there has been no evidence for a change in the rate of esterification of free fatty acid following drug treatment (Renaud et al., 1978). The β -oxidation capacity of mitochondria has been reported to be enhanced by clofibrate (Lazarow and De Duve, 1976). Thus, it has been suggested that FABP plays an important part in the uptake and metabolism of free fatty acid in the liver.

It has been demonstrated that the levels of hepatic acetyl CoA carboxylase and fatty acid synthetase are affected by different nutritional factors. It has been shown that rat liver acetyl CoA carboxylase activity increases at the time of weaning (Lockwood et al., 1970). This increase has been related to a change from a high fat to a high carbohydrate diet.

A decrease in enzyme activity has been found to occur in fasted rats (Numa et al., 1961; Bortz and Lynen, 1963). Activity of acetyl CoA carboxylase is known to increase on re-feeding the fasted animals with a high-carbohydrate diet (Goodridge, 1973a; Craig et al., 1972; Gibson et al., 1972). The rates of both synthesis and degradation of acetyl CoA carboxylase are altered by the nutritional and hormonal states of the

animal (Nakanishi and Numa, 1970). These authors have shown that changes in the enzyme activity were accompanied by proportional changes in the amount of immunochemically-reactive protein. The relative rates of synthesis of acetyl CoA carboxylase as measured by the incorporation of [^{3}H] leucine were decreased 1.9 and 1.7 fold by fasting and in diabetes, respectively. However, the rate of enzyme degradation is the same in normal, refed and diabetic rats (half-life 59h), but is accelerated in fasted rats (half-life 31h). They suggested that the former animals are in a steady state, whereas the latter are adjusting to a new environment and this adjustment results in accelerated enzyme degradation. A specific polysome fraction which is involved in the synthesis of liver acetyl CoA carboxylase has been identified (Nakanishi et al., 1976; Tanabe et al., 1976). These authors have shown that the binding of [^{125}I] - anti-acetyl CoA carboxylase to the isolated polysomes from fasted rats is two-fold lower than to polysomes from normal animals, whereas this binding is four-fold higher when the polysomes are isolated from liver of a re-fed rat. Thus, the hepatic content of acetyl CoA carboxylase synthesising polysomes is closely correlated to changes in acetyl CoA carboxylase synthesis due to different nutritional states; the translational process is not greatly affected.

A number of hormonal factors have been implicated in the long-term regulation of the acetyl CoA carboxylase content of rat liver. It has been suggested that insulin stimulates synthesis of the enzyme and glucagon prevents the synthesis induced by carbohydrate feeding (Volpe and Vagelos, 1976).

2. Fatty acid synthetase

In the presence of NADPH, fatty acid synthetase catalyses the formation of fatty acid from acetyl CoA and malonyl CoA. This cytoplasmic enzymes has been resolved into seven subunits, each with a separate enzymic capability:

1. Acetyl CoA ACP transacylase
2. Malonyl CoA ACP transacylase
3. β -Ketoacyl ACP synthase
4. β -Ketoacyl ACP reductase
5. β -Hydroxybutyryl ACP dehydrase
6. Enoyl ACP reductase
7. Fatty acyl - S ACP thioesterase

The following reactions catalysed by the subunits are as follows:

Acetyl - S - CoA + ACP - SH $\xrightarrow{1}$ Acetyl - S - ACP + CoA - SH

Malonyl - S - CoA + ACP - SH $\xrightarrow{2}$ Malonyl - S - ACP + CoA - SH

Acetyl - S - ACP + malonyl - S - ACP $\xrightarrow{3}$ Acetoacetyl - S - ACP + CO_2 + ACP - SH

Acetoacetyl - S - ACP + NADPH + H^+ $\xrightarrow{4}$ D(-) - β- Hydroxybutyryl - S - ACP + $NADP^+$

D (-) - β- Hydroxybutyryl - S - ACP $\xrightarrow{5}$ Crotonyl - S - ACP + H_2O

Crotonyl - S - ACP + NADPH + H^+ $\xrightarrow{6}$ Butyryl - S - ACP + $NADP^+$

Fatty acyl - S - ACP + H_2O $\xrightarrow{7}$ fatty acid + ACP - SH

(ACP = Acyl carrier protein).

The major fatty acid produced is palmitate, This is thought to be a consequence of the high specificity of the thioesterase for 16 and 18 carbon acyl CoA derivatives compared to the low specificity for the shorter chain derivatives (Kumar et al., 1972). The molecular weight of rat liver fatty acid synthetase is about 500,000 (Kumar et al., 1972).

Hepatic fatty acid synthetase exhibits Km values for acetyl CoA of 4.4×10^{-6} M for malonyl CoA of 10×10^{-6} M (Nepokroeff et al., 1975) and for NADPH the Km is 4.0×10^{-5} M (Volpe and Kishimoto, 1972). It has been found that both acetyl CoA and malonyl CoA competitively inhibit the enzyme activity at high concentrations (Katiyar and Porter, 1974).

Recently, it has been shown that chicken liver fatty acid synthetase is irreversibly inactivated by malonyl CoA and by acetyl CoA plus malonyl CoA (Srinivasan and Kumar, 1981). These authors have demonstrated that the rate of inactivation is controlled by the conformational state of the enzyme. $NADP^+$ protects the enzyme against inactivation by acetyl CoA and malonyl CoA. Inactivation results from the enhanced covalent binding of malonyl CoA groups at multiple sites on the enzyme in addition to those required for fatty acid synthesis. The liver enzyme is also competitively inhibited by palmitoyl CoA and long-chain free fatty acids (Knoche et al., 1973). Bovine serum albumin protects fatty acid synthetase from the effect of the inhibitor (Knoche et al., 1973). The inhibition occurs at a palmitoyl CoA concentration of 20×10^{-6} M (Hsu et al., 1969). Other authors believe that the inhibition of fatty acid synthetase by long-chain acyl CoA is non-specific and is due to the detergent properties of the compound (Dorsey and Porter, 1968).

It has been demonstrated that rat liver fatty acid synthetase is inactivated by the substrate analogue chloroacetyl CoA. The overall loss of enzyme activity results from the inhibition of condensation-CO_2 exchange activity of the enzyme complex (Kumar et al., 1980). It is suggested that the data is consistent with a proposition that fatty acid synthetase consists of two functionally identical subunits of 250,000 molecular weight.

It has been suggested that CoA is required for the termination of the fatty acid synthetase reaction (Linn and Srere, 1980). These authors have shown that highly purified rat liver fatty acid synthetase is completely inhibited when assayed in the presence of a co-enzyme A - depleting system such as that catalysed by phosphotransacetylase, acetyl CoA synthetase, or ATP citrate lyase and the addition of free CoA causes a reversal of this inhibition (Linn and Srere, 1980; Linn et al., 1980; Sedgwick and Smith, 1981). In the absence of CoA, the rate of elongation of acyl moieties on both native fatty acid synthetase and fatty acid synthetase lacking the chain-terminating thioesterase I component was reduced 100-fold and the addition of CoA promoted elongation of acyl - S - multienzyme thioester without affecting the

release from the enzyme (Sedgwick and Smith, 1981).

The enzyme activity appears to be stimulated by Pi and various organic phosphates (Wakil et al., 1957). The most effective stimulator is Fru 1,6 - P_2 but the levels of this effector required to produce a significant activation are far above the physiological levels. Kinetic studies show that the effect of the Fru 1,6 - P_2 was to decrease the Km for NADPH whilst reversing the inhibition by malonyl CoA (Plate et al., 1968).

Long-chain acyl CoA have also been reported to inhibit fatty acid synthetase, but again the physiological significance of this effect is doubtful and the general conclusion drawn is that it is acetyl CoA carboxylase and not fatty acid synthetase which is the critical enzyme in the acute regulation of lipogenesis (Lane et al., 1974).

Nutrional factors are also known to be important in the adaptive changes in fatty acid synthetase activity; for example, fasting causes a lowering of enzyme activity (Craig et al., 1972) and re-feeding fasted animals with a high-carbohydrate, low-fat diet results in the induction of enzyme synthesis leading to a great increase in the level of the enzyme in the liver. The nature of the dietary carbohydrate is important on the fatty acid synthetase activity. Thus, fructose feeding increases fatty acid synthetase by 2-3 fold more than glucose feeding (Bruckdorfer et al., 1972a; Volpe and Vagelos, 1974). This difference has been ascribed to the fact that fructose metabolism to pyruvate is more rapid than glycolysis from glucose and that the induction of fatty acid synthetase is the result of elevated levels of the precursors of fatty acids.

Fatty acid synthetase activity is low in diabetic rats but is restored to normal after insulin administration (Lakshmanan et al., 1972 Craig and Porter, 1973). The increase in enzyme activity appears to be the result of an increase in the rate of enzyme synthesis (Lakshmanan et al., 1972) Other authors have found no evidence that insulin is necessary for the regulation of liver fatty acid synthetase activity. Volpe and Vagelos, (1974) demonstrated an increase in liver enzyme activity following fructose feeding in both normal and diabetic rats. These authors suggest that it is the carbohydrate intake which is causing

the increase in fatty acid synthetase rather than a specific insulin effect. It has been shown that fructose-feeding results in high levels of hepatic fatty acid synthetase and plasma triacylglycerol, but low adipose fatty acid synthetase and plasma insulin levels (Bruckdorfer et al., 1972a). Thus, these authors did not find any correlation between plasma insulin levels and hepatic fatty acid synthetase activity, but there was a correlation between the latter and plasma triacylglycerol levels when the animals were fed on fructose or sucrose. Then the rate of hepatic lipogenesis may become an important factor in determining plasma triacylglycerol levels.

The induction of liver fatty acid synthetase in fasted-re-fed rats can be inhibited by glucagon and theophylline (Volpe and Marasa, 1975) and by cAMP (Lakshmanan et al., 1972). They suggested that the enzyme activity is regulated by the relative levels of insulin and glucagon in the blood. The effects of insulin and glucagon are regarded to be due to changes in the rate of enzyme synthesis (Lakshmanan et al., 1972; 1975) and this is a reflection of the translatable fatty acid synthetase mRNA content of the liver (Pry and Porter, 1981).

It has been demonstrated that hepatic fatty acid synthetase activity increases in hyperthyroid animals (Volpe and Kishimoto, 1972; Baquer et al., 1976). Neither adrenalectomy nor hydrocortisone administration have any effect on the liver enzyme but adipose tissue fatty acid synthetase activity is decreased in animals treated with the hormone and increased in adrenalectomised animals (Baquer et al., 1976). This change in the activity of the enzyme in adipose tissue is due to change in the rate of enzyme synthesis. It has been demonstrated that, although adrenalectomy of normal rats has no effect on liver fatty acid synthetase activity (Volpe and Maraza, 1975), similar treatment of diabetic rats results in restoration of the decreased fatty acid synthetase which is found in livers of diabetic animals (Volpe and Maraza, 1975).

The presence of polyunsaturated fat in the diet is also known to reduce the levels of hepatic fatty acid synthetase (Bruckdorfer et al., 1972b). In this context Flick et al., (1977) have shown that the induction of hepatic fatty acid synthetase is markedly reduced by feeding linoleate; an effect which is reflected both in a decrease in the half-life of the enzyme and in a decreased rate of synthesis of the

enzyme.

3. ATP-citrate lyase (E.C.4.1.3.8), ATP; citrate oxaloacetate-lyase (CoA - acetylating and ATP-dephosphorylating).

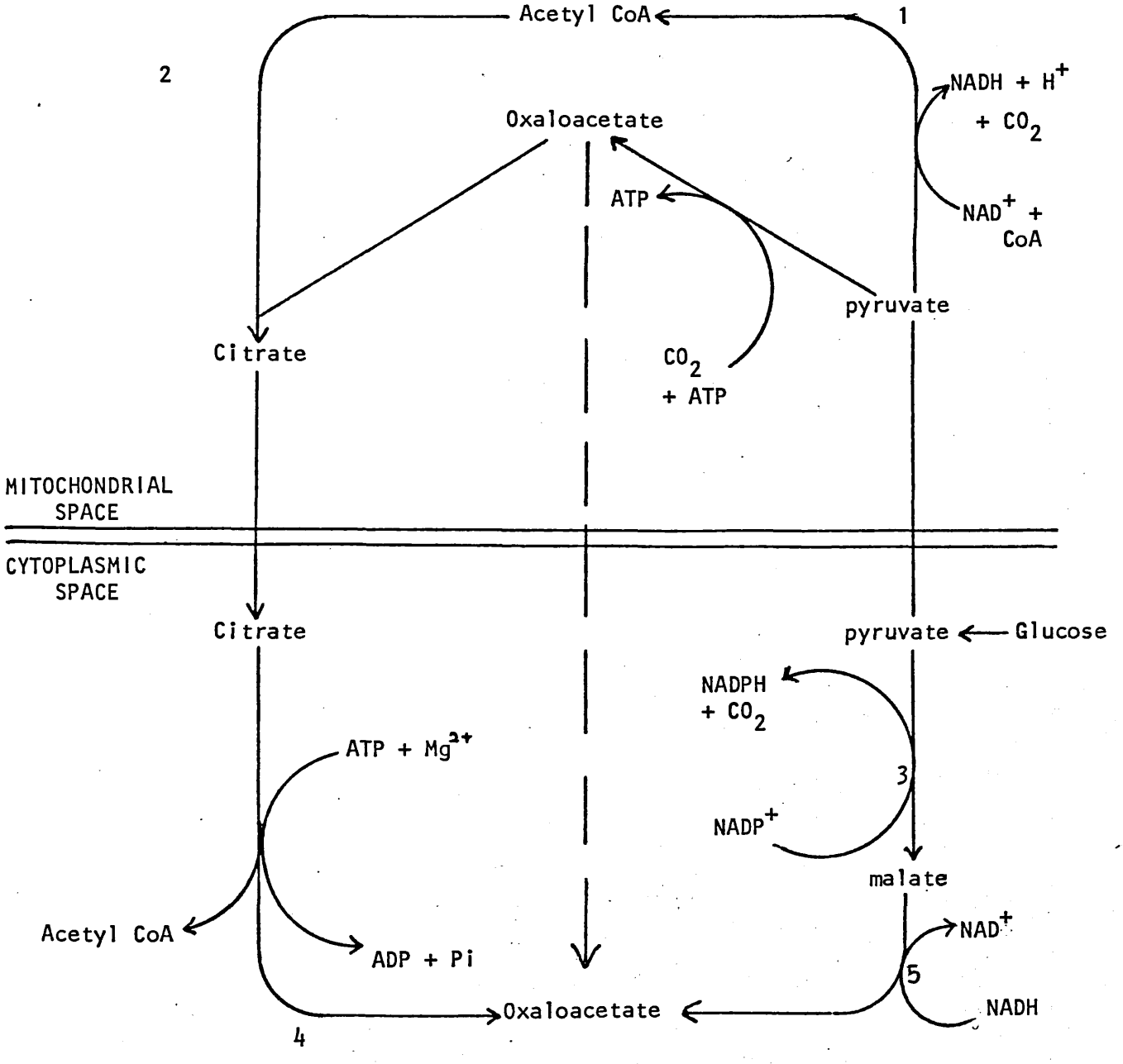
ATP-citrate lyase, also known as citrate-cleavage enzyme, is a key lipogenic enzyme providing cytosolic acetyl CoA for fatty acid synthesis from citrate produced in the mitochondria (Kornacker and Lowenstein, 1965a).

The enzyme catalyses the following reaction:

$$\text{Citrate} + \text{ATP} + \text{CoA} \longrightarrow \text{acetyl CoA} + \text{oxaloacetate} + \text{ADP} + P_i$$

Translocation of acetyl CoA from mitochondria into cytoplasm is an obligatory process in de novo fatty acid synthesis. There are two hypotheses for the mechanism of this transfer. Firstly, that free acetate leaves the mitochondria and is then converted to acetyl CoA by acetyl CoA synthase. Secondly, that citrate generated by the action of mitochondria is used to generate acetyl CoA in the cytoplasm by the action of ATP-citrate lyase (Lowenstein, 1968) (Fig. 5). Acetyl CoA synthetase and ATP-citrate lyase are both present in rat liver (Kornacker and Lowenstein, 1965a; Barth et al., 1971; 1972), but the level of the latter is much higher, suggesting that the citrate shuttle is the most important mechanism for acetyl CoA transfer from mitochondria to cytoplasm, although it has been reported (Endemann et al., 1982) that ketone bodies may contribute significantly to lipogenesis via a cytoplasmic acetoacetyl CoA synthase.

ATP-citrate lyase has a Km of 5.8×10^{-4} M for citrate (Spector, 1972). The specific enzyme activity is reported to be 2.4 μmol/min/g liver (Plowman and Cleland, 1967). It has been shown that Mg^{2+} stimulates the enzyme activity and that ADP is a competitive inhibitor K_i 1.71×10^{-4} M (Walsh and Spector, 1968; 1969). The enzyme is susceptible to oxidation of the sulphydryl group at the active site and EDTA is also needed to prevent enzyme inactivation (Walsh and



Enzymes

1. pyruvate dehydrogenase
2. citrate synthase
3. malate dehydrogenase ($NADP^+$)
4. ATP-citrate lyase
5. NAD^+-linked malate dehydrogenase

FIG. 5 Scheme for the generation of cytoplasmic acetyl CoA from pyruvate showing the involvement of the mitochondria.

Spector, 1969).

The activity of citrate-cleavage enzyme varies in accordance with the nutritional state of the animal. The activity of the enzyme is suppressed on starvation and is restored on re-feeding (Kornacker and Lowenstein, 1965b). These authors have shown that the increase in enzyme activity that occurs on re-feeding starved animals depends on the diet. The enzyme activity is highest in animals fed on diets rich in carbohydrate and low in fat, and lowest in animals fed on diets high in fat or in alloxan-diabetic animals. These authors have shown that diets high in glucose or fructose elevate the activity of citrate-cleavage enzyme in normal animals, whereas only the diet high in fructose does so in diabetic animals. The specific activities of ATP-citrate lyase increased significantly in rats fed on the fructose-based diet (Pearce, 1980). A high-fat diet containing polyunsaturated fatty acids results in the conversion of the enzyme into a catalytically inactive form (Schwartz and Abraham, 1981).

The activity of ATP-citrate lyase is under hormonal regulation. Acute insulin administration to the rat or to isolated perfused normal liver causes an increase in enzyme activity (McCormick et al., 1978) and these authors have also demonstrated that the effect of chronic hyperinsulinaemia in young rats is an increase in rat liver citrate-cleavage enzyme over a 6-day period. Yen et al.,(1976) found a higher-enzyme activity in obese mice with hyperinsulinism, in comparison to that in lean mice with lower plasma insulin level. They suggested that there is a positive correlation between the plasma insulin level and ATP-citrate lyase activity. Addition of insulin (1 μM) to cultured hepatocytes increases ATP-citrate lyase activity by 2.6-fold, whereas glucagon (0.1 μM) decreases the activity by 68% (Spence et al., 1979) Bt_2 cAMP (0.1 μM) mimicked the effect of glucagon and glucagon supplementation of culture medium already containing Bt_2 cAMP had no additive effect. The increase in enzyme activity caused by insulin is the result of new enzyme protein synthesis as was shown by the increased incorporation of radioactivity into ATP-citrate lyase immunoprecipitated from extracts of hepatocytes (Spence et al., 1979). These authors have also shown that, when hepatocytes isolated from thyroidectomized rats were placed in culture, they exhibited decreased levels of ATP-citrate

lyase activity which could be restored by the addition of 3,3',5-triiodo-L-tyronine (10 μM) to the culture medium.

ATP-citrate lyase is reported to be phosphorylated via a glucagon stimulated cAMP-dependent protein kinase (Janski et al., 1979; Ranganathan et al., 1980, 1982) and by an insulin-stimulated cAMP-independent mechanism (Alexander et al., 1982) but neither modification results in a change in the regulatory or catalytic properties of the enzyme. It is suggested that the enzyme may be subjected to hormonal influences which alter its susceptibility to degradation (Osterlund et al., 1980; Vogel and Bridger, 1981).

B. Regulation of lipogenesis

1. Hormonal control

The biosynthesis of fatty acid is regulated by both nutritional and hormonal factors. These factors initiate short-term as well as adaptive mechanisms for controlling fatty acid synthesis (Goodridge, 1975). Lipogenesis has been reported to be diminished in the insulin-deficient, diabetic animal and insulin has been reported to stimulate lipogenesis both in vivo and in vitro (Hers, 1977). Insulin has been reported to increase fatty acid synthesis by isolated rat hepatocytes (Geelen and Gibson, 1975; Müller et al., 1976; Geelen et al., 1978b). It has been shown that physiological levels of insulin (1 to 10 ng/ml and higher) stimulates the incorporation of $[1\text{-}^{14}C]$ acetate and $^{3}H_2O$ into fatty acids in cultured rat hepatocytes (Geelen and Gibson, 1976). Insulin has also been shown to stimulate fatty acid synthesis in freshly isolated hepatocytes from livers of neonatal chicks (Goodridge, 1973c).

Since those reports a number of papers showing the short-term stimulation of lipogenesis by insulin in isolated hepatocytes (Müller et al., 1976; Geelen et al., 1978b, Witters et al., 1979b; Assimacopolous-Jeannet et al., 1981) and in perfused liver (Assimacopolous-Jeannet et al., 1977) have appeared. These changes have been correlated to apparent changes in acetyl CoA carboxylase levels (Geelen et al., 1978b; Witters et al., 1979b; Assimacopolous-Jeannet et al., 1981). Little is known about the substrate availability for fatty acid synthesis, glucose has been reported to be a poor precursor

of fatty acids whereas glycogen and lactate are much better precursors (Clark et al., 1974; Bloxham et al., 1977). The effects of insulin could be related to the availability of lactate and pyruvate, since the hormone is known to promote glycolysis. Generally, lipogenesis is measured by the incorporation of $[1 - {}^{14}C]$ acetate, L-$[U - {}^{14}C]$ lactate, or $^{3}H_2O$ into fatty acids. Fatty acid synthesis by isolated liver cells as assayed by the latter method is dependent on the availability of lactate and pyruvate (Harris, 1975). There is a lag in fatty acid synthesis which reflects the time required for lactate and pyruvate to accumulate. It is of interest to note that inclusion of high concentrationsof lactate and pyruvate in the hepatocyte incubation medium results in a loss of the insulin response, suggesting that fatty acid synthesis is maximally stimulated under these conditions. This implies that the stimulation of lipogenesis by insulin may simply be the result of lactate accumulation. However, Assimacopolous-Jeannet et al., (1977)have also found that lipogenesis from $[1 - {}^{14}C]$ acetate is also stimulated by the hormone in a perfused liver system, suggesting that control is also exerted at a post-acetyl CoA step in fatty acid synthesis, presumably acetyl CoA carboxylase. Thus, lactate or pyruvate may stimulate lipogenesis independently by their roles as substrates. More recently Rognstad and Katz (1980) have estimated lipogenesis from $^{3}H_2O$ and $[3 - {}^{14}C]$ lactate by isolated hepatocytes from fasted-re-fed rats in the presence and absence of added lactate. Glucagon inhibition of lipogenesis from both substrates is overcome by the addition of unlabelled lactate to the medium, suggesting that the major effect of the hormone is, in fact, to control glycolytic flux.

The rate of hepatic lipogenesis from $^{3}H_2O$ has been reported to be increased in the perfused mouse liver by glucose (Assimacopolus-Jeannet et al., 1977). These authors have shown that the rate of lipogenesis in insulin pre-treated animals was higher than that of animals which were treated with anti-insulin serum prior to the perfusion. It has also been reported that when rat liver is perfused with a low concentration of free fatty acid (0.3mM) then insulin has no effect on the rate of lipogenesis. High concentrations of free fatty acid (1.9mM) resulted in an inhibition of lipogenesis which could be partially overcome by addition of insulin to the perfusate (Topping and Mayes, 1976).

Glucagon antagonises insulin effects in fatty acid synthesis. Administration of glucagon inhibits fatty acid synthesis by a short-term as well as by an adaptive mechanism (Lakshmanan et al., 1972; Lee et al., 1973, Volpe and Vagelos, 1974; Goodridge, 1975). Both effects appear to be mediated via cAMP (Bricker and Levy, 1972; Lakshmanan et al., 1972).

Glucagon inhibits fatty acid synthesis by isolated hepatocytes (Goodridge, 1973c; Geelen and Gibson, 1975; Müller et al., 1976; Cook et al., 1977; Geelen et al., 1978b) and blocks the insulin effect in rat hepatocytes cultures (Geelen and Gibson, 1976).

Glucagon has also been reported to inhibit fatty acid synthesis by perfused mouse liver at hormone concentrations greater than 10^{-9}M (Ma et al., 1978). This concentration is about two orders of magnitude higher than that required for the stimulation of glycogen breakdown. These authors proposed that the action of glucagon on hepatic fatty acid biosynthesis could be secondary to depletion of glycogen (Ma et al., 1978). However, hepatic fatty acid synthesis as measured by incorporation of tritium from ^3H_2O into fatty acids was inhibited 60% by glucagon in vivo within 15 min after injection of the hormone. This inhibition was correlated to a lowering of hepatic malonyl CoA levels. However, the activity of acetyl CoA carboxylase and the synthesis of cholesterol were not affected by glucagon treatment (Cook et al., 1977). The conclusion drawn by these authors is that the only explanation for these observations was that acetyl CoA carboxylase was inhibited in vivo by an unknown mechanism. Cook et al.,(1978) also reported that malonyl CoA levels could be increased in isolated hepatocytes by glucose and by lactate plus pyruvate and decreased by glucagon and oleate.

Glucagon has been reported to change the direction of fatty acid matabolism from synthesis to oxidation in isolated rat hepatocytes (McGarry et al., 1978). The inhibition of fatty acid synthesis was shown to be largely offset by addition of lactate plus pyruvate, but glucagon did exert some inhibitory effect on lipogenesis in the presence of these substrates. The changes in cellular citrate and malonyl CoA levels indicated that glucagon exerts its inhibitory effect on fatty acid synthesis at two levels; firstly, the blockade of glycolysis and secondly, by partial inhibitionof acetyl CoA carboxylase. A central

role is postulated for malonyl CoA in altering the balance between fatty acid synthesis and oxidation since this intermediate is a potent inhibitor of carnitine acyltransferase. The antiketogenic action of insulin may be mediated entirely via changes in tissue malonyl CoA (McGarry et al., 1978). They propose that in the fed animal the insulin/glucagon ratio and malonyl CoA levels are high and fatty acid synthesis is rapid, whereas fatty acid oxidation is reduced to a minimum. In the fasted or diabetic animal insulin/glucagon ratio and malonyl CoA levels are low and fatty acid oxidation predominates.

Guynn et al., (1972) have reported that the rate of fatty acid synthesis is directly related to the concentration of malonyl CoA in vivo, but these authors could find no correlation between the rate of lipogenesis and the hepatic contents of citrate, ATP, ADP, glucose, glucose 6-phosphate or sn-glycerol 3-phosphate. These authors concluded that the short-term control of fatty acid synthesis in vivo probably results from an inhibition of acetyl CoA carboxylase by long-chain acyl CoA which accumulates under conditions where lipogenesis is inhibited.

The concentration of malonyl CoA is correlated in a number of situations with the rate of lipogenesis which, in turn, is dependent on the supply of precursors such as glycogen, glucose, lactate and pyruvate (Guynn et al., 1972; Cook et al., 1977; McGarry et al., 1978). It has been shown that lipogenesis is inhibited in perfused liver (Mayes and Topping, 1974) or hepatocytes (McGarry et al., 1978) from fed rats by increased concentrations of non-esterified fatty acids. It has been reported that, in hepatocytes from meal-fed rats, malonyl CoA could be increased by glucose or by lactate plus pyruvate and decreased by both glucagon and oleic acid (Cook et al., 1978).

When chick hepatocytes metabolising glucose and acetate as sole exogenous substrates are treated with Bt_2 cAMP or glucagon, the level of cellular citrate and the rate of incorporation of D-[U-^{14}C] glucose, [1-^{14}C] acetate, or ^3H_2O into fatty acids decrease concomitantly by over 90%. Most of the citrate (> 75%) is found in the cytoplasmic compartment where acetyl CoA carboxylase is localised (Watkins et al.,1977).

It is proposed that regulation of fatty acid synthesis by Bt_2 cAMP is due, in part, to changes in the citrate level (Clarke et al., 1979). These authors have also shown that fatty acid synthesis from [1 - ^{14}C] acetate is partially inhibited by Bt_2 cAMP in the presence of fructose, lactate and pyruvate, despite a high citrate level, indicating that the site of action of the cyclic nucleotide involves the regulation of the utilisation of cytoplasmic acetyl CoA. Incorporation of D - [U - ^{14}C] fructose, [2 - ^{14}C] pyruvate or D - [U - ^{14}C] lactate into fatty acid is similarly depressed by Bt_2 cAMP. However, the effect of glucagon and of Bt_2 cAMP on cellular citrate levels in the rat liver cell appears to differ from that in the chick liver cell. The large decrease in total cellular citrate level caused by glucagon and Bt_2 cAMP, which accompany decreased fatty acid synthesis from [1 - ^{14}C] acetate and ^3H_2O in chick liver cells, is not observed in rat hepatocytes (Lane et al., 1974, Harris, 1975; Cook et al., 1978) Another possible mechanism for the effect of glucagon on lipogenesis suggested by Müller and Jeanrenaud (1978) is that in the presence of glucagon or cAMP hepatic lipogenesis might be inhibited by intracellular accumulation of fatty acyl CoA, derived from increased cAMP-mediated triacylglycerol breakdown (Müller and Jeanrenaud, 1978). These authors have also found that the inhibition of lipogenesis by glucagon was accompanied by a significant decrease in the activity of acetyl CoA carboxylase assayed in the presence and absence of added citrate (Müller and Jeanrenaud, 1978).

Hopkirk and Bloxham (1977; 1979) have found that diurnal rhythm of lipogenesis occurs in meal-fed rats in vivo, and also in hepatocytes isolated from these animals. The increase in fatty acid synthesis is clearly correlated with periods of feeding. At least some of the increased flux of carbon into fatty acids is derived from glycogen, which suggests that regulation of glycolysis can play a role in the control of fatty acid synthesis.

Postle and Bloxham (1980) have shown that glucocorticoids exert a permissive action on the expression of the glucagon inhibition of lipogenesis, and they suggest that the effect of glucagon may be mediated via changes in pyruvate kinase, which becomes insensitive to glucagon in adrenalectomized animals despite the action of cAMP-dependent protein kinase on pyruvate kinase being retained. Bloxham

and York (1976) have also correlated changes in lipogenesis with flux through phosphofructokinase.

A number of other hormones have been shown to exert a short-term effect on hepatic lipogenesis Angiotensin II, adrenaline (Ma et al., 1977) and vasopressin (Ma and Hems, 1975; Hems, 1977) inhibited lipogenesis in perfused mouse liver. These hormones do not apparently exert their effects via changes in hepatic cAMP levels (Hems, 1977) but, possibly, via changes in Ca^{2+} concentration (Assimacopoulos-Jeannet et al., 1981). The effect of vasopressin was noted to occur in perfused mouse liver but not in perfused rat liver (Kirk and Hems, 1979). The reason for this discrepancy is not clear. It is of interest however, that the stimulatory effect of vasopressin on lipogenesis by isolated hepatocytes is abolished by raising the lactate and pyruvate concentration in the medium (Assimacopoulos-Jeannet et al., 1981)

2. Inhibition of lipogenesis by free fatty acids

De novo synthesis of fatty acid by the liver is inhibited by a high level of dietary fat (Jeffcoat and James, 1977; Jeffcoat et al., 1979). This inhibition is probably the result of adaptive changes in acetyl CoA carboxylase and fatty acid synthase in response to the availability of fatty acyl CoA (Volpe and Vagelos, 1976; Flick et al., 1977; Halperin et al., 1972). Dietary linoleate has been shown to markedly inhibit the induction of fatty acid synthesis in rat liver (Flick et al., 1977). In addition, it is known that an inverse relationship exists between plasma free fatty acid concentration and the rate of fatty acid synthesis.

It is well known that exogenous free fatty acid inhibits lipogenesis by various cell cultures by short-term and long-term regulation (Goodridge et al., 1974; Volpe and Vagelos, 1976).

Goodridge (1973c) found that albumin-bound free fatty acids effectively inhibited fatty acid synthesis from $\left[1 - {}^{14}C\right]$ acetate by isolated chick hepatocytes, and he suggested that fatty acid synthesis is regulated by the intracellular level of long-chain acyl CoA which

is known to increase under these conditions (Goodridge et al., 1974). A similar inhibition of lipogenesis by long-chain fatty acids has been found in rat hepatocytes; stearic acid is the most efficient inhibitor (Nilsson et al., 1974) despite the fact that this is utilised at a lower rate than other fatty acids (Sundler et al., 1974). The degree of inhibition by various fatty acids (Nilsson et al., 1974) has been correlated to the inhibition of ACC by the corresponding acyl CoA derivatives (Numa et al., 1963).

The physiological role of long-chain acyl CoA in the regulation of lipogenesis has been questioned on the basis of difficulties associated with the detergent properties of palmityl CoA and other long-chain acyl CoAs. However, the change in hepatic lipogenic activity in fasted or diabetic conditions correlated to the changes in palmityl CoA have strengthened the case for the physiological role of these CoA derivatives in the negative feedback inhibition of lipogenesis (Goodridge, 1973b; Hsu et al., 1975; Block and Vance, 1977).

An inverse logarithmic relationship has been observed between lipogenesis and serum FFA concentration in the perfused liver (Mayes and Topping, 1974; Topping and Mayes, 1976). This suppression in lipogenesis with increasing concentration of FFA is similar to the diminution observed in active liver pyruvate dehydrogenase in the presence of FFA (Wieland et al., 1972). Therefore, it was proposed that changes in activity of this enzyme might be a regulatory point in the supply of acetyl CoA for hepatic lipogenesis (Mayes and Topping, 1974). It was also observed that, at high concentrations of serum FFA, insulin antagonised their anti-lipogenic action (Topping and Mayes, 1976). In liver perfused with increasing FFA concentrations, total adenine nucleotides are significantly decreased and $[ATP]/[AMP]$ ratio increased; pyruvate dehydrogenase activity is negatively correlated with $[ATP]/[AMP]$ and $[ATP]/[ADP]$ ratios (Topping et al., 1977). These authors show that insulin modifies the effects of FFA on pyruvate dehydrogenase. The ability of insulin to increase pyruvate dehydrogenase activity may be accounted for by an anti-lipolytic effect of the hormone which would effectively cause a decreased availability of intracellular FFA (Topping and Mayes, 1972).

Lipogenesis by isolated hepatocytes is also controlled by VLDL and and chylomicron fractions isolated from human plasma and rat serum

(Lakshmanan et al., 1977). Fatty acid synthesis is inhibited by about 50% after incubation with the triacylglycerol-rich lipoprotein fractions. Chylomicron remnants, produced by the action of lipoprotein lipase, caused a significant inhibition of [1 - ^{14}C] acetate and $^{3}H_2O$ incorporation into fatty acids by isolated hepatocytes (Koizumi et al., 1979).

It is also apparent that VLDL binds to a high-affinity site on the hepatocytes. It is suggested that the specific interaction leads to hydrolysis of triacylglycerol, the free fatty acid formed enter the cell and are converted to acyl CoAs which, in turn, cause a feedback inhibition of ACC (Lakshmanan et al., 1977). Koizumi et al., (1979), however, found a greater inhibition of lipogenesis by chylomicron remnants than by chylomicrons and they suggest that it is the degree of catabolism and the amount of chylomicron remnant which are important in inhibition of fatty acid synthesis.

3. Source of carbon for fatty acid synthesis

It has been found that in hepatocytes prepared from livers of ad libitum and meal-fed rats, fructose, at concentration below 10mM, is a better precursor of fatty acids than glucose but the reverse is true at higher concentrations. Also, lactate carbon is a much better fatty acid precursor than glucose carbon when both substrates are present at physiological concentrations (Clark et al., 1974). Data obtained following perfusion of mouse liver with L - [U - ^{14}C] lactate and [U - ^{14}C] glucose shows that circulating glucose at concentrations less than 17 mM is not a major carbon source for newly synthesised fatty acid whereas lactate (10 mM) markedly stimulates fatty acid synthesis and contributes extensive carbon to lipogenesis (Salmon et al., 1974). It has also been shown that the rate of fatty acid synthesis by isolated hepatocytes is increased when substrates such as glucose, fructose, DHA, lactate and pyruvate are used whereas xylitol and glycerol are inhibitory (Harris, 1975). The effects of fructose and DHA on lipogenesis are presumably related to the relative ease of their conversion to pyruvate.

The de novo synthesis of fatty acids from [1 - ^{14}C] acetate is stimulated by fructose (11 mM), glycerol (20 mM), DHA (10 mM), lactate

(10 mM) and pyruvate (10 mM) in isolated hepatocytes prepared from neonatal chicks (Goodridge, 1973c). However, the stimulatory effects of fructose and DHA are not observed when $^{3}H_2O$ was used as a substrate for the estimation of fatty acid synthesis (Goodridge, 1973c).

Bloxham et al., (1977) claim that lactate is a major source of carbon for fatty acid synthesis in rat hepatocytes at low concentrations of the substrate and possibly the only source at higher concentrations. These authors have shown a poor correlation between incorporation of $^{3}H_2O$ and $[U - ^{14}C]$ glucose and a lack of enhancement of $[^{3}H]$ incorporation into fatty acid by increasing glucose concentration, suggesting that external glucose carbon is not a major substrate for fatty acid synthesis. The preference for lactate in the rat is of physiological significance since, in meal-fed rats, there is a dramatic rise in hepatic-portal lactate concentration (10-15 mM) following ingestion of a meal demonstrating the rapid availability of lactate for lipogenesis (Bloxham et al., 1977). These authors have found that at low extracellular lactate concentrations, tritium incorporation into glyceride fatty acids is higher than L - $[U - ^{14}C]$ lactate and this difference may be accounted for by conversion of an intrahepatic precursor, such as glycogen, to fatty acids. The possible role of glycogen as a precursor for lipogenesis has also been suggested by Salmon et al.,(1974). The utilisation of the endogenous precursor, therefore, must be masked by high extracellular lactate (20 mM) when the incorporation of L - $[U - ^{14}C]$ lactate and of $^{3}H_2O$ are virtually identical (Bloxham et al., 1977). These authors have also demonstrated that a fall in hepatic glycogen during hepatocyte incubation could be related to the rate of lipogenesis. Müller and Jeanrenaud (1978) have also shown that lipogenesis from $^{3}H_2O$ is markedly stimulated in the presence of glucose, fructose or lactate whereas it is not stimulated by acetate.

A number of glucose precursors, including glycerol, lactate and fructose and DHA are antiketogenic and this may account for the stimulation of lipogenesis. Dihydroxyacetone stimulated the rate of lipogenesis by about 37% in the absence of oleate (Harris, 1975; Williamson and Whitelaw, 1978). It has been shown that addition of oleate inhibits lipogenesis by about 65%, but DHA relieves this inhibition (Mayes and Topping, 1974). This suggests that there is not

a simple reciprocal relationship between ketogenesis and lipogenesis and that the distribution of carbon between the two pathways is controlled by at least two factors.

It has been reported that high concentrations of fructose cause inhibition of lipogenesis (Clark et al., 1974) which is probably related to the depletion of ATP (see section I, C). Fructose in a concentration of 15 mM inhibits lipogenesis from ^3H_2O by 75% in isolated liver cells from fed rats (Selmer and Grunnet, 1976). Three possible links between ATP and lipogenesis exist: ATP-citrate lyase, acetyl CoA synthase and ACC. The increase in acetyl CoA (Thurman and Scholz, 1973) and in citrate (Sestoft et al., 1972) concentration observed when fructose is metabolised makes ACC the most likely candidate for the inhibited step in fatty acid synthesis during fructose metabolism. It is, however, unlikely that the decreased lipogenesis is the result of inhibition of acetyl CoA carboxylase by decreased ATP levels because of the high affinity of the enzyme for ATP (Alberts and Vagelos, 1972). The mechanism of the inhibition remains a mystery.

III. TRIACYLGLYCEROL METABOLISM

The liver occupies a central role in the metabolism of glycerolipid. It synthesises triacylglycerols and phosphoglycerides for intrahepatic use as an energy reserve and also manufactures these important glycerolipids for export in the form of lipoproteins via the blood to other tissues. Hepatic triacylglycerol synthesis requires long-chain acyl CoA which may be derived either from de novo fatty acid synthesis or from circulating FFA produced by the action of the enzyme long-chain acyl CoA synthase. The glycerol moiety of the triacylglycerol is thought to be mainly derived from sn-glycerol 3-phosphate.

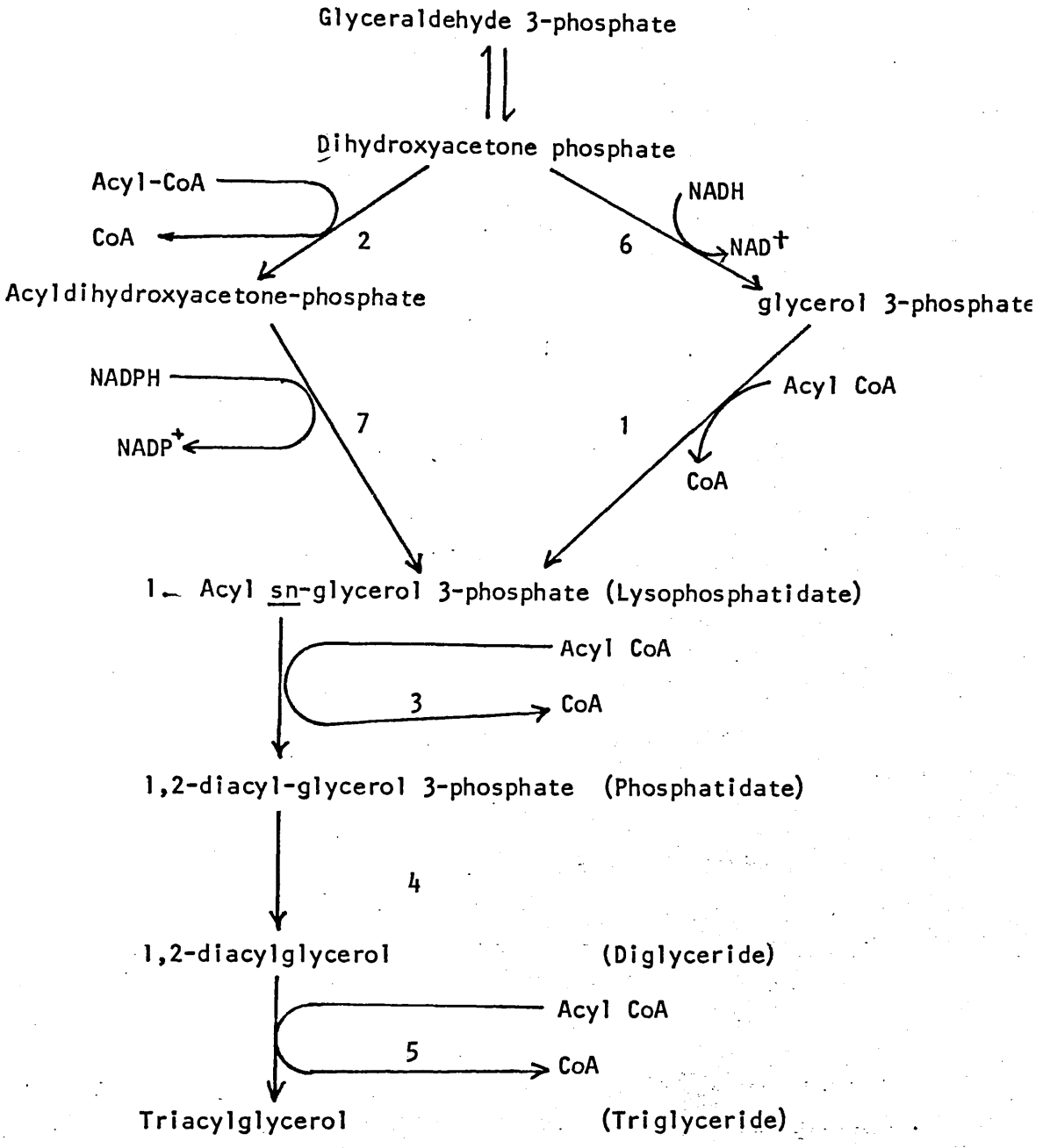
A. Triacylglycerol synthesis

1. Biosynthesis of 1-acylglycerol phosphate

The major pathway for the biosynthesis of the glycerol moiety of the lipids has long been considered to be via sn-glycerol 3-phosphate produced from dihydroxyacetone phosphate (DHAP) by the action of the sn-glycerol 3-phosphate dehydrogenase or from glycerol by glycerokinase.

However, evidence has been produced for another pathway involving acylation of DHAP. The proposed pathway for the synthesis of phosphatidate is illustrated in Fig. 6 (Pollock et al., 1975).

Acyldihydroxyacetone phosphate was first discovered as a lipid which was rapidly labelled by ^{32}Pi or by γ-[^{32}P]ATP in guinea pig liver mitochondria (Hajra and Agranoff, 1967; 1968). This rapid labelling was due to the dephosphorylation and rephosphorylation of endogenous acyldihydroxyacetone phosphate in mitochondria (Hajra et al., 1968). Acyldihydroxyacetone phosphate was shown to be biosynthesised by the direct acylation of DHAP with long-chain acyl CoA (Hajra, 1968). It has been found that the reduction of acyldihydroxyacetone phosphate requires an NADPH - dependent, mitochondrial dehydrogenase (Hajra and Agranoff, 1968). When the rates of formation of phosphatidic acid from DHAP are compared, either in the presence of NADH (sn-glycerol 3 - phosphate pathway) or in the presence of NADPH (acyldihydroxyacetone phosphate pathway), it is found that phosphatidic acid is formed more rapidly via DHAP than via sn-glycerol 3-phosphate (Pollock et al., 1975). The same conclusion is reached when [4 - ^{3}H] NADH and [4 - ^{3}H] NADPH are used as substrates to compare the two pathways (Agranoff and Hajra, 1971; Hajra, 1973). Using specifically labelled glycerol, it has been shown that a significant amount of glycerolipid is synthesised via the DHAP pathway by rat liver slices (Manning and Brindley, 1972; Bowley et al., 1973). However, when isolated rat liver parenchymal cells are incubated with [U - ^{14}C, 2 - ^{3}H] glycerol, glycerides are produced with $^{3}H/^{14}C$ ratios in the glycerol moieties similar to that of intracellular sn-glycerol 3 - phosphate (Rognstad et al., 1974). This indicates that isolated liver cells show less dependence on the acyldihydroxyacetone phosphate pathway than that claimed for liver slices (Okuyama and Lands, 1970; Manning and Brindley, 1972) since the conversion of sn-glycerol 3-phosphate to DHAP would result in the loss of ^{3}H. The approach of Okuyama and Lands (1970) and Manning and Brindley (1972) has also been criticised by Curstedt (1974) and Curstedt and Sjövall (1974), who found that, in the presence of [1,1 - $^{2}H_2$] ethanol, the deuterium labelling at C - 2 of the glycerol moiety of phosphatidylcholine was 2-3 times higher than that at C - 2 of the total hepatic sn-glycerol 3-phosphate pool. This indicated that a specific pool of sn-glycerol 3-phosphate might be used for synthesis of phosphoglycerides in the liver. If this is the case then it is difficult to evaluate the relative



Enzymes

1. Glycerol 3-phosphate acyltransferase (E.C.2.3.1.15)
2. Dihydroxyacetone phosphate: AcylCoA transferase (E.C.2.3.1.42)
3. Acyl-glycerol 3-phosphate acyltransferase
4. Phosphatidate phosphohydrolase (E.C.3.1.3.4)
5. Diacylglycerol acyltransferase
6. Glycerol 3-phosphate dehydrogenase (E.C.1.1.1.8)
7. Acyl dihydroxyacetone phosphate reductase (E.C.1.1.1.101)

Fig. 6 The glycerol phosphate and acyldihydroxyacetone phosphate pathways for the de novo synthesis of glycerolipids.

importance of the pathway via acyldihydroxyacetone phosphate by comparing the $^{3}H/^{14}C$ ratios of the phosphoglycerides with that of total hepatic sn-glycerol 3-phosphate; therefore, these authors have concluded that the acyldihydroxyacetone phosphate pathway is of little importance in the synthesis of phospholipids in the liver. An estimation of rates of phosphatidic acid synthesis from DHAP or sn-glycerol 3-phosphate in homogenates of various tissues, including liver, has shown that the DHAP enters into phosphatidic acid more rapidly via acyldihydroxyacetone phosphate, suggesting a significant contribution of the pathway via acyldihydroxyacetone phosphate *in vitro* (Pollock et al., 1975) despite the fact that sn-glycerol 3-phosphate dehydrogenase and sn-glycerol 3-phosphate acyltransferase are more active than the dihydroxyacetone phosphate: acyl CoA transferase. However, the *in vivo* concentration of sn-glycerol 3-phosphate in liver is 134 nmoles/g and that of DHAP is 17 nmoles/g (Greenbaum *et al.*, 1971). This, together with the evidence of the kinetic properties of dihydroxyacetone phosphate acyltransferase and sn-glycerol 3-phosphate acyl transferase (Schlossman and Bell, 1977), suggest that the pathway via DHAP has a minor role *in vivo*. Schlossman and Bell (1977) have also shown that liver microsomes have a single enzyme capable of acylating both DHAP and sn-glycerol 3-phosphate. These authors have shown that, assuming that 70% of the weight of hepatocytes is aqueous and that there is no compartmentalization of substrates sn-glycerol 3-phosphate and dihydroxyacetone phosphate, the concentration of the substrates and the activity of the enzyme involved would mean that the ratio of microsomal sn-glycerol 3-phosphate and DHAP acylation would be greater than 84:1: thus, the microsomal sn-glycerol 3-phosphate pathway for phospholipid and triacylglycerol synthesis would be expected to be predominant in liver cells *in vivo*.

2. sn-glycerol 3-phosphate acyltransferase (E.C.2.3.1.15), or acyl-CoA: L-glycerol 3-phosphate acyltransferase (I) and acyl-CoA: 1-acylglycerol 3-phosphate acyltransferase (II).

The first step in the formation of glycerides and phosphoglycerides is the acylation of sn-glycerol 3 -phosphate catalysed by the sn-glycerol 3-phosphate acyltransferase system. This enzyme catalyses the conversion of acyl CoA and sn-glycerol 3-phosphate to 1-acyl-sn-glycerol 3-phosphate (Lysophosphatidate) and subsequently to 1,2-Diacylglycerol 3-phosphate

(Phosphatidate) Manroy et al., 1973).

The acylation of sn-glycerol 3-phosphate has been shown to occur in both mitochondria (Manroy et al., 1973); Sánchez et al, 1973; Davidson and Stana-Cev, 1974) and microsomes (Yamashita et al., 1973). Half of the total capacity for sn-glycerol 3-phosphate acylation of hepatocytes is localised in the outer mitochondrial membrane (Manroy et al., 1973). It has been shown that both 1-acyl-sn-glycerol 3-phosphate and phosphatidic acid accumulate in mitochondria.

The activities of GPAT in the mitochondrial and microsomal fractions have been reported to be associated with two separate enzymic proteins following a study of kinetic properties and differential inhibition by SH-blockers (Abu-Issa and Cleland, 1969). The microsomal GPAT has an apparent Km of 0.14 mM for sn-glycerol 3-phosphate with a V_{max} of 2.8 nmol/min/mg (Schlossman and Bell, 1977). The optimum pH is between 7.5 - 8.0 and sulphydryl group protectors, such as cysteine and 2-mercaptoethanol stimulate the enzyme activity (Abu-Issa and Cleland, 1969; Husbands and Lands, 1970). The best substrate for acylating sn-glycerol 3-phosphate is an acyl CoA with 15-18 carbon atoms (Abu-Issa and Cleland, 1969). Acyl CoAs at higher concentrations ($>10 \times 10^{-6}$ M) inhibit the enzyme activity by a mechanism which is thought to be due to the detergent effect of the acyl CoA (Zahler and Cleland, 1969).

Both microsomal and mitochondrial dihydroxyacetone phosphate acyltransferase show similar specificity to the corresponding glycerol phosphate acyl-transferase, suggesting that the DHAP and sn-glycerol 3-phosphate are acylated by the same enzyme (Bremer et al., 1976).

Liver microsomal GPAT activity has been shown to be influenced by various dietary states. It has been reported that sn-glycerol 3-phosphate level in the liver is elevated by fructose or glycerol administration (Nikkilä, 1974). This is suggested to lead to the stimulation of plasma triacylglycerol formation in the liver. The total hepatic GPAT activity has been reported to be decreased after 48h fasting and to be increased when the fasted animals are re-fed a fat-free or a carbohydrate rich diet (Vavnecka et al., 1969; Bremer et al., 1976).

However, other investigators did not find any change in specific activity of GPAT after a fasting period, although the specific activity of the enzyme is increased following a 7-day period on a high fat or on a high-carbohydrate diet (Fallon and Kemp, 1968).

There has been very little research on the hormonal regulation of sn-glycerol 3-phosphate acyltransferase activity in the rat liver. Experiments using isolated adipocytes have shown that adrenalin (1 μM) decreases the sn-glycerol 3-phosphate acyltransferase activity, while insulin increases the activity. When fructose and insulin are included in the incubation medium of adipocytes there is a decrease in sn-glycerol 3-phosphate acyltransferase activity (Sooranna and Saggerson, 1976). Hepatic GPAT has been shown to be decreased in diabetic rats (Bates and Saggerson, 1977) and by adrenalectomy in fasted animals (Bates and Saggerson, 1979). The activity of dihydroxyacetone phosphate acyltransferase/total liver has been shown to be decreased by 44% in adrenalectomised animals and that injection of cortisol restores the activity to levels found in sham-operated animals (Bates and Saggerson, 1981).

In the intact liver (Åkesson, 1970; Åkesson et al., 1970a) and in liver slices (Hill et al., 1968) phosphatidic acids and, therefore, diacylglycerols are synthesised with high positional specificity. Saturated fatty acids occupy mainly position 1 and unsaturated fatty acids are predominantly esterified to position 2.

In intact liver cells, incubated with [^{3}H] glycerol only a small amount of phosphatidate accumulates (Sundler et al., 1974) and kinetic studies using intact animals injected with [^{3}H]glycerol (Åkesson et al., 1970b) have demonstrated that phosphatidate is formed from glycerol

before the formation of diacylglycerols. This evidence suggests that the control point in the regulation of triacylglycerol synthesis in liver is before the formation of phosphatidic acid.

3. Role of phosphatidate phosphohydrolase (E.C.3.1.3.4) in the regulation of glycerolipid biosynthesis

The formation of 1,2-diacylglycerol from 1,2 - diacylglycerol 3 - phosphate (phosphatidic acid) can be accomplished by the enzyme phosphatidate phosphohydrolase (PPH). Phosphatidate is an intermediate in the biosynthesis of diacylglycerol, triacylglycerol, phosphatidyl-ethanolamine, phosphatidyl choline and CDP-diacylglycerol. PPH, considered to be the rate limiting enzyme in the synthesis of triacyl-glycerols (Lamb and Fallon, 1974a; Fallon et al., 1977; Brindley, 1978b). PPH has been found in several different animal tissues. PPH activity is found primarily in the microsomal and supernatant fractions of liver, adipose tissue and intestine and also, to some extent, in the mitochondrial fraction (Smith et al., 1967; Lamb and Fallon 1974a). The physiologically important substrate appears to be the particulate-bound phosphatidate (Smith et al., 1967). The method for the preparation of this substrate, developed by Fallon et al., (1975), involves the labelling of phosphatidate in a microsomal preparation. It has been shown that microsomes from rat liver have at least two active forms of PPH (Caras and Shapiro, 1975), the non-specific form with a high Km (0.3×10^{-3}M) for phosphatidate and a specific form with a low Km (0.03×10^{-3}M) for the substrate.

There are several lines of evidence which support an important role for PPH in the regulation of neutral-lipid formation by liver or adipose tissue. The reaction rate of PPH under optimum conditions in vitro is the lowest in the overall synthetic pathway in liver microsomal fractions (Lamb and Fallon, 1974a). PPH activity has been shown to be 0.6 - 0.8 nmol/min/mg of microsomal protein when radio-labelled particulate-bound phosphatidate is used as substrate. The overall rate of glycerolipid formation by these microsomal preparations is approximately 1.0 nmol/min/mg of microsomal protein but the optimum rate of microsomal GPAT is 5-7 fold higher. Addition of the soluble supernatant results in a 5-20 fold increase in triacylglycerol synthesis (Lamb and Fallon, 1974a). Induction of increased liver triacylglycerol

formation by administration of a 75% fructose diet is accomplished by an equivalent rise in the activity of the microsomal PPH (Fallon et al., 1977). These changes in lipid metabolism occur simultaneously over a 10-day period (Lamb and Fallon, 1974b). Further, the phosphatidate content of liver microsomal preparation is diminished by approximately 15% and diacylglycerol increased by approximately 90% under these dietary conditions. The effect of sucrose, glycerol, sorbitol or ethanol intubation on hepatic PPH activity in rat liver is an increase in specific activity of PPH in comparison to control animals intubated with 0.15 M NaCl. Glucose intubation was ineffective. The greatest increase was observed in both soluble and microsomal PPH activity of rats treated with ethanol (Sturton et al., 1978). Either 75% glucose or 75% fructose given in diets for a period of 60h resulted in a 3-fold increase in PPH activity in both the microsomal and supernatant fractions in comparison with chow fed animals (Lamb and Fallon, 1974b). These changes can be correlated to the increase in triacylglycerol synthesis which occurs on carbohydrate feeding and to the change in the ratio of neutral lipid to polar lipid synthesis observed using rat liver homogenates or microsomal fractions (Lamb and Fallon, 1974b).

An increased supply of saturated and monounsaturated fatty acids to the liver, such as in starvation, stress and obesity has also been shown to be accompanied by an increase in the soluble PPH activity (Glenny et al., 1978). These authors have suggested that ingestion of diet rich in fructose or glucose results in an increased synthesis of saturated or monounsaturated fatty acids which, in turn, may give rise to an increase in PPH activity. The increase in liver triacylglycerol content after partial hepatectomy is also accompanied by a corresponding increase in PPH activity, but actinomycin-D administration results in a lowering of PPH activity yet does not prevent triacyglycerol accumulation (Mangiapane et al., 1973). Several drugs which cause substantial inhibition of PPH in vitro also inhibit neutral lipid formation when administered to the intact animal (Brindly and Bowley, 1975). A series of 1,3-bis(substituted phenoxy)-2-propanones inhibit microsomal PPH in vitro. The inhibition ranges from 8 to 92% of total activity of the enzyme. In animals fed on laboratory chow supplemented with these agents the incorporation of [^{14}C] glycerol into hepatic triacylglycerol is decreased by 3 - 75%. Agents which caused a 50% or greater

decrease in PPH in vitro also lowered hepatic triacylglycerol formation, measured in vivo by more than 60%. Although each of these drugs caused some inhibition of GPAT activity, the correlation between enzyme inhibition and decreased hepatic triacylglycerol formation was better for PPH. Studies of the products of liver microsomal preparations or homogenates from sn - [1,3 - ^{14}C]glycerol 3-phosphate have shown a rapid incorporation of radioactivity in phosphatidate and a much slower rise in neutral-lipid radioactivity (Lamb and Fallon, 1974a; 1974b; Fallon et al., 1975).

In addition, in obese rodents an increased capacity for triacylglycerol biosynthesis by both adipose tissue and liver is accompanied by a substantial rise in PPH activity (Fallon et al., 1977) as indicated by the change in the ratio of neutral-lipid to phosphatidate formed; as expected, the increase in PPH activity exceeded the rise in sn-glycerol 3 - phosphate esterification. However, there are some contradictory reports, for example, from studies in the perfused liver, the intact animal and hepatocytes grown in monolayer culture (Lamb et al., 1976). It has been shown that the administration of [^{14}C] glycerol to animals or added to intact hepatocyte preparations does not result in an accumulation of radioactivity in liver phosphatidate. The radiolabel appears promptly in triacylglycerol and complex phospholipids (Fallon et al., 1977).

An increase of between 180 and 320% in the hepatic soluble PPH activity has been observed after 40h fasting, while GPAT levels are lower. However, glyceride synthesis by liver homogenates was found to be unchanged or slightly increased in the starved animals (Vavrecka et al., 1969).

It has been shown that the particulate PPH activity is inhibited by incubation of adipocytes with noradrenalin, but it is not clear if this is a direct effect of the hormone on the enzyme or an indirect effect via the stimulation of lipolysis (Cheng and Saggerson, 1978). Activities of PPH and DGAT are increased in microsomal fractions isolated from liver perfused with Bt_2cAMP. These effects are associated with a decrease in microsomal triacylglycerol, diacylglycerols and phosphatidate synthesis from [U - ^{14}C] glycerol 3-phosphate. The output of triacylglycerol is decreased by the cyclic nucleotide but ketogenesis and

glucose output are stimulated (Soler-Argilaga et al., 1978a). Thus, these authors have suggested that Bt_2 cAMP inhibits hepatic microsomal synthesis of triacylglycerol at a step prior to the formation of phosphatidate, presumably at the GPAT step.

Cortisol administration resulted in an increase in soluble PPH level and an increase in the relative proportion of $[^{14}C]$ glycerol incorporated into triacylglycerol (Pritchard et al., 1977; Glenny et al., 1978; Glenny and Brindley, 1978). It has also been reported that thyroxine increases the relative rate of triacylglycerol synthesis (Glenny and Brindley,1978). Glucagon and insulin, however, have no significant effect on the soluble PPH activity (Lehtonen et al., 1979). These authors have also described the induction of soluble hepatic PPH activity by cortisol both in vivo and in an isolated perfused rat liver. Recently it has been shown that Mg^{2+}-dependent PPH activity increased in the microsomal and decreased in the soluble fraction of isolated fat cells incubated for short periods with lipolytic hormones or agents such as adrenaline, cyclic AMP, theophylline and Bt_2cAMP (Moller et al., 1981). The increase in microsomal activity ranged from 30 to 134% with adrenaline and to almost 200% with Bt_2cAMP. The effect of adrenaline was inhibited by the β-adrenergic antagonist propranolol while the α -antagonist phentolamine enhanced it (Moller et al., 1981). These authors strongly suggest that the fat cell PPH is controlled through the β-adrenergic receptor and the activity of adenylate cyclase. These authors have also speculated that the activation of microsomal PPH by lipolytic stimuli may represent a mechanism whereby fatty acid release from adipose tissue by hormone sensitive lipase may be modulated and intracellular fatty acid accumulation maybe counteracted during accelerated lipolysis in adipose tissue (Moller et al., 1981).

4. Diacylglycerol acyltransferase (E.C.3.1.20)

Diacylglycerol acyltransferase (DGAT) catalyses the conversion of 1,2 - diacylglycerol to triacylglycerol. This enzyme has been isolated from both microsomal and mitochondrial fractions of different mammalian tissues. Its activity has been shown to be 0.4 nmol/min/mg protein in rat liver microsomal fraction (Fallon et al., 1975). The activity of DGAT can be affected by different nutritional states (Fallon et al., 1975; Coleman and Bell, 1976). It has been found that rat liver

homogenate DGAT activity is increased by fasting (Vavrecka et al., 1969). This increase in enzyme activity correlated with an increase in triacylglycerol/diacylglycerol ratio from 0.7 to 1.0. High fructose diets fed to rats for 11 days resulted in the accumulation of diacylglycerol (Fallon et al., 1975). In addition, DGAT activity measured with microsomal-bound diglyceride was increased 2-fold. It has been found that sucrose, corn oil and lard feeding for a period of 14 days resulted in an increase in hepatic DGAT activity in rats when compared to starch-fed controls. Fat feeding is more effective than sucrose in maintaining the enzyme activity (Glenny et al., 1978). It has been shown that the specific activity of rat adipocyte DGAT increased in microsomal fraction from precursor cells grown in culture in the presence of added insulin (Roncari et al., 1979). It has been shown that the stimulatory effect of fatty acids on triacylglycerol synthesis and VLDL secretion in isolated rat hepatocytes may be a consequence of enhanced activity of DGAT, the only enzyme that is exclusively concerned with the synthesis of triacylglycerol (Haagsman et al., 1981b).

B. Regulation of triacylglycerol synthesis and secretion

1. Effect of free fatty acid on triacylglycerol secretion

Hepatic triacylglycerol synthesis is controlled by the level of FFA in the liver. It has been shown that increasing the concentration of FFA in the perfusate of isolated liver leads to an increase in the rate of secretion of VLDL (Mayes and Felts, 1966; 1967; Mayes, 1970). There is a reciprocal relationship between the esterification of FFA and their oxidation to CO_2 and ketone bodies (Topping and Mayes, 1972). At any given concentration of FFA the livers from fed rats oxidised less, and esterified and exported more of FFA as VLDL compared to livers from starved rats. In livers from fed rats at low concentrations of FFA most of the intake into the liver is esterified and exported as triacylglycerol in VLDL. However, as the FFA concentration is raised, the fractional esterification is decreased although the total esterification is increased (Topping and Mayes, 1972). Enhancement of VLDL-triacylglycerol secretion by the liver results from increased synthesis of triacylglycerol in a small precursor pool, which is located in the smooth endoplasmic reticulum. There is also a large pool of

cytoplasmic triacylglycerol (floating fat) which is turned over at a slow rate(Bar-on et al., 1971; Kuksis et al., 1975; Mayes, 1976). When labelled FFA are taken up by the liver, this small microsomal pool contains triacylglycerol of high specific radioactivity compared with the floating fat fraction (Mayes, 1976). The function of the large precursor pool appears to be in the storage of the triacylglycerol which is not exported as VLDL. Hydrolysis of the storage triacylglycerol and resynthesis in the microsomal pool must occur before secretion of the stored triacylglycerol can take place (Bar-on et al., 1971). (See Fig. 7). The quantity of VLDL secreted by the liver in the normal fed animal in vivo is determined by the necessity to secrete triacylglycerol. It has been postulated that the output of the VLDL in the intact animal is directly proportional to the concentration of FFA in the plasma (Heimberg et al., 1974). Triacylglycerol synthesis (Ontko, 1972; Groener and Van Golde, 1978; Haagsman et al., 1981) and secretion of VLDL-triacylglycerol (Kempen, 1980; Haagsman and Van Golde, 1981) by isolated hepatocytes are also stimulated by the addition of fatty acids to the incubation medium.

It has been found that in the perfused rat liver from fed rats VLDL secretion is directly related to endogenous fatty acid synthesis, if exogenous fatty acid is not supplied. There is a high correlation between the amount of fatty acid synthesised and the esterified fatty acid secreted in such a perfused system (Windmueller and Spaeth, 1967). This correlation has also been found in isolated hepatocytes from meal fed rats except that the secretion of newly synthesised triacylglycerol shows a lag phase of about 30 min. These authors conclude that de novo triacylglycerol synthesis controls the VLDL-triacylglycerol release by a 'push' mechanism (Beynen et al., 1981). This is in line with the well-known stimulatory effect of fatty acids on the secretion of triacylglycerols by the perfused liver (Havel et al., 1962; Nestel and Steinberg, 1963; Kohout et al., 1971) and by isolated hepatocytes (Kempen, 1980; Haagsman and Van Golde, 1981). Addition of fatty acid to isolated hepatocytes also stimulates triacylglycerol synthesis from $[^3H]$ glycerol, whereas phospholipid synthesis is influenced to a lesser degree (Ontko, 1972; Sundler et al., 1974). However, Mayes and Topping (1974) found that increased concentration of serum FFA did not alter significantly the rate of VLDL fatty acid secretion by perfused rat liver, but there was a considerable decrease in the proportion of the

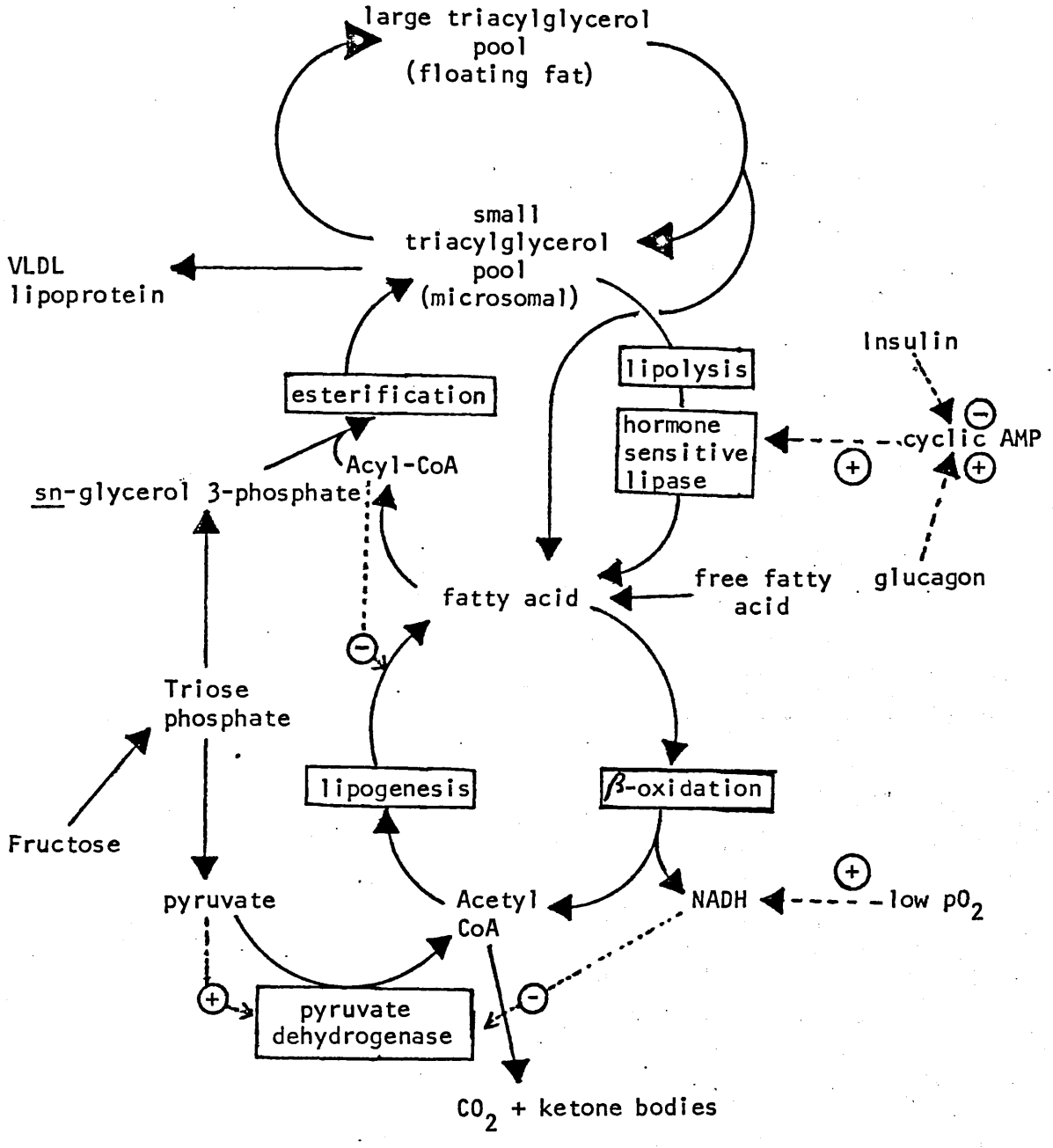


Fig. 7 The effect of free fatty acids, insulin, pO_2 and fructose on the secretion of VLD-lipoproteins in the liver. Proposed scheme by Mayes (1972).

VLDL fatty acid which was synthesised de novo as the FFA concentration increased.

The type of long-chain fatty acid metabolised by the liver has a considerable effect on the rate of formation and the composition of the VLDL. It has been shown that the output of triacylglycerol from the perfused liver decreased as the degree of unsaturation of fatty acid increased, but the output increased with the carbon chain length (Kohout et al., 1971; Dave and Mayes, 1979). It has been demonstrated that VLD-lipoproteins formed from oleate were larger and contained less cholesterol and phospholipids per molecule of triacylglycerol than VLD-lipoproteins formed from palmitate (Heimberg and Wilcox, 1972). Large lipoproteins are cleared from the circulation at a faster rate than smaller lipoproteins and thus the nature of the triacylglycerol fatty acids may have an effect on the concentration of plasma VLDL-triacylglycerol (Mayes, 1976). It has been observed that when equimolar quantities of the palmititic (16:0) oleic (18:1) or linoleic (18:2) acids were infused, the output of triacylglycerol was in the order of 18:1 = 18:2 $>$ 16:0 (Goh and Heimberg, 1977). The release of triacylglycerol in primary monolayer cultures of rat hepatocytes is greater in the presence of unsaturated acids (16:1, 18:1 and 18:2) than the saturated acids (16:0 and 18:0) (Lamb et al., 1977).

When long-chain fatty acids are infused in equimolar amounts as an albumin complex, the rate of triacylglycerol fatty acid output in VLDL for each fatty acid is as follows: oleate (18:1) $>$ linoleate 18:2 $>$ linolenate (18:3) $>$ arachidonate (24:5) $>$ stearate (16:1) $>$ palmitate (16:0) $>$ myristate (14:0) (Dave and Mayes, 1979).

This data shows that unsaturated fatty acids promote more triacylglycerol secretion from the liver.

2. Effect of pO_2 redox state of the liver tissue

When whole blood is perfused through the isolated liver the pO_2 has been shown to decrease without any change in O_2 consumption (Mayes and Felts, 1976). This leads to increases in the sn-glycerol 3-phosphate/dihydroxyacetone phosphate, lactate/pyruvate, 3-hydroxybutyrate/acetoacetate

and $NADH/NAD^+$ ratios. The more reduced redox state is associated with a marked shift in balance between oxidation and esterification of FFA in favour of oxidation. This is accompanied by a corresponding decrease in secretion of VLDL-triacylglycerol (Mayes, 1976). The increased $NADH/NAD^+$ ratio may cause inactivation of pyruvate dehydrogenase, increased fatty acid oxidation and thus a decrease in the rate of esterification of fatty acid. An increase in the NADH concentrations will favour the formation of sn-glycerol 3-phosphate whereas a decrease in NADH will provide more NAD^+ available for the conversion of pyruvate to acetyl CoA and hence increase the rate of fatty acid synthesis (Mayes, 1976).

It has been proposed that the availability of sn-glycerol 3-phosphate is a regulatory factor in fatty acid esterification (Exton and Park, 1967; Mayes and Felts, 1967; Van Tol, 1974; Debeer et al., 1981). A strong stimulatory effect of glycerol on esterification and a low sn-glycerol 3-phosphate content of isolated hepatocytes from starved animal strongly suggest that sn-glycerol 3-phosphate availability may be a limiting factor in triacylglycerol synthesis in isolated rat hepatocytes (Debeer et al., 1981; Declercq et al., 1982b). These authors have shown that the variations in sn-glycerol 3-phosphate content from 0.1 - 0.3 $\mu mol/10^8$ cells significantly alter rates of esterification in isolated hepatocytes. There is no significant difference in the relationship between triacylglycerol synthesis by hepatocytes from fed and fasted animals and the sn-glycerol 3-phosphate content of the cells incubated under different conditions. However, the latter is lower in cells from fasted animals. There is also a 40% decrease in V_{max} of GPAT on fasting (Declercq, 1982b).

3. Hormones and triacylglycerol secretion

The major limiting factor in the secretion of VLDL-triacylglycerol appears to be the availability of FFA. Thus, the factors which influence the plasma FFA levels, for example the effect of hormones on adipose tissue, will indirectly affect VLDL secretion.

The process of glyceride synthesis and secretion in the liver is known to be regulated directly by hormones, especially insulin and

glucagon (Hems, 1975). It has been reported that lipogenesis is impaired in diabetic animals (Mayes and Felts, 1967; Heimberg et al., 1969), at the same time plasma FFA is increased but the secretion of VLDL is suppressed. Topping and Mayes (1972) showed that whole blood containing elevated levels of insulin directly affects the metabolism of FFA in perfused liver by increasing the rate of esterification and decreasing the rate of oxidation. Secretion of triacylglycerol-fatty acid in VLDL and the incorporation of [^{3}H] acetate into VLDL triacylglycerol are also stimulated. However, Nikkilä (1974) found no direct effect of insulin on perfusate triacylglycerol levels in perfused liver and he suggested that the differences between his work and that of Topping and Mayes (1972) is that the latter authors used whole blood taken from rats given a glucose load as a source of rat insulin. Woodside and Heimberg (1976) found that although plasma triacylglycerol secretion is suppressed in vivo by anti-insulin serum, addition of insulin in vitro did not significantly affect triacylglycerol secretion by perfused livers from rats treated with the serum. Glucose, on the other hand, stimulated triacylglycerol secretion, whereas insulin and glucose together depressed the rate of secretion.

Salmon and Hems (1976) have also reported that the presence of insulin exerts little effect on net triacylglycerol secretion by perfused mouse liver, but that hormone inhibited a lipase resulting in a significantly greater retention of triacylglycerol fatty acids in the liver compared to untreated controls.

Hepatocytes in suspension secrete VLDL which is very similar to that found in vivo (Kempen, 1980). Insulin has been reported to stimulate the incorporation of [9,10 $^3H_2$] palmitate into triacylglycerol by isolated hepatocytes, but the increase is only of the order of 12% (Beynen et al., 1980). These authors have attempted to correlate this increase to increased (by 18%) sn-glycerol 3-phosphate level in isolated hepatocytes treated with insulin. The same group were unable to show any affect of insulin on the release of pre-labelled VLDL-triacylglycerol. When hepatocytes are incubated with ^3H_2O, the secretion of newly-synthesised triacylglycerol in the VLDL shows a lag phase of about 30 min. Insulin stimulates the secretion of the newly synthesised VLDL but the stimulation observed is very small. Perfusion of liver with glucagon or Bt_2cAMP increases the oxidation of FFA and decreases their

conversion into VLDL-triacylglycerol and the secretion of triacylglycerol (Van Harken et al., 1969; Heimberg et al., 1969; Weinstein et al., 1973). One suggested mechanism (Topping and Mayes, 1972) for this effect is that glucagon stimulates the hormone-sensitive lipase which hydrolyses the triacylglycerol in the small microsomal triacylglycerol pool (see Fig. 5). The positive effect of insulin observed by these workers may be related to the inhibition of this lipase. These hormonal effects would account for both the decrease in triacylglycerol export and the net decrease in esterification and increase in oxidation of fatty acids observed.

The effect of Bt_2cAMP on triacylglycerol synthesis has been studied by investigating the microsomal glycerolipid biosynthesis after perfusion of liver with the cyclic nucleotide. The incorporation of sn- [U-^{14}C] glycerol 3-phosphate into glycerolipid is reduced by the treatment, but PPH and DGAT were increased. This suggests that inhibition occurs at a step prior to the formation of the phosphatidate, presumably GPAT (Soler-Argilaga et al., 1978a).

The secretion of VLDL-triacylglycerol by isolated hepatocytes has also been reported to be depressed by both glucagon and Bt_2cAMP. Thus, it is likely that glucagon exerts its effect via changes in intracellular cAMP, presumably leading to increased protein phosphorylation by cAMP-dependent protein kinase.

The mechanism of action by which Bt_2cAMP diminishes the hepatic output of triacylglycerol may include inhibition of esterification of long-chain fatty acylCoA to triacylglycerol; depression of formation and release of the VLDL associated with transport of triacylglycerol; increase in the rate of transport of fatty acids from the cytoplasm into the mitochondria by altering the activity of CAT I, or by increasing the availability of free carnitine or both; direct stimulation of mitochondrial pathways for oxidation of fatty acids and for formation of ketone bodies; and any combination of these factors (Heimberg et al., 1969; 1978).

The effect of Bt_2cAMP has also been observed in liver perfusion experiments. The net output of triacylglycerol by liver (Heimberg et al., 1974) and the incorporation of [^{14}C] fatty acid into perfusate

triacylglycerol is inhibited by glucagon (Heimberg et al., 1974). The effects of the cyclic nucleotide are relatively small during the first hour of perfusion, but thenafter become extensive. This is in contrast to the effect on glucose output which occurs during the first hour. The explanation for this time lag is, presumably, related to some other change which precedes the effect on triacylglycerol output. In addition, Müller and Jeanrenaud (1978) found that triacylglycerol secretion by isolated hepatocytes was inhibited by glucagon but at higher concentrations of the hormone (above 10^{-8}M) than were required to stimulate glycogenolysis and lipogenesis. This is again indicative that the action of the hormone on triacylglycerol output may be indirect.

Geelen et al., (1978a) showed that glucagon had no effect on the formation of phosphoglycerides from exogenous fatty acids by isolated hepatocytes, but caused a marked decrease in the rate of triacylglycerol synthesis. Indeed, later work (Geelen et al., 1979) suggested that phospholipid synthesis may be increased by the hormone. Haagsman et al., (1981) have concluded that microsomal DGAT is regulated by glucagon; they showed a 53% decrease in enzyme activity in hormone-treated hepatocytes. They found a similar decrease in activity when the microsomal preparation was treated with ATP and Mg^{2+} in the presence of supernatant, suggesting that a phosphorylation of the enzyme could account for the changes in enzyme activity observed.

Ca^{2+} has also been implicated in the regulation of triacylglycerol synthesis. The uptake of Ca^{2+} by hepatic microsomes resultsin a decrease in the biosynthesis of phosphatidate and other glycerolipids. Ca^{2+}-depletion of hepatocytes results in diminished response of the cells to both glucagon and cAMP. Rates of triacylglycerol synthesis were decreased and uptake of $[^{14}C]$ oleate was reduced in Ca^{2+}-depleted hepatocytes (Soler-Argilaga et al., 1978a,b).

The inhibition of triacylglycerol synthesis by glucagon in glycogen-depleted hepatocytes from fed rats has been shown to be related to a lowering of cellular sn-glycerol 3-phosphate content of the cells (Declercq, 1982a).

Adrenalectomy has been reported to impair the synthesis and secretion of triacylglycerol fatty acid by perfused liver systems

(Klausner and Heimberg, 1967; Kirk et al., 1975; 1976). Normal release of triacylglycerol is restored by steroid treatment of the adrenalectomized animal. Kirk et al., (1976) have suggested that adrenalectomy results in the preferential inhibition of triacylglycerol synthesis; phospholipid synthesis remaining unaffected. This is in accord with the work of Glenny and Brindley (1978) who found that cortisol treatment of the intact rat increased the relative rate of triacylglycerol synthesis in rat liver and increased the flux from phosphatidate to diacylglycerol. This was correlated to an increase in the soluble PPH.

Glucocorticoids are known to raise plasma triacylglycerol levels in rats (Afolabi et al., 1976), an effect which involves both increased hepatic triacylglycerol output and an impaired VLDL clearance by the adipose tissue (Bagdade et al., 1976).

It is well known that the output of triacylglycerol by perfused liver from female rats exceeds that in the male (Watkins et al., 1972). Furthermore, triacylglycerol output by perfused liver from ovariectomized rats is reduced but is restored by oestrogen treatment (Weinstein et al., 1974; Watkins et al., 1972). It appears that larger VLDL particles are secreted by the female livers (Soler-Argilaga et al., 1976). The mechanism of action of oestrogen is not clear, but it is suggested that oestrogen may act by diminishing the response to factors that increase the intracellular cAMP levels (Weinstein et al., 1979).

Glenny and Brindley (1978) reported that thyroxine also increases the relative rate of triacylglycerol synthesis from $[^3H]$ glycerol while not affecting either the accumulation of the radioactive isotope in phosphatidate or the activity of soluble PPH.

Laker and Mayes (1981) also reported that the thyroid status of rats influenced hepatic lipid metabolism. Synthesis and secretion of triacylglycerol was decreased in hyperthyroid rats; at the same time more oleate was oxidised to CO_2 and ketone bodes.

4. Nutritional control of hepatic triacylglycerol output

High carbohydrate diets are known to enhance the hepatic output of VLDL-triacylglycerol. The fatty acid of VLDL-triacylglycerol may

originate from circulating FFA derived from mobilization of adipose tissue-triacylglycerol from the diet, or from de novo hepatic lipogenesis (Steinberg, 1963). Perfused livers of fructose-fed rats secrete twice as much oleate - [^{14}C] triacylglycerol as control animals (Schonfeld and Pfleger, 1971).

Ingestion of 75% fructose increased the rate of DHAP esterification and the serum triacylglycerol concentrations (Lamb and Fallon, 1977). Diets containing fructose are reported to increase the hepatic formation of triacylglycerol via changes in the activity of GPAT and PPH (Lamb and Fallon, 1974a; Fallon et al.,1977).

The rates of secretion of VLDL in rats adapted to a high-fat diet decreased by 40% (Kalopissis et al., 1980). The reasons for this decrease in VLDL secretion in vivo are not clear, since non-esterified fatty acids are known to enhance VLDL secretion rates by the perfused liver (Heimberg et al., 1969) and by cultured hepatocytes (Dashti, et al., 1980). A possible explanation could be that fat-feeding lowers VLDL production through inhibition of hepatic lipogenesis (Ogiwara et al., 1978). A high degree of correlation exists between the rates of hepatic lipogenesis and VLDL secretion (Windmueller and Spaeth, 1966; 1967). In perfused livers from fed rats, release of triacylglycerol is highly correlated with the rate of fatty acid synthesis. However, as the concentration of FFA is raised, lipogenesis is progressively inhibited and the contribution of newly synthesised fatty acid to VLDL-triacylglycerol falls (Mayes and Topping, 1974).

In the starved state hepatic lipogenesis from carbohydrate via acetyl CoA is decreased and FFA are the major source of triacylglycerol fatty acids in VLDL (Mayes, 1976). It has been shown in isolated hepatocytes that fasting strongly decreases the incorporation of [2 -^{3}H] glycerol via sn-glycerol 3-phosphate into triacylglycerols, whereas the formation of phospholipids is much less affected (Groener and Van Golde, 1977). It has also been reported that triacylglycerol output and lipogenesis vary in parallel in several metabolic states (Mayes and Felts, 1967; Assimacopoulos-Jeannet et al., 1974; Bloxham et al., 1977; Holt et al., 1979). Incorporation of exogenous fatty acids into hepatic VLDL is greatly decreased in fat-fed rats as is

their hepatic esterification to triacylglycerols and phospholipids. Furthermore, lipogenesis is strongly inhibited after fat-feeding and the endogenous triacylglycerol pool contributes less to the VLDL-triacylglycerols than in the control animals. Thus, it appears that fatty acid from exogenous, as well as from endogenous sources, are not readily available as substrates for VLDL-triacylglycerol synthethis in the fat-fed rats, suggesting that fatty acid availability at the assembly point could be a limiting factor of VLDL secretion (Kalopissis et al., 1981).

There is evidence that ingesting saturated fats increases the concentration of serum triacylglycerol whereas polyunsaturated fats have the opposite effects (Macdonald, 1971; Bruckdorfer et al., 1972a,b). There is also evidence that the rate of synthesis of triacylglycerol in the liver is influenced by the composition of dietary fat (Brindley, 1978a).

RESULTS AND DISCUSSION

There have been several reports (Macdonald and Roberts, 1965; Nikkilä and Ojala, 1965; Bar-on and Stein, 1968; Mukherjee et al., 1969; Macdonald, 1973) that dietary fructose causes a greater increase in fasting serum triacylglycerol levels compared to an equicaloric amount of glucose. In addition, both fructose and sucrose in the diet of rats cause an elevation of serum triacylglycerol levels (Macdonald and Braithwaite, 1964; Shiff et al., 1971).

Tay (1977) found that male rats had significantly higher levels of serum triacylglycerol than female animals and that a sucrose diet increased the levels of serum triacylglycerol to a greater extent than glucose in both sexes. No significant sex or dietary differences in serum phospholipid were observed. However, Tay (1977) was unable to clarify whether the differences were in rates of synthesis or in the rates of clearance of the lipid. The work of Hashemi-Dezfully (1979) on hepatic lipogenic enzymes in animals kept on similar dietary regimes indicated that the sex differences in serum triacylglycerol observed were the result of differences in rates of triacylglycerol clearance, but that sucrose in the diet elevated lipogenic enzymes to a greater extent than glucose, indicating that the fructose component may also stimulate fatty acid synthesis and, possibly, hepatic triacylglycerol output.

Further evidence that fructose can influence triacylglycerol secretion was obtained from studies on perfused rat liver by Topping and Mayes (1972), who found that the ketose increased secretion of VLDL-triacylglycerol and decreased oxidation of FFA. It has been found that perfusion of liver with fructose results in an increase in sn-glycerol 3-phosphate levels (Wieland and Matschinsky, 1962; Woods et al., 1970), and the latter group suggested that fructose enhances FFA esterification because it is readily converted to sn-glycerol 3-phosphate. Pereira and Jangaard (1971) have come to a similar conclusion from the results of their work with isolated liver slices. In view of the effects of ischaemia on triacylglycerol synthesis in liver slices and perfused liver, the aim of the present study was to investigate the short term effects of fructose on fatty acid and triacylglycerol synthesis using well-oxygenated isolated hepatocytes as a model system.

A. Hepatocyte viability

The use of isolated liver slices in metabolic studies has been subject to a number of criticisms, mainly relating to the viability of the cells (Krebs et al., 1973). Isolated hepatocytes have been shown to be superior to liver slices in a number of important respects. For example, the adenine nucleotide content of isolated hepatocytes is much closer to the value found in vivo than to that found in liver slices incubated for a similar period under the same conditions (Krebs et al., 1973).

It was therefore decided to examine the metabolic fate of fructose and glucose in isolated hepatocytes and to examine the effects of the hexoses on lipogenesis and triacylglycerol synthesis in isolated liver cells. Most metabolic studies on hepatocytes have been concentrated on the regulation of gluconeogenesis by cells from fasted animals and there have been relatively few studies on lipid metabolism using cells from fed animals. Hopkirk and Bloxham (1979), however, found a considerable diurnal variation in lipogenesis from ^3H_2O by isolated hepatocytes; the increase in the rate of fatty acid synthesis followed a period of feeding

In the present study isolated hepatocytes were routinely isolated at 0.900 from animals which were fed on normal laboratory chow, Dixon 86. This is a high carbohydrate, low fat diet which would be expected to give high rates of lipogenesis since the level of lipogenic enzymes in animals on the diet are higher than in animals fed on another type of chow, Dixon CDD[R], which has a higher fat content (Hashemi-Dezfully, 1979).

The trypan blue exclusion test is widely used as an index of cell viability. In the experiments described in this thesis viabilities of approximately 90% were generally found and hepatocytes with a viability index of less than 80% were discarded. The viability declined somewhat to about 50% over a 4h incubation period and this was paralleled by leakage of lactate dehydrogenase into the incubation medium. Perhaps a better indication of hepatocyte viability is the ATP content of the cells. After a 1h pre-incubation this value was 2.45 ± 0.095 SEM. A further 1h incubation resulted in no significant change in the level of

the nucleotide (Table I). These values are close to those found in vivo (Krebs et al., 1973). It is clear that a high concentration (10 mM) of fructose depletes hepatocytes ATP levels over a 60 min period whereas a physiological concentration of the ketose has relatively little effect. Further incubation of hepatocytes for 180 min results in a diminution of the nucleotide levels in both fructose- and glucose-treated cells. The depletion of ATP by fructose is a well-known phenomenon (Van den Berghe, 1978) and is the result of a rapid metabolism of the ketose by ketohexokinase. A more recent study in the laboratory (Mapungwana, 1982) confirmed the depletion of ATP in fructose-treated cells from fed rats. The depletion is indeed very rapid; in the presence of 10 mM fructose the level decreases profoundly over an initial 10 min period and then increases gradually for up to 60 min. The depletion at lower fructose concentrations is very much less marked and glucose (10 mM) has no effect on hepatocyte ATP content.

The metabolic competence of the hepatocytes was also tested by measuring rates of gluconeogenesis from L - [U-^{14}C] lactic acid. The values obtained were approximately 10 µmole/h/g, comparable to those found by other workers (Wagle and Ingebretsen, 1975; Elliot et al., 1976) taking into account the observation that gluconeogenesis from lactate is proportional to substrate concentration up to 20 mM lactate (Tay, 1977). A typical example is shown in Fig. 8 which shows that the rate of gluconeogenesis was linear for up to 60 min. In addition, when glucagon was added to the cells at zero time there was a 2-fold stimulation of gluconeogenesis from lactate. This is generally regarded as a stringent test for hepatocyte viability, since such a stimulation requires the integrity of the plasma membrane and the integrated functions of different subcellular compartments, i.e. mitochondria, cytosol and endoplasmic reticulum.

Glucose output by isolated hepatocytes incubated with 2.5 mM fructose was also shown to be sensitive to glucagon (10^{-6}M). Hormone treatment resulted in a 2.3-fold increase in glucose output from 0.81 µmols /min/g hepatocyte to 1.83 µmols/min/g hepatocyte. The increased output of glucose probably arises from increased glycogenolysis and increased gluconeogenesis. It is clear that the hepatocytes are metabolically competent for at least 60 min of incubation after the 60 min pre-incubation, but after this time there may be a gradual

Table I ATP Concentrations in isolated hepatocytes

Substrate	Time (min)	µmoles of ATP/g cells	n
NONE ADDED	0	2.45 ± 0.095	3
NONE ADDED	60	2.57 ± 0.140	4
Fructose (2mM)	60	2.31 ± 0.127	3
Fructose (10mM)	60	1.52 ± 0.120	3
Glucose (9mM)	180	1.07 ± 0.056	8
Fructose(9mM)	180	1.15 ± 0.076	9

Isolated hepatocytes from fed rats were preincubated for 60 min and then incubated with the appropriate substrate at a concentration of 32 mg (wet weight) hepatocytes per 1 ml. The cells were extracted with $HClO_4$ (2% w/v final concentration) and the neutralised extracts used for ATP analysis.

Each value represents the mean ± SEM of results

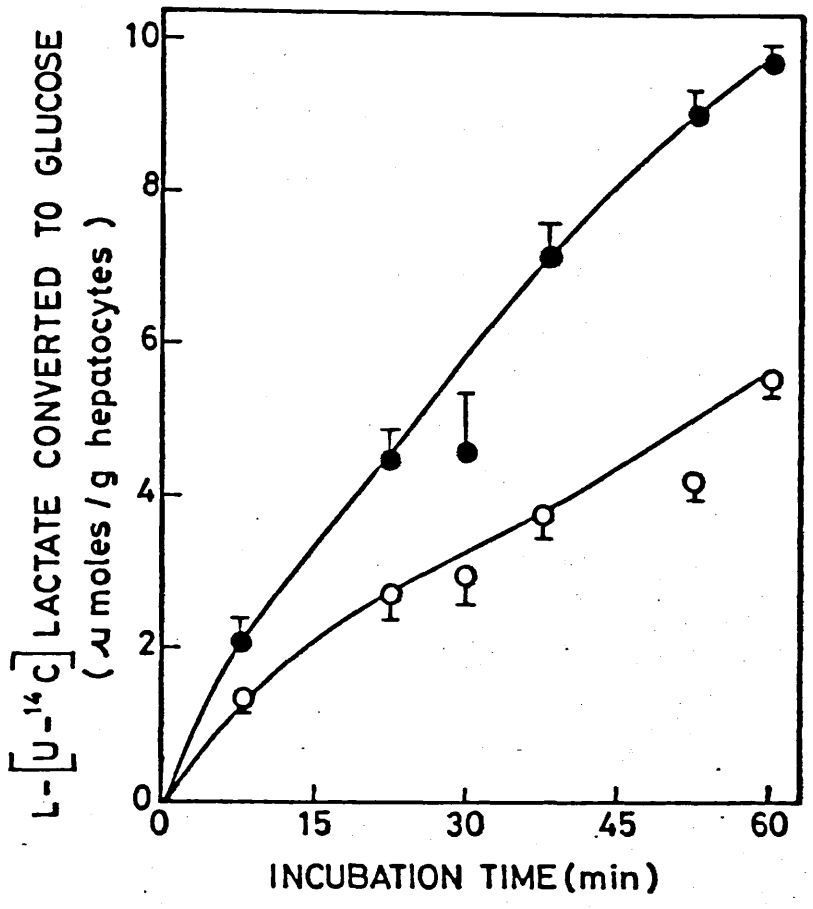


Fig. 8 Rate of gluconeogenesis from L - [U-^{14}C] lactate.

Isolated hepatocytes (4.0×10^6 cells/ml) from fed rats were preincubated for 1h without substrate and then with lactate (2mM) (o) for various times. Glucagon (10 μM) (•) was added at zero time. Experimental details described in Section VI. B. 3c.

Each point represents the mean ± SEM of triplicate results with one hepatocyte preparation.

deterioration in the cells as indicated by the ATP contents and trypan blue exclusion of the hepatocytes.

B. Incorporation of hexose into total lipid

An initial study on the incorporation of [^{14}C] hexoses into total lipid by isolated hepatocytes showed that the rates of incorporation from both D-[U-^{14}C] fructose and D-[U-^{14}C] glucose were linear for at least 2h at concentrations of 1 mM and 9 mM added hexose (Fig. 9). The difference in reaction rates suggest that fructose is a better precursor of total lipid than glucose. A higher incorporation of radioactivity from fructose into liver triacylglycerol has been reported to occur in vivo (Bar-on and Stein, 1968: Marhama and Macdonald, 1972; 1973; Wusteman and Macdonald, 1977) and in isolated liver slices (Pereira and Jangaard, 1971). These observations can be readily explained by the considerable dilution of [^{14}C] glucose by endogenous glucose, especially in vivo. With the isolated hepatocytes preparation, this can be taken into account by estimating the endogenous glucose level. The other explanation for the different rates of utilization of glucose and fructose is the ease of conversion of the latter into sn-glycerol 3-phosphate. Pereira and Jangaard (1971) have claimed that the rapid conversion of fructose to sn-glycerol 3-phosphate may play a role in fructose-induced hypertriglyceridaemia. This possibility was further investigated using isolated hepatocytes as a model system.

The utilization of fructose for total lipid synthesis (Fig. 10) was found to be greater than the utilization of glucose, even taking into account the dilution of the radioactivity by endogenous glucose. This difference in the rates of utilization is evident at all concentrations of hexose up to 10 mM. This provides evidence that the difference in incorporation is not due to isotope dilution but is the result of a more rapid metabolism of the ketose.

However, since isolated hepatocytes from fed rats were used in these experiments, it is clear that glycogen breakdown occurred which can change the concentration of glucose in the hepatocyte medium. The rate of glucose output was 0.83 µmole/min/g liver and was linear for a 60 min incubation period (Fig. 11). At the beginning of the incubation period there was a level of 1.6 mM glucose in the hepatocyte plus medium.

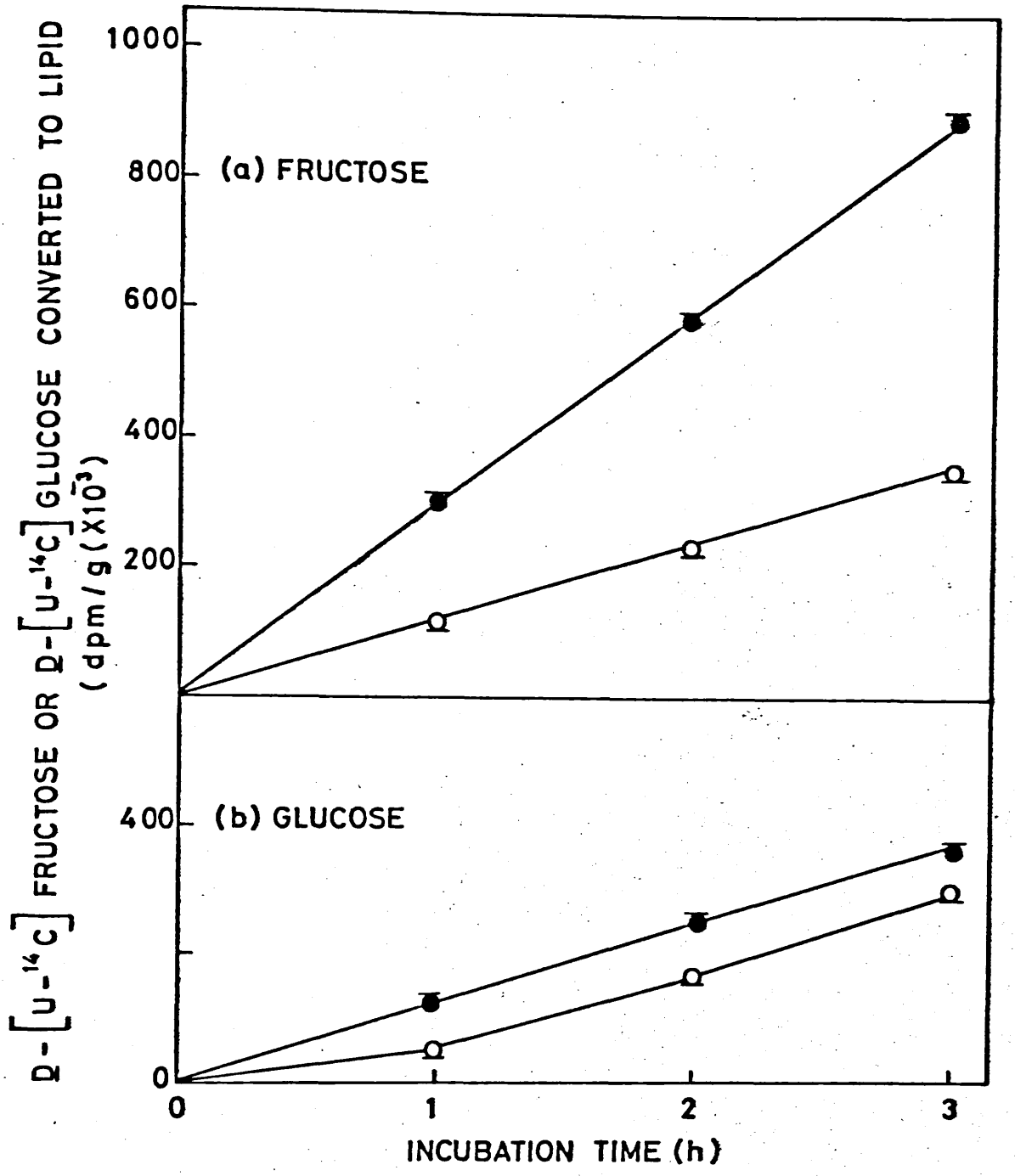


Fig. 9 Incorporation of (a) D - [U-^{14}C] fructose and (b) D - [U-^{14}C] glucose into total lipid by isolated hepatocytes.

Hepatocytes were prepared from fed rats and incubated in KRB + Ca^{2} + BSA in the presence of added substrates at a concentration of 9mM (•) or 1mM (o). (Incubation vol = 1ml, 4.92×10^{6} cells/ml.) Reaction was terminated by the addition of 6% $HClO_4$ and the lipid extracted by Folch solvents as described in Materials and Methods Section VI.B.5b. Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

D-[U^{14}C]fructose (9×10^{5} dpm/assay)
D-[U^{14}C]glucose (9×10^{5} dpm/assay)

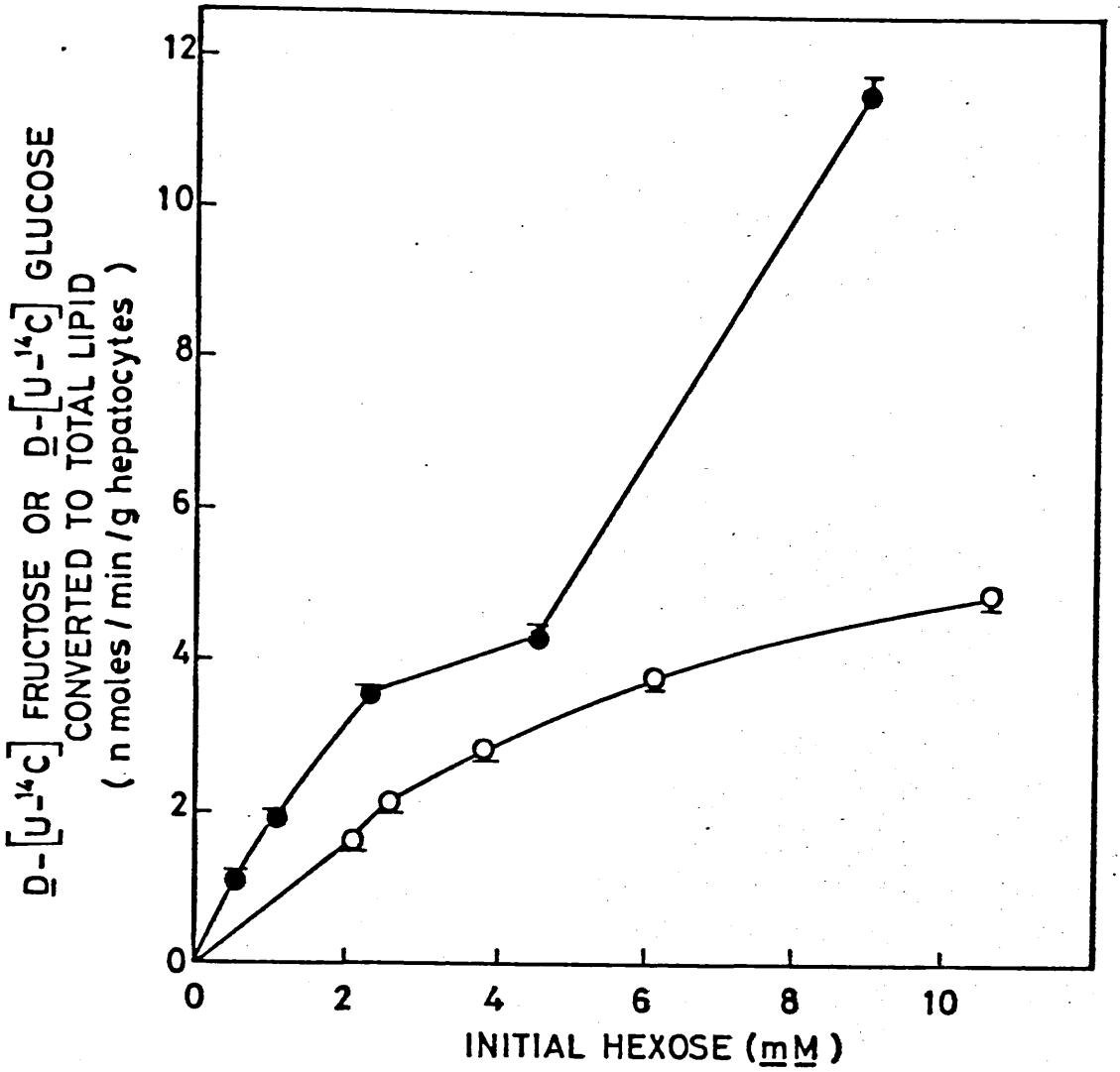


Fig. 10 Incorporation of D - [U-^{14}C] fructose (•) and D - [U-^{14}C] glucose (o) into total extractable lipid by isolated hepatocytes.

Isolated hepatocytes (4.4×10^6 cells/ml) from fed rats were preincubated at 37° for 1h in KRB + Ca^{++} + BSA (1.5%) and then with various concentrations of hexose for 2h.

Experimental techniques are described in Materials and Methods Section VI.B.5b.

Each point represents the mean ± SEM of the results with 4 hepatocyte preparations.

This value changed to 3.2 mM after 60 min incubation. Thus, the incorporation of glucose into total lipid may be underestimated. However, addition of fructose to the hepatocytes also causeda further increase in glucose levels (Fig. 11) which could also result in isotopic dilution of glycolytic intermediates. The concentration dependence of the effect of fructose is shown in (Fig. 12). Fructose stimulates glucose output at all concentrations up to 10 mM. There are two possible explanations for this phenomenon. Firstly, gluconeogenesis from fructose could contribute glucose (Zahlten et al., 1973; Foster and Blair, 1978) and secondly, it is known that incubation of hepatocytes with fructose can lead to an increased glucose output as a result of the elevation of phosphorylase a activity (Van der Werve and Hers, 1977). One method of minimizing the differential isotope dilution effect is to use isolated hepatocytes from fasted animals, although in this condition a much reduced synthesis of triacylglycerol would be expected (Groener and Van Golde, 1977). Fig. 13 shows that the basal glucose output by these hepatocytes is 0.124 ± 0.035 S.D. (n = 3) μmole/min/g. This is much reduced compared to the cells from fed animals. Incubation of the fasted hepatocytes with fructose, however, resulted in a rapid glucose output 3.02 ± 0.05 S.D. (n = 3) with the glucose concentration reaching 5.6 mM after 30 min incubation. The reduced glucose output in fasted hepatocytes has also been reported by Foster and Blair (1978) and Pilkis et al. (1976) and appears to be due to glycogen depletion during fasting. Thus the radioactive dilution by endogenous glucose is much reduced in cells isolated from fasted animals. However, the difference in incorporation of D - [U-^{14}C] fructose and D - [U-^{14}C] glucose persists in these hepatocytes. When the hepatocytes were incubated with fructose (9 mM) the rate of fructose incorporation into total lipid was 3.5 ± 1.9 S.D. (n = 8) nmole/min/g, whereas at the same concentration of glucose the rate of glucose incorporation was 1.9 ± 0.8 S.D. (n = 22) nmole/min/g. One explanation for this difference is the ease of conversion of fructose to sn-glycerol 3-phosphate in isolated hepatocytes compared to that of glucose, but since the dilution by endogenous intermediates is reduced it is also possible that fructose increases the rate of lipid synthesis.

A further study on the fate of the label in the various fractions which constitute the total lipid fraction was carried out with hepatocytes from fed animals. The various lipid classes were separated

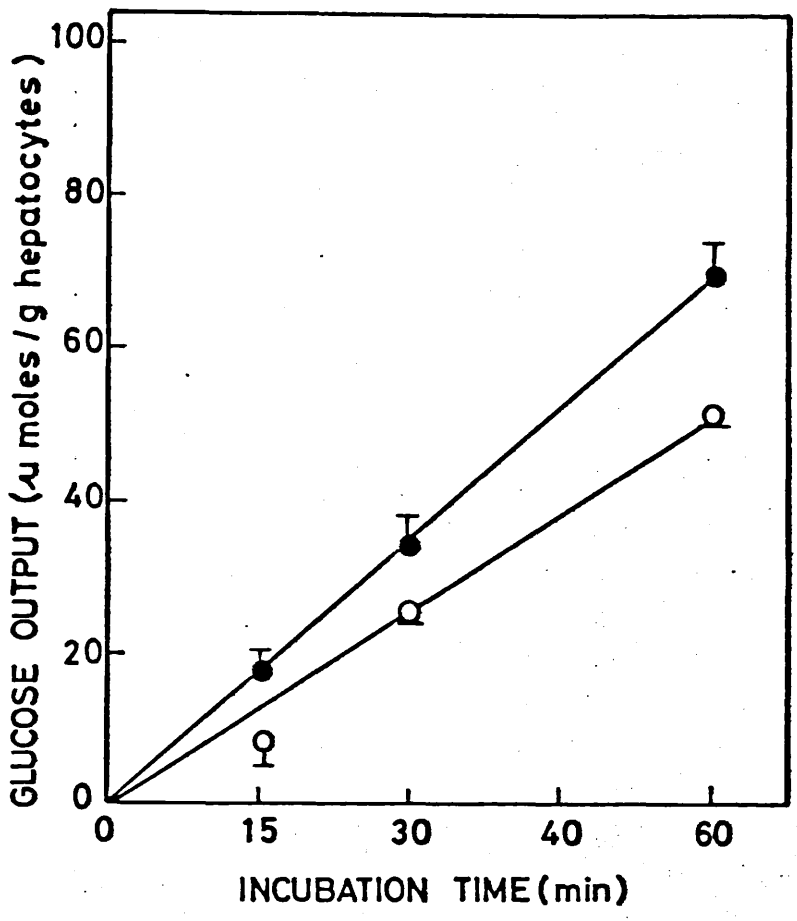


Fig. 11 Effect of fructose on glucose output.

Isolated hepatocytes (4×10^6 cells/ml) from fed rats were preincubated for 1h without substrate and then with (•) or without (o) fructose (1mM) for various times. Experimental methods are described in Section VI B.4b.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

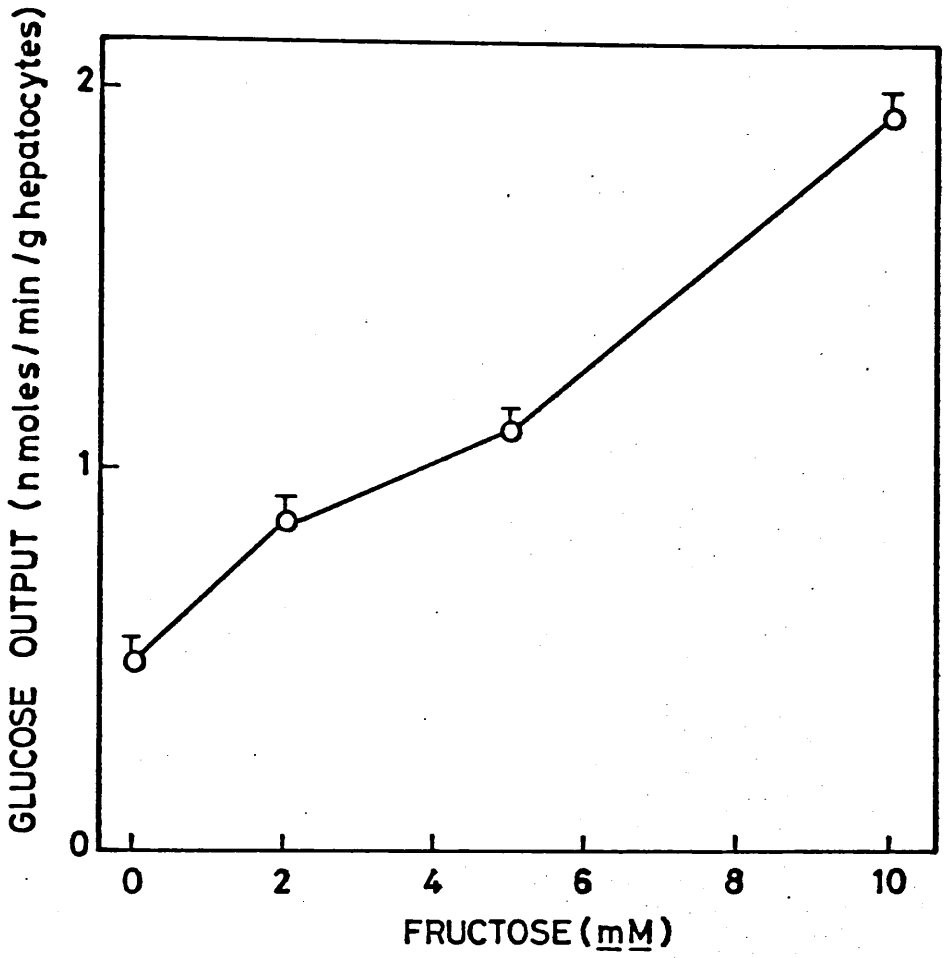


Fig. 12. Effect of various concentrations of fructose on glucose output.

Isolated hepatocytes (10 x 10^6 cells/ml) were preincubated at 37^o for 20 min and then in presence of fructose for 30 min.

Experimental techniques are described in Section VI.B.4b.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

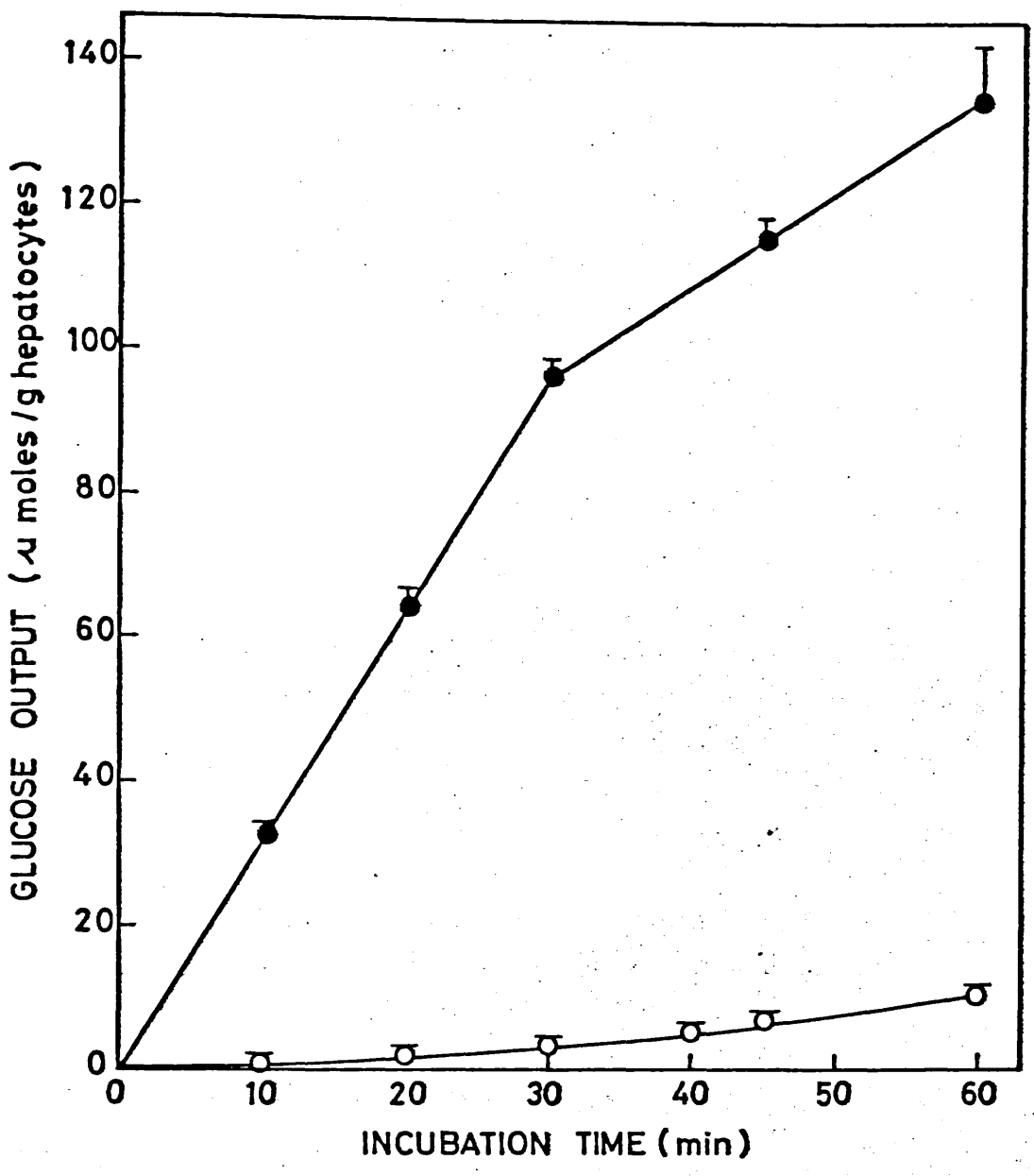


Fig. 13 The effect of fructose on glucose output by isolated hepatocytes from 24h fasted rats.

Isolated hepatocytes (7×10^6 cells/ml) from 24h fasted rats were incubated with (•) and without (o) fructose (10 mM) for various times.

Experimental details are described in Section VIB.4b.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

by thin-layer chromatography (TLC) and the distribution of radioactivity in the various fractionswas examined. Most of the radioactivity was found to be associated with either phospholipid or the triacylglycerol fractions (Table II). In the case of the D - [U-^{14}C] fructose a greater proportion of the label was associated with the triacylglycerol than with the phospholipid fraction, but the reverse was true for D - [U-^{14}C] glucose (Table II). Geelen et al. (1978) also found a preferential synthesis of phosholipid in cultured hepatocytes incubated with D - [U-^{14}C] glucose.

In a further experiment the total lipid was fractionated and the fate of the [^{14}C] hexose of the triacylglycerol fraction was determined. Only 13% was associated with the fatty acid fraction and the remainder (87%) was found in the glycerol moiety in the case of hepatocytes incubated with D - [U-^{14}C] fructose. With hepatocytes incubated with D - [U-^{14}C] glucose an even smaller proportion (7%) was found in the fatty acid fraction of the triacylglycerol. It is clear from this work that most of the label associated with the total lipid fraction is found in the glycerides and that most of this is associated with the glycerol moiety. These findings are comparable to those of other authors who used intact animals and cultured hepatocytes (Chernick and Scow, 1964; Bar-on and Stein, 1968; Maruhama and Macdonald, 1973; Geelen et al., 1978a). It is clear, therefore, that most of the incorporation into the total lipid is due to the ease of conversion of fructose to sn-glycerol 3-phosphate, a substrate for both triacylglycerol and phospholipid synthesis. It is known that perfusion of liver with fructose leads to an elevated sn-glycerol 3-phosphate level in the tissue (Exton and Park, 1969; Burch et al.,1970; Woods et al., 1970). Under the conditions used in this study an increase was found in the sn-glycerol 3-phosphate content of the hepatocytes from 0.20 ± 0.02 SEM (n = 3) to 0.42 ± 0.03 SEM (n = 3) μmol/g cells when hepatocytes were incubated for 60 min with 10 mM fructose. Further studies in this department (Mapungwana and Davies unpublished results) revealed that the elevation of sn-glycerol 3-phosphate was also found at low concentrations of fructose (1 mM) and that these changes are achieved without a significant change in the lactate/pyruvate ratio which can also affect the sn-glycerol 3-phosphate content of the cells (See also Table IV).

The present study also shows that comparatively little [^{14}C] fatty

Table II. Incorporation of radioactivity into various lipid fractions.

Hepatocytes (8×10^6 cells/ml; 0.5 ml) from fed rats were incubated with either D - [U-^{14}C] fructose or D - [U-^{14}C] glucose for 1 h. The total lipid extract was fractionated by thin-layer chromatography (see Section VI 5b).

The results are expressed as the percentage of the total lipid radioactivity in each fraction.

	Precursor (9mM)	
Fraction	D - [U-^{14}C]fructose	D - [U-^{14}C] glucose
Phospholipid	39	48
Triacylglycerol	50	29
Total glycerolipid	89	77
Cholesterol	8	13
Cholesterol ester	3	10

acid is synthesised from either D - [U-^{14}C]glucose or D - [U-^{14}C] fructose. This finding is presumably the result of the greater isotopic dilution which occurs during glycolysis and fatty acid synthesis whereas the conversion of fructose and, to a lesser extent, glucose to sn-glycerol 3-phosphate involve fewer enzymatic reactions. Furthermore, it has been shown that glucose at low concentrations is a relatively poor carbon source for fatty acid synthesis (Clark et al., 1974; Salmon et al., 1974; Hems, 1975; Bloxham et al., 1977) whereas lactate is a much better precursor (Clark et al., 1974; Salmon et al., 1974; Hems, 1975; Hems, 1977) and that fructose (15 mM) is a potent inhibitor of fatty acid synthesis (Selmer and Grunnet, 1976). Thus, the conditions used in the present study are not conducive to maximal rates of fatty acid synthesis from hexoses. However, the data obtained in the present study points to the conclusion that fructose is a better precursor for fatty acid synthesis than glucose. This is in accord with the observation of Woods and Krebs (1971) and Salmon et al., (1974) which suggest that fatty acid synthesis is related to glycogen depletion. The utilization of either glycogen or of fructose for the synthesis of fatty acid are both independent of the regulatory enzyme, glucokinase, which is thought to regulate glucose utilization by hepatocytes (Crisp and Pogson, 1972; Bontemps et al., 1978). Indeed, one would expect that fructose would be more readily converted to fatty acid than glycogen since the metabolism of the latter requires flux through two further regulatory enzymes, glycogen phosphorylase and phosphofructokinase. Hopkirk and Bloxham (1979) have provided evidence that the regulation of hepatic pyruvate kinase may be related to the regulation of fatty acid synthesis in the fed rat. It is known that fructose 1-phosphate, which accumulates in livers of animals fed with fructose (Tay, 1977) or in livers perfused with 10 mM fructose (Kjerulf-Jensen, 1942; Heinz, 1968; Burch et al., 1969; 1970; Woods et al., 1970), is an activator of pyruvate kinase (Eggleston and Woods, 1970). This would suggest that the accumulation of FIP, which also occurs in isolated hepatocytes incubated with fructose (Mapungwana and Davies, 1982) would result in a high rate of glycolysis from fructose, and thus a higher rate of incorporation from D - [U-^{14}C] fructose into fatty acids compared to the incorporation of D - [U-^{14}C] glucose.

It should be noted that the rate of incorporation of label from [^{14}C] hexose into lipid does not represent the total amount of lipid

synthesised since the synthesis of both sn-glycerol 3-phosphate and of fatty acid from endogenous sources is not taken into account by this method of assay. For example, glycerol released from triacylglycerol during lipolysis may be rephosphorylated by hepatic glycerokinase and this could dilute the sn-glycerol 3-phosphate pool.

C. Effect of added oleate on the incorporation of hexose into total lipid.

Hepatic triacylglycerol and phospholipid can arise either from the plasma FFA or from long-chain fatty acid synthesised by the liver (Mayes, 1976). Therefore, in the absence of added exogenous fatty acid the utilization of $[^{14}C]$ hexose for the synthesis of glycerolipid is dependent on either de novo fatty acid synthesis or on the availability of endogenous fatty acid synthesis or on the availability of endogenous fatty acid arising from the breakdown of the pool of triacylglycerol in the hepatocyte. It was decided, therefore, to examine the incorporation of $[^{14}C]$ hexoses under conditions where synthesis of triacylglycerol would be stimulated by exogenous fatty acid. FFA in the form of an oleate-albumin complex was added to the hepatocyte preparation and the incorporation of D - $[U\text{-}^{14}C]$ glucose and D - $[U\text{-}^{14}C]$ fructose into total lipid was examined. It was found that the rate of incorporation of the hexoses was also linear in the presence of added oleate (2 mM) (data not shown).

It was observed that oleate (1 mM) added to the incubation medium had a marked stimulatory effect on the incorporation of label from D - $[U\text{-}^{14}C]$ fructose into total lipid (Fig. 14a) but comparatively little effect on the incorporation of label from D - $[U\text{-}^{14}C]$ glucose (Fig. 14b). A statistical analysis of the data indicated that addition of oleate (1 mM) increased the incorporation of D - $[U\text{-}^{14}C]$ fructose into total lipid significantly ($P < 0.005$) at all concentrations of fructose examined. D - $[U\text{-}^{14}C]$ glucose incorporation, on the other hand, is only significantly increased by addition of FFA in the presence of 9 mM glucose. Fig. 15 shows the effect of increasing oleate concentration on the incorporation of $[^{14}C]$ hexose into total lipid. It is clear that as the oleate levels are raised more incorporation occurs from both labelled precursors but it appears that the effect is more pronounced with fructose. Inhibition of glucose incorporation was

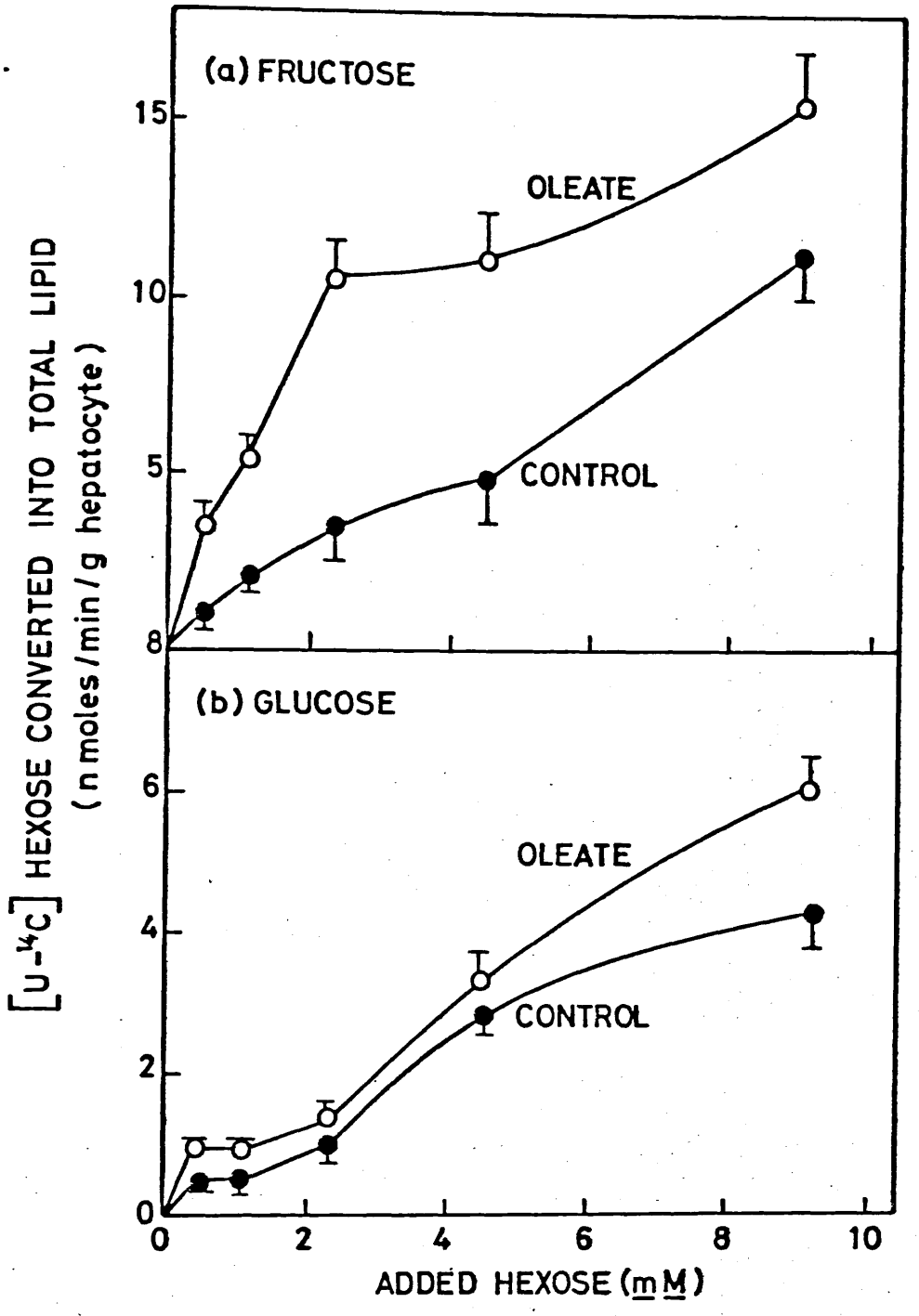


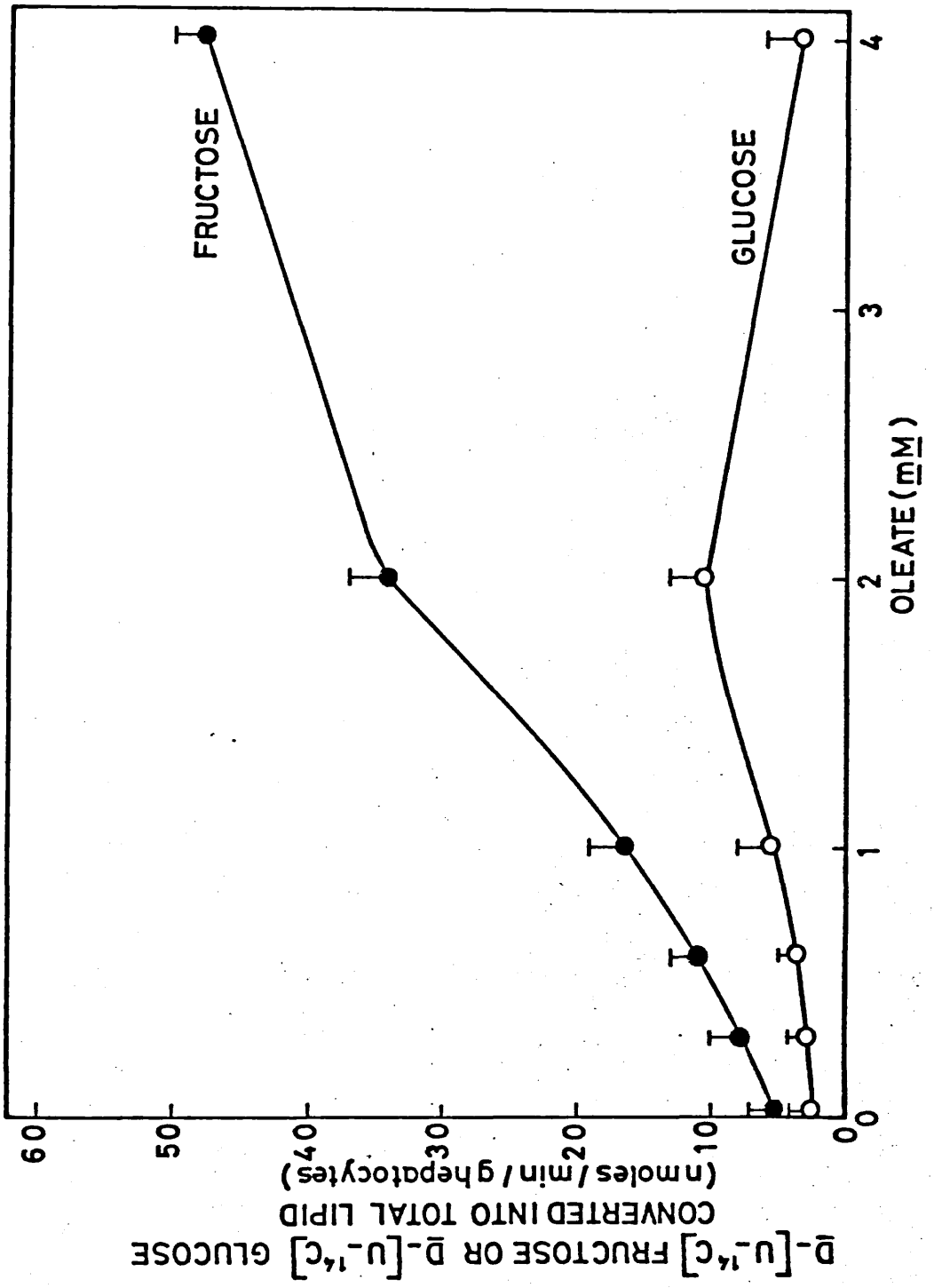
Fig. 14 Effect of sodium oleate (1mM) on the incorporation of D - [U-^{14}C] fructose and D - [U-^{14}C] glucose into total lipid by isolated hepatocytes from fed animals incubated with the appropriate substrates for 2h. Experimental details are described in Section IIB. 5b.

Each point represents the mean ± SEM of the results with 4 hepatocyte preparations.

Fig. 15 Effect of sodium oleate - BSA complex on the incorporation of hexoses into total lipid.

Isolated hepatocytes (4×10^6 cells/ml) from fed rats were incubated for 2h with D - [U-^{14}C] fructose (•) or D - [U-^{14}C] glucose (o) and sodium oleate - BSA complex. The total BSA content of the incubation medium was maintained at 1.5%. Experimental details are described in Section VI B.5b and VI V.7.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.


D-[U-14C] FRUCTOSE OR D-[U-14C] GLUCOSE
CONVERTED INTO TOTAL LIPID
(n moles / min / g hepatocytes)
FRUCTOSE
GLUCOSE
OLEATE (mM)
0
1
2
3
4
10
20
30
40
50
60

observed with an unphysiological concentration (4 mM) of the oleate. The increase in the rate of incorporation presumably reflects an increased flux through sn-glycerol 3-phosphate as a result of increased glycerolipid synthesis. If isotopic dilution of the intermediates is ignored, then the rate of flux from D - [U-^{14}C] fructose into glycerolipid reaches a maximum level of approximately 34 nmoles/min/g hepatocytes in the presence of 2 mM oleate. This is comparable with maximal rates of triacylglycerol output by liver perfused with oleate observed by Heimberg et al., 1969: 1974) and of oleate esterification in isolated hepatocytes (Williamson et al., 1980).

A preliminary study on the fate of the label in the triacylglycerol fraction indicated that the increase in incorporation was totally accounted for by an increased incorporation into the glycerol moiety (Table III) and, as expected, a percentage decrease in the amount of [^{14}C] hexose converted to fatty acid was observed. This is consistent with the observations of Mayes and Topping (1974) who found that oleate inhibits lipogenesis by perfused liver and of Williamson and Whitelaw (1978) who showed that the fatty acid inhibits lipogenesis from $^{3}H_2O$ by 65% in isolated hepatocytes from fed rats, but there is little evidence that addition of exogenous oleate to hepatocytes results in a substantial inhibition of fatty acid synthesis from hexoses in the results shown in Table III. In the present study, the increased utilization of fructose and, to a lesser extent, glucose for the synthesis of the glycerol moiety of the glycerolipid following the addition of oleate must be the result of increased glycerolipid synthesis. It follows that incorporation of label from D - [U-^{14}C] fructose into triacylglycerol is a good indication of the amount of triacylglycerol synthesis occurring. The results shown in Fig. 14 are consistent with the observation of Ontko (1972) that fructose stimulates oleate esterification by isolated hepatocytes. The results of Ontko (1972) indicated that fructose exerts its effect by entering the glycolytic sequence at the triose phosphate level and thus inhibiting ketogenesis by competition with fatty acid oxidation and that this results in an increased availability of long chain FFA for esterification. Later work by Prager and Ontko (1976) using isolated mitochondria and a high-speed supernatant from rat liver indicated that the effect of fructose in the inhibition of ketogenesis was exerted at the β-oxidation of long-chain FFA at a site beyond fatty acid activation and acyl carnitine

Table III. Estimation of radioactivity in glycerol and fatty acid moieties of triacylglycerol following incubation of hepatocytes with $[^{14}C]$ hexoses.

Hepatocytes (8×10^6 cells/ml; 0.5 ml) from fed rats were pre-incubated without substrates and then with D - $[U-^{14}C]$ fructose (9mM) and D - $[U-^{14}C]$ glucose (9mM) in presence and absence of oleate (2mM) for 2h. The triacylglycerol fraction was separated by TLC and then subjected to saponification as described in Materials and Methods (Section VI 5d).

Incubation	Incorporation into glycerol moiety *	Incorporation into fatty acid moiety *	% label incorporated into fatty acid fraction
D $[U-^{14}C]$ fructose	13.4	2.04	13.2
D $[U-^{14}C]$ glucose	2.6	0.21	7.5
D $[U-^{14}C]$ fructose + oleate	168.0	1.75	1.0
D $[U-^{14}C]$ glucose + oleate	12.3	0.22	1.8

*nmoles of hexose incorporated /min/g hepatocytes

formation. In this context, Benito et al., (1979) have shown a significant inverse correlation between esterification and ketogenesis in hepatocytes isolated from rats in a wide variety of physiological states. However, there are few indications in the literature on the specific effects of fructose on triacylglycerol synthesis other than it may inhibit ketogenesis and thus indirectly stimulate esterification. If it is assumed that fatty acids have no effect on the conversion of fructose to sn-glycerol 3-phosphate, it is clear from the present study that fructose stimulates triacylglycerol synthesis from oleate. Recent evidence by Declerq et al., 1982b suggests a correlation between sn-glycerol 3-phosphate levels and triacylglycerol synthesis which gives a clue as to the mode of action of fructose in the present study.

Fig. 16 shows the results of a typical experiment demonstrating the effect of increasing oleate concentrations on the distribution of label from D - $[U\text{-}^{14}C]$ fructose into the various lipid classes. There are two major products, phospholipid and triacylglycerol, which account for more than 80% of the total lipid. At each concentration of oleate the majority of the label was associated with the triacylglycerol fraction and a smaller proportion with the phospholipid fraction. With increasing levels of oleate there was an increased incorporation into triacylglycerol with a maximum level at 2 mM oleate. As the concentrations of oleate are increased the proportion of the total lipid associated with the triacylglycerol fraction increases, indicating that triacylglycerol synthesis is favoured compared to phospholipid synthesis.

The incorporation of D - $[U\text{-}^{14}C]$ glucose into triacylglycerol as used by Geelen et al., (1978a) is not a good measure of triacylglycerol synthesis because of the isotopic dilution by endogenous glucose and because of the relatively slow metabolism of the hexose to sn-glycerol 3-phosphate, since there are two regulatory enzymes involved. The use of $[^{14}C]$ glycerol as a precursor, on the other hand, is also subject to some criticism since this can effect the NADH/NAD ratio within the cells (see Table IV) and also the intracellular pool of sn-glycerol 3-phosphate is vastly increased from 0.28 ± 0.1 SEM (n = 3) µmoles/g hepatocytes to unphysiological levels 6.5 ± 1.47 SEM (n = 3) by incubating the cells for 60 min with 10 mM glycerol. It has been shown in this laboratory that concentrations as low as 1 mM glycerol can produce an elevation in sn-glycerol 3-phosphate content to levels greater than 1 µmole/g hepatocytes (Mapungwana and Davies, 1982).

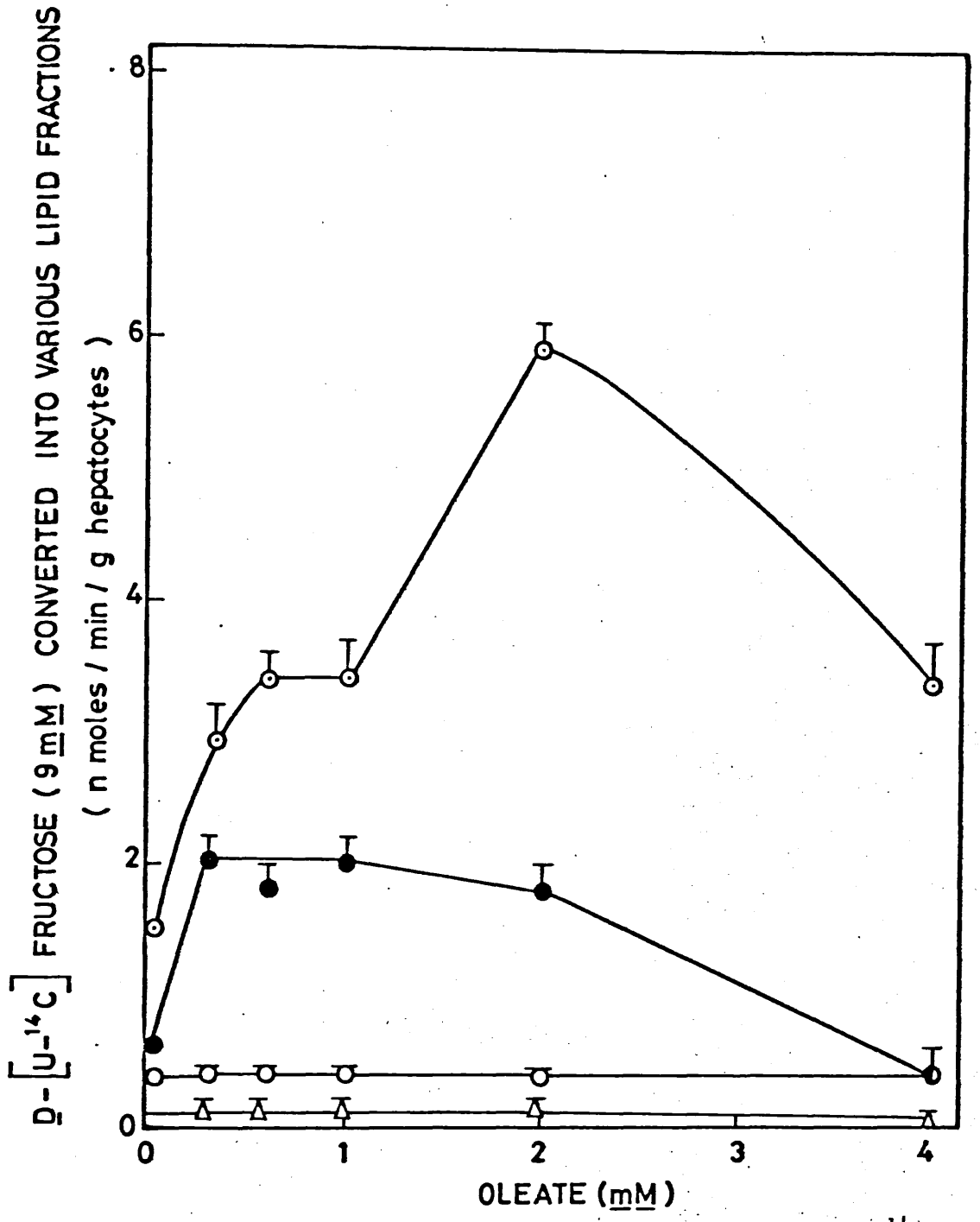


Fig. 16 Effect of sodium oleate on the incorporation of D - [U-^{14}C] fructose (9mM) into various lipid fractions separated by TLC. Isolated hepatocytes (4.0 x 10^6 cells/ml) were preincubated at 37° and then incubated in presence of D - [U-^{14}C] fructose (9mM) and various concentrations of sodium oleate for 2h. Symbols: (●) phospholipid; (o) cholesterol; (◉) triacylglycerol; (Δ) cholesterol ester. Experimental details in Materials and Methods Section VI.B.5c. Each point represents the mean ± SEM of three determinations with one hepatocyte preparation.

Table IV. The effect of fructose (10mM) and glycerol (10mM) on lactate and pyruvate levels in isolated hepatocytes

	μmole per g hepatocytes		
	Control	Fructose (10mM)	Glycerol (10mM)
Lactate	27.8 ± 12.2	126.8 ± 8.5	15.5 ± 0.33
Pyruvate	3.7 ± 0.62	13.0 ± 0.37	0.62 ± 0.05
Lactate/pyruvate ratio	7.6 ± 0.85	9.8 ± 0.59	25.5 ± 2.15

Isolated hepatocytes (8×10^6 cells/ml., 0.5 ml) were incubated with an equal volume of the appropriate substrate for 60 min. Aliquots of the cells were removed and extracted with perchloric acid and the neutralized extracts were assayed for lactate and pyruvate as described in Materials and Methods (Section VI 4C). All the values are means ± SEM (n = 3).

However, the results in Table IV are in accordance with the evidence presented by Lamb et al., (1977) who used [1, 3 ^{14}C] glycerol to estimate fatty acid esterification in isolated hepatocytes cultured in a primary monolayer and by Sundler et al., (1974) who found that triacylglycerol synthesis from [^{3}H] glycerol by isolated hepatocytes was markedly stimulated by fatty acid but that the effect on phospholipid synthesis was less apparent. It is of interest to note that glycerolipid synthesis from both fructose and glucose is inhibited at high concentration of oleate (see Fig. 16). This is presumably because of the deleterious effect of high levels of FFA on hepatocytes (Krebs and Söling, 1976) resulting in damage to the triacylglycerol synthesising system. In this context it is of interest to note that Lamb et al., (1977) did not find any inhibition of glycerolipid synthesis by cultured hepatocytes at concentration up to 2 mM palmitate but they did observe inhibition of the appearance of triacylglycerol in the medium at this concentration.

The values for total lipid synthesis were lower than those obtained using D - [U-^{14}C] glucose as a precursor but similar in that increasing oleate levels up to 2 mM had a stimulatory effect on the incorporation of label into the glycerolipids. No stimulation of either cholesterol or cholesterol ester synthesis was observed with either fructose or glucose (Fig. 16 and 17). It is interesting, however, that with the aldose the major labelled product is phospholipid and relatively little label is incorporated into the triacylglycerol whatever the concentration of oleate used. This contrasts with the results obtained for fructose. This difference is also seen in the results presented in Fig. 18 which show that with increasing oleate levels, the proportion of label from fructose associated with the triacylglycerol increases to > 70% whereas with glucose the level of incorporation remains comparatively low (a maximum of 35%), the data for glucose-treated hepatocytes are in agreement with the results of Groner and Van Golde (1977) who measured phospholipid and triacylglycerol synthesis from [2-^{3}H] glycerol by isolated rat hepatocytes and found that the rate of incorporation into the former fraction was greater than into the latter. Fasting the animals for up to 48 h accentuated the difference. These authors did not examine the effect of exogenous fatty acid on the rates of incorporation but triacylglycerol synthesis was much more susceptible to fasting/refeeding than phospholipid synthesis. Since the experiment shown in Fig. 16 and Fig. 17 were run in parallel using the same sample of hepatocytes,

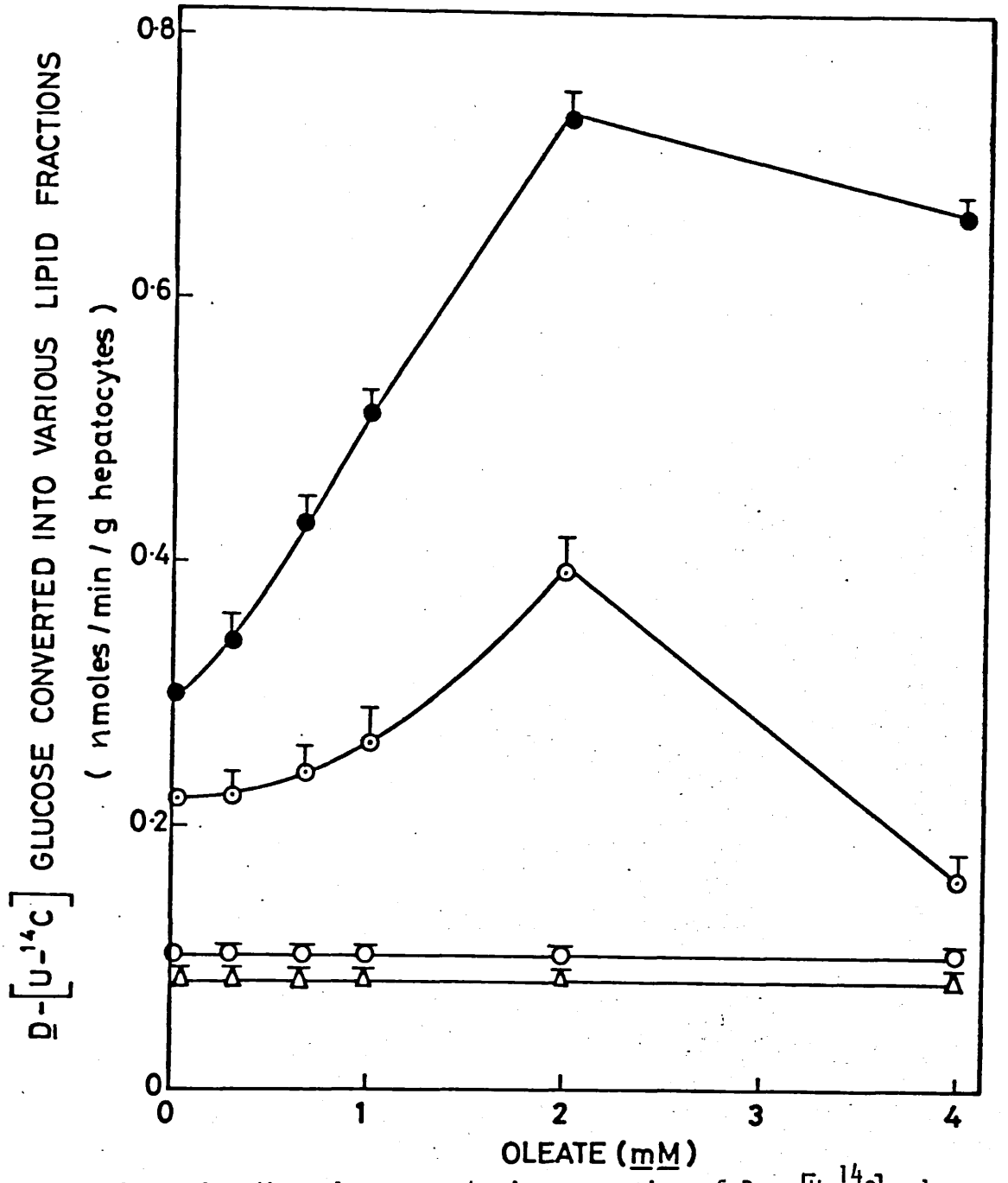


Fig. 17 Effect of sodium oleate on the incorporation of D - [U-^{14}C] glucose (9mM) into various lipid fractions.

Isolated hepatocytes (4.0 x 10^6 cells/ml) were preincubated at 37° for 2h and then in presence of D - [U-^{14}C] glucose and various concentrations of sodium oleate - BSA complex for 2h.

Symbols: (●) phospholipid; cholesterol (o); triacylglycerol (⊙); cholesterol ester (Δ).

Experimental technique described in Section VI.B.5b.

Each point represents the mean ± SEM of three determinations with one hepatocyte preparation.

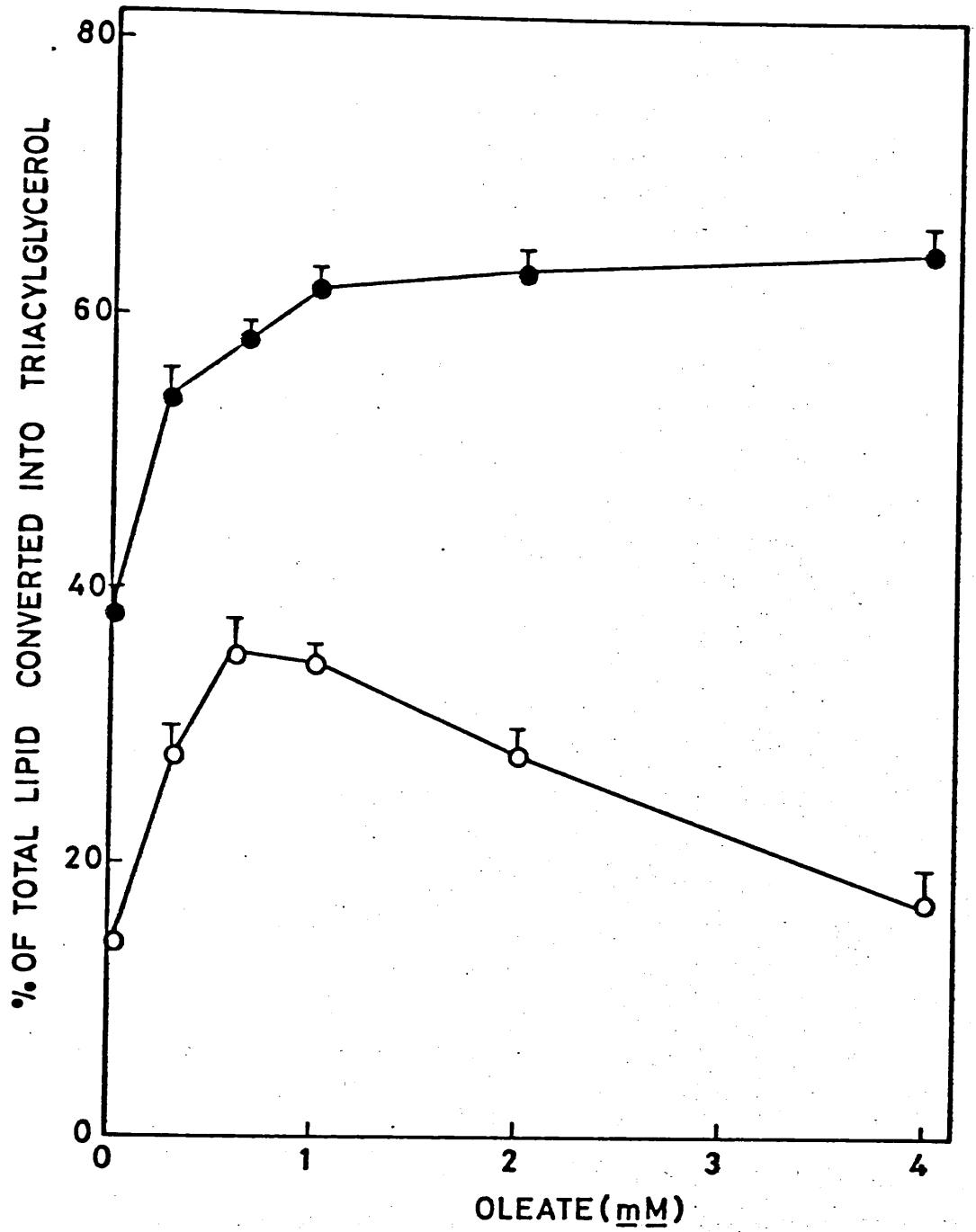


Fig. 18 Effect of sodium oleate concentration on the percentage of the total lipid incorporated from D - [U-^{14}C] fructose or D - [U-^{14}C] glucose (9mM) into the triacylglycerol.

Isolated hepatocytes (4.0 x 10^6 cells/ml) from fed rats were preincubated for 1h without substrate and then with D -[U-^{14}C] fructose (•) or D - [U-^{14}C] glucose (o) and various concentrations of sodium oleate for 2h.

Experimental details described in Section VI.B.5b.

Each point represents the mean ± SEM of three determinations with one hepatocyte preparation.

The results suggest a specific effect of fructose on triacylglycerol synthesis relative to phosphoglyceride synthesis. The pathway for the incorporation of label into the glyceride fractions follows the same route to phosphatidic acid (see Fig. 4). It is clear that a greater incorporation occurs from fructose than from glucose, again reflecting the ease of conversion of the former to triosephosphate but the difference in relative rates of synthesis of phospholipid and triacylglycerol must be the result of differences in the response of the cells to fructose and glucose. It is likely, therefore, that fructose, or a metabolite which changes in concentration as a result of fructose metabolism, is regulating triacylglycerol synthesis either by stimulation of one of the enzymes unique to the synthesis of the triacylglycerol, PPH and diacylglycerol: Acyl CoA acyltransferase, or by inhibition of phosphoglyceride synthesis from phosphatidic acid. Assuming that fatty acid has no effect on the conversion of fructose to sn-glycerol 3-phosphate then it is clear from Fig. 14a and Fig. 16 that addition of oleate in the presence of fructose results in an increased flux through sn-glycerol 3-phosphate into the glycerolipids, notably triacylglycerol. It is of interest in this context that Groener and Van Golde (1977) found that fasting resulted in a decrease in both triacylglycerol and phospholipid synthesis from $[2^3H]$ glycerol by isolated hepatocytes and that sucrose-refeeding rats for 24h resulted in a specific stimulation of triacylglycerol synthesis.

Cooper and his co-workers (Abrams and Cooper, 1976; Lipkin et al., 1978; Palmer et al., 1978) have found considerable rates of hepatic triacylglycerol output in starved animals in vivo. The triacylglycerol arises from plasma FFA and the recycling of plasma triacylglycerol. In addition, it is well-known that plasma FFA are elevated and hepatic ketogenesis increased in the starved condition (Amatruda et al., 1978). Therefore, it was of interest to examine if the effect of fructose on glycerolipid synthesis was also found in hepatocytes from starved animals. Table V shows that in the absence of added oleate there is a significant difference ($P < 0.005$) between the incorporation of D - $[U\text{-}^{14}C]$ fructose into glycerolipid by hepatocytes from fed animals and cells from rats fasted for 24h. A similar result was also observed with D -$[U\text{-}^{14}C]$ glucose ($P < 0.005$) in agreement with the data obtained by Groener and Van Golde (1977). However, addition of oleate (1mM) to the hepatocytes resulted in a statistically significant stimulation of incorporation of label from both hexoses in both fed and fasted animals (Table V). With oleate, however, no significant difference between the incorporation of

Table V. Effect of fasting on total lipid synthesis from $[^{14}C]$ hexoses in the presence or absence of oleate (1mM).

The isolated hepatocytes (4×10^6 cells/ml; 1.0 ml) were incubated with either D - $[U-^{14}C]$ glucose or D - $[U-^{14}C]$ fructose in the presence or absence of sodium oleate - BSA complex as described in Section IV, 5.

	nmoles hexose/min/g hepatocytes converted to lipid			
	Glucose (9 mM)	Fructose (9 mM)	Glucose (9 mM) + oleate (1 mM)	Fructose (9 mM) + oleate (1 mM)
Fasted (24h)	1.9 ± 0.8 (n = 22)	3.5 ± 1.9 (n = 8)	7.5 ± 2.6 (n = 8)	11.6 ± 4.4 (n = 8)
Fed	4.2 ± 2.3 (n = 28)	10.6 ± 6.7 (n = 28)	6.0 ± 2.4 (n = 9)	15.6 ± 4.7 (n = 14)

The results are expressed as nmoles hexose utilized per min per g liver ± S.D. Number of animals used are given in parenthesis.

A statistical analysis of the data is given in Table Va.

Table Va. Statistical analysis of the data in Table V.

Comparison		Statistical significance P	
Glucose fed	V fasted	< 0.005	S
Fructose fed	V fasted	< 0.005	S
Glucose + oleate fed	V fasted	< 0.10	N.S.
Fructose + oleate fed	V fasted	< 0.10	N.S.
Fed Glucose	V Glucose + oleate	< 0.05	S
Fed Fructose	V Fructose + oleate	< 0.005	S
Fasted Glucose	V Glucose + oleate	< 0.005	S
Fasted Fructose	V Fructose + oleate	< 0.005	S

N.S. = Not significant

S = Significant

label by hepatocytes from fed and fasted animals was found. The explanation for this finding is not clear; it is possible that there are no differences in the maximal rate of oleate esterification by fed and fasted animals. It is likely that the difference in D - [U-^{14}C] fructose incorporation into total lipid, in the absence of oleate, in the fasted and fed animals reflect the total synthesis of glycerolipids since it would be expected that in the fasted state less glycerolipid would be synthesised and more of the endogenous fatty acid would undergo ketogenesis and oxidation. It appears that exogenous fatty acid has the effect of stimululating fatty acid esterification in hepatocytes from both fasted and fed animals. The stimulation of esterification by fructose may be related to the known antiketogenic effect of the sugar (Mayes, 1962; Exton and Edson, 1964; Mayes and Felts, 1967). A similar but less marked stimulation with D - [U-^{14}C] glucose as a precursor possibly reflects the fact that glucose does exert some antiketogenic effect (Exton and Edson, 1964).

Fig. 19 shows the effect of increasing oleate concentrations on the incorporation of [^{14}C] hexose into total lipid by hepatocytes from 24h fasted rats. Again, the incorporation is increased with increasing fatty acid concentration with a maximum rate observed at 2mM; 4mM oleate was found to have an inhibitory effect on the reaction. This is comparable to the effect found in hepatocytes from fed animals (cf. Fig. 16 and Fig. 17). With hepatocytes from fasted rats a significant difference between D - [U-^{14}C] fructose and D - [U-^{14}C] glucose incorporation was observed at all concentrations of oleate examined except 4mM oleate (Fig. 19). Again, this reflects the comparative ease of conversion of the ketose to sn-glycerol 3-phosphate but the similarities in the result for fed and fasted hepatocytes indicate that incorporation into total lipid reflects the stimulation by oleate of sn-glycerol 3-phosphate esterification in both conditions. The rate of incorporation observed in this experiment (Fig. 19) and in hepatocytes from fed rats (Fig. 15) indicates that there is no significant difference in the rate of flux from fructose into glycerolipid - glycerol in fed and fasted animals if exogenous oleate is added to the hepatocytes (see also Table V).

Fig. 20 shows the result of one experiment with an animal fasted for 48h before isolation of hepatocytes. Again, stimulation of [^{14}C]

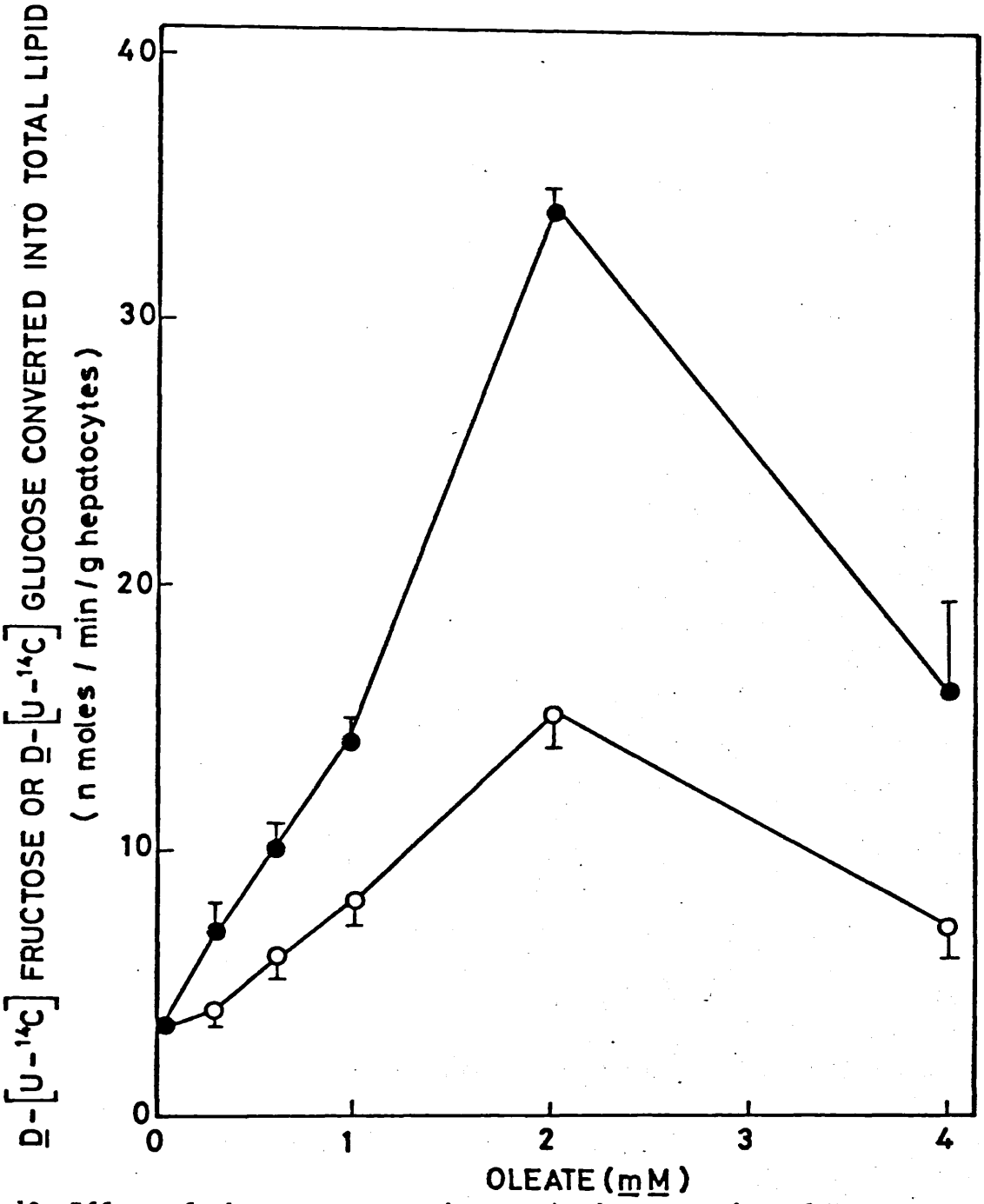


Fig. 19 Effect of oleate concentration on the incorporation of Hexoses into total lipid by hepatocytes from 24h fasted rats.

Hepatocytes (5.0×10^6 cells/ml) from 24h fasted rats were pre-incubated in KRB + Ca^{++} + BSA (1.5%) and then incubated with 9mM fructose (•) or glucose (o) and various concentrations of sodium oleate in KRB + Ca^{++} + BSA (1.5%) for 2h.

Experimental details given in Materials and Methods Section VI.B.5b.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

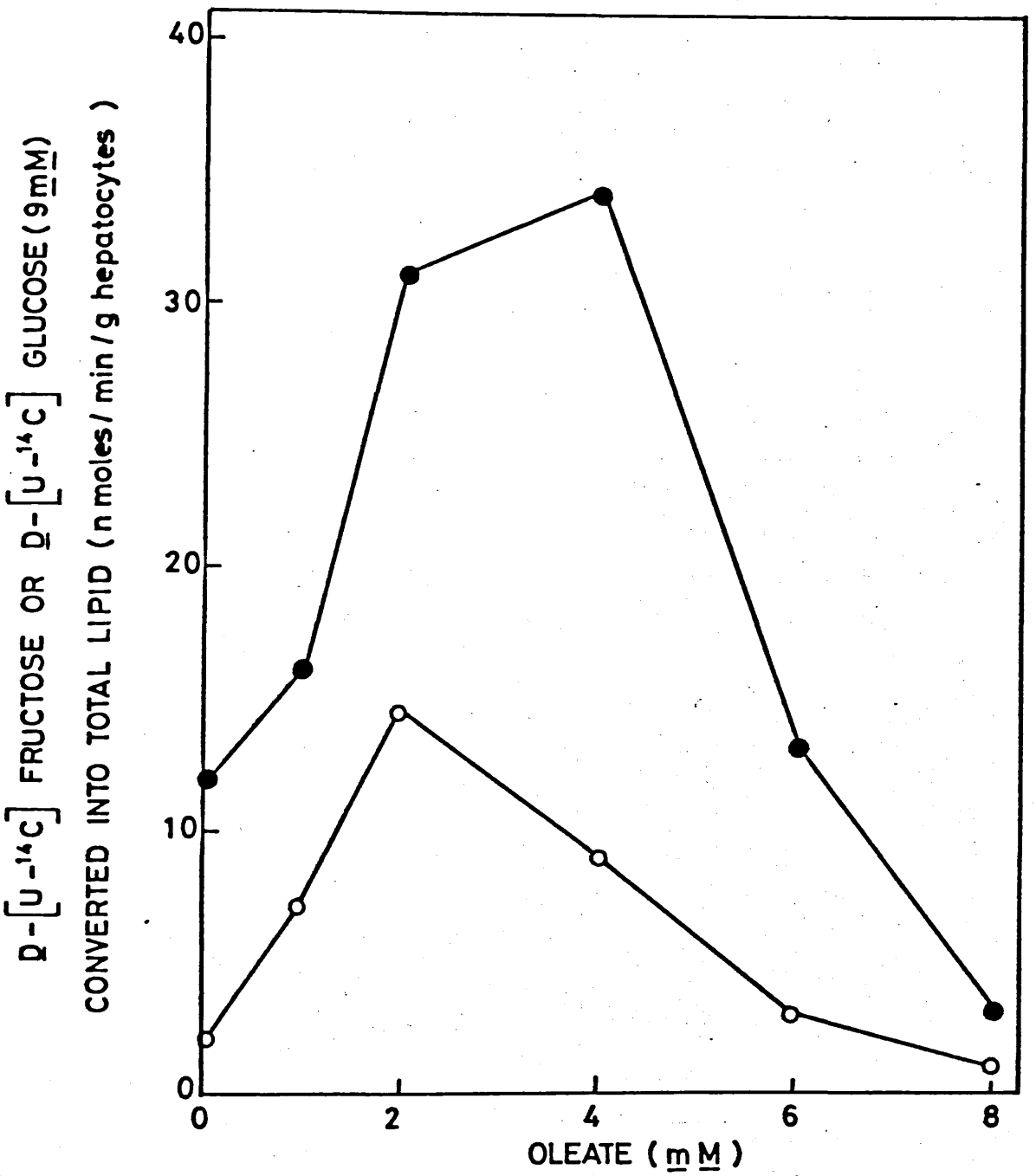


Fig. 20 Effect of oleate on the incorporation of hexoses into total lipid by isolated hepatocytes from 48h fasted rats.

Isolated hepatocytes (4.0×10^6 cells/ml) from 48h fasted rats were preincubated in KRB + Ca^{++} + BSA (1.5%) and then incubated with 9 mM fructose (•) or glucose (o) and various concentrations of sodium oleate for 2h.

Experimental details described in Section VI.B.5b.

hexose incorporation was found at low oleate levels and inhibition at high oleate levels. Indeed, the incorporation of both D $[U\text{-}^{14}C]$ glucose and D - $[U\text{-}^{14}C]$ fructose were almost completely inhibited at 8mM oleate, emphasising the harmful effect of the high concentration of the fatty acids on the hepatocytes (Krebs and Söling, 1976). A similar result with very high oleate levels was observed in an experiment with hepatocytes from fed animals (results not shown).

To summarize the results in section V B and C, it is clear that the incorporation of D - $[U\text{-}^{14}C]$ fructose and to a lesser extent D - $[U\text{-}^{14}C]$ glucose into total lipid gives a good indication of the glycerolipid accumulation by isolated hepatocytes. The differences in incorporation between fed and fasted hepatocytes indicate a greater utilisation of acyl CoA for glycerolipid synthesis in the fed state. Addition of oleate to the hepatocyte results in a substantial stimulation of fructose incorporation and, in addition, the ketose stimulates the synthesis of triacylglycerol whereas glucose appears to favour phosphoglyceride synthesis.

D. Effect of insulin and glucagon on $[^{14}C]$ hexose incorporation into lipid.

The various differences observed between fed and fasted animals can be explained by changes in hormone levels, affecting lipid metabolism either directly or via an indirect effect exerted by changes in carbohydrate metabolites brought about by these hormones. The major hormones which are thought to regulate triacylglycerol synthesis in the short-term are insulin and glucagon.

Triacylglycerol synthesis in liver is rapidly responsive in the isolated perfused liver to both insulin (Topping and Mayes, 1972) and glucagon (Van Harken et al., 1969, Heimberg et al., 1969; Klausner et al. 1978; Soler-Argilaga et al., 1978b). Christiansen (1977) claims that glucagon inhibits $[1\text{-}^{14}C]$ palmitate esterification by isolated hepatocytes and Geelen et al., (1978a) have observed glucagon inhibition of triacylglycerol synthesis as estimated by the incorporation of D - $[U\text{-}^{14}C]$ glucose into the lipid by cultured hepatocytes. However, they were unable to detect a hormonal effect when triacylglycerol synthesis was measured using $[2\text{-}^{14}C]$ glycerol as a substrate..

In the present study an attempt was made to examine the effects of insulin and glucagon on glycerolipid synthesis. Isolated hepatocytes from fed animals were capable of responding to glucagon as evidenced by examining the effect of glucagon on gluconeogenesis from L - [U-^{14}C] lactate (see Fig. 8). Table VI shows the effect of insulin and glucagon on the incorporation of D - [U-^{14}C] fructose and D - [U-^{14}C] glucose into total lipid. Little or no effect of former hormone was observed, although glucagon (10^{-7}M) reduced somewhat the incorporation of D - [U-^{14}C] glucose into total lipid. This inhibition was not statistically significant. This is in contrast to the data of Geelen et al., (1978a) who found a considerable inhibition of glycerolipid synthesis in cultured hepatocytes by glucagon but they also were unable to show any effect of insulin. In the present study the use of higher concentrations of hormones to combat the rapid metabolism of the hormones by the cells did not result in the appearance of a hormonal response.

Table VII shows the effect of insulin and glucagon on the incorporation of D - [U-^{14}C] fructose into triacylglycerol in the presence of exogenous fatty acid. As shown in previous experiments there is a stimulation of the incorporation of label from D - [U-^{14}C] fructose into triacylglycerol by the addition of oleate (2mM) indicating that this is a true reflection of the rate of triacylglycerol synthesis. Glucagon (1μM) appears to inhibit the incorporation of label by 40%; the difference is statistically significant. Insulin appears to have no effect on triacylglycerol synthesis. Soler-Argilaga et al., (1978b) have shown an inhibition by glucagon of oleate incorporation into triacylglycerol which is Ca^{2+} dependent. However, these workers used a lower concentration of oleate (0.5mM) and incubated for a shorter time interval. Thus, in the present study no significant short-term effect of insulin on either glycerolipid or triacylglycerol synthesis was observed.

E. Effect of fructose and glucose on [1-^{14}C] oleate esterification

The data obtained using D - [U-^{14}C] fructose as a precursor suggest that the sugar may stimulate glycerolipid, especially triacylglycerol, synthesis (see Section V A and B). It was therefore decided to use [1-^{14}C] oleate as a precursor for triacylglycerol synthesis and

Table VI. Effect of insulin and glucagon on the incorporation of $[^{14}C]$ hexoses into total lipid by hepatocytes.

Hepatocytes (4×10^6 cells/ml, 1.0ml) from fed rats were preincubated and then incubated with D - $[U\text{-}^{14}C]$ fructose (9 mM) and D - $[U\text{-}^{14}C]$ glucose (9 mM) in presence or absence of hormones for 2h.

	Fructose or glucose incorporated into total lipid nmoles/min/g hepatocytes				
	Control	Insulin		Glucagon		
		10^{-7}M	10^{-6}M	10^{-7}M	10^{-6}M	10^{-5}M
Fructose	12.05 ± 1.10 (n = 5)	14.2 ± 0.92 (n = 5)	14.6	14.32 ± 0.55 (n = 5)	13.8	14.3
Glucose	3.45 ± 0.85 (n = 4)	3.1 ± 0.64 (n = 4)	2.9	2.5 ± 0.18 (n = 4)	3.0	2.6

The results are expressed as nmoles hexose utilized per min per g hepatocytes ± S.D. Number of animals are given in parenthesis.

Table VII. Effect of insulin and glucagon on the incorporation of D-[U-^{14}C] fructose into triacylglycerol in the presence of 2mM oleate.

Isolated hepatocytes (4×10^6 cells/ml., 1.0ml) from fed rats were preincubated and then incubated with D -[U-^{14}C] fructose (9mM) and oleate (2 mM) in presence and absence of glucagon (1 μM) and insulin (1 μM) for 2h. The triacylglycerol was separated from total lipid by thin-layer chromatography.

Experimental details are described in Section VI 5C.

Fructose incorporated into triacylglycerol nmoles/min/g hepatocytes			
Fructose	Fructose + oleate	Fructose + oleate + glucagon	Fructose + oleate + insulin
1.2 ± 0.38 (n = 4)	14.0 ± 4.05 (n = 4)	7.9 ± 4.2 (n = 4)	14.25 ± 5.27 (n = 4)

The values are expressed as means ± S.D.

Statistical significance

	P	
Fructose V Fructose + oleate	< 0.005	S
Fructose V Fructose + oleate + glucagon	< 0.02	S
Fructose + oleate V Fructose + oleate + insulin	N.S.	

to examine the effect of added fructose on oleate esterification. Fig. 21 shows that the rate of oleate utilization is linear for up to 3h under the conditions used in these experiments but it appears that the nature of the added hexose has little or no effect on the rate of utilization. This is also shown in Fig. 22 where the effects of added hexose on oleate utilization at various concentrations of the fatty acid were examined. The utilization of the fatty acid was proportional to the oleate concentration. The rates of utilization in these experiments (0.30 - 0.50 μmoles/min/g hepatocytes at 2mM oleate) is of the same order as those found in rat liver perfused with 1mM palmitate (Kondrup, 1979) and with isolated hepatocytes from female rats incubated with 1mM oleate (Whitelaw and Williamson, 1977; Williamson et al., 1980).

Triacylglycerol synthesis was also found to be linear for up to 120 min in the presence of 0.5 mM oleate (Fig. 23) and 2 mM oleate (results not shown). Williamson et al., (1980) found that in the presence of 1 mM oleate about 50% of the uptake by hepatocytes was accounted for by the esterification of the fatty acid. This contrasts with the data obtained in the present study where only 4.2% of the oleate was converted to triacylglycerol at this concentration of fatty acid. However, Williamson et al., (1980) used female rat hepatocytes and also estimated total esterified fatty acid.

Fig. 24 clearly shows that the proportion of the fatty acid esterified increases with an increasing concentration of oleate since the utilization of the fatty acid is proportional to its concentration (Fig. 22). Fig. 24 also shows that the addition of hexoses to the hepatocytes results in an even greater proportion of the fatty acid being esterified. This stimulation was more pronounced at the higher concentrations of oleate. At 2mM oleate in the presence of fructose (9 mM) 20% of the total utilized fatty acid was esterified to triacylglycerol, in the case of glucose (9 mM) this value was 18%. It is clear from these results that the rate of triacylglycerol synthesis is dependent on the hexose concentration in the hepatocyte medium.

It appeared from this experiment that both fructose and glucose were stimulating triacylglycerol synthesis from added oleate although the ketose appeared to be more potent in this respect. This is confirmed by the results shown in Table VIII. It is clear that in the presence of 2 mM oleate that fructose increases triacylglycerol synthesis from

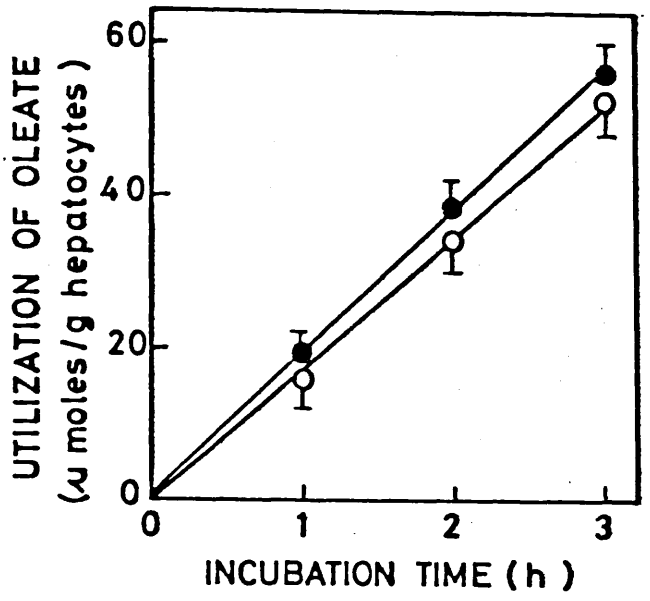
Fig. 21 Time course for the utilization of [1-^{14}C] oleate (2mM) by isolated hepatocytes from fed rats.

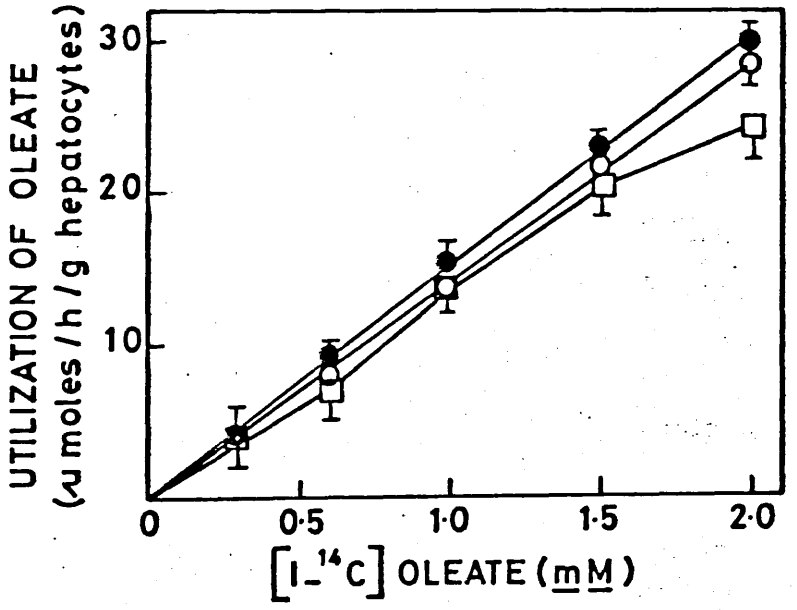
Hepatocytes (4.0 x 10^6 cells/ml) from fed rats were pre-incubated and then incubated with [1-^{14}C] oleate with either 9mM fructose (•) or 9mM glucose (o) for appropriate times. Oleate utilization was determined by assaying the radioactivity in the fatty acid fraction after separation by TLC (Section VI.B.8).

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

Fig. 22 Effect of various oleate concentrations on the utilization of fatty acid by isolated hepatocytes from fed rats.

Hepatocytes (4.0 x 10^6 cells/ml) from fed rats were pre-incubated and then incubated with [1-^{14}C] oleate for 2h in the absence of added hexose (□), with 9mM glucose (o) or with 9mM fructose (•). Oleate utilization was determined as described in Fig. 21.


UTILIZATION OF OLEATE
(μ moles / g hepatocytes)
60
40
20
0
0
1
2
3
INCUBATION TIME (h)


UTILIZATION OF OLEATE
(μ moles / h / g hepatocytes)
30
20
10
0
0·5
1·0
1·5
2·0
[1-14C] OLEATE (mM)

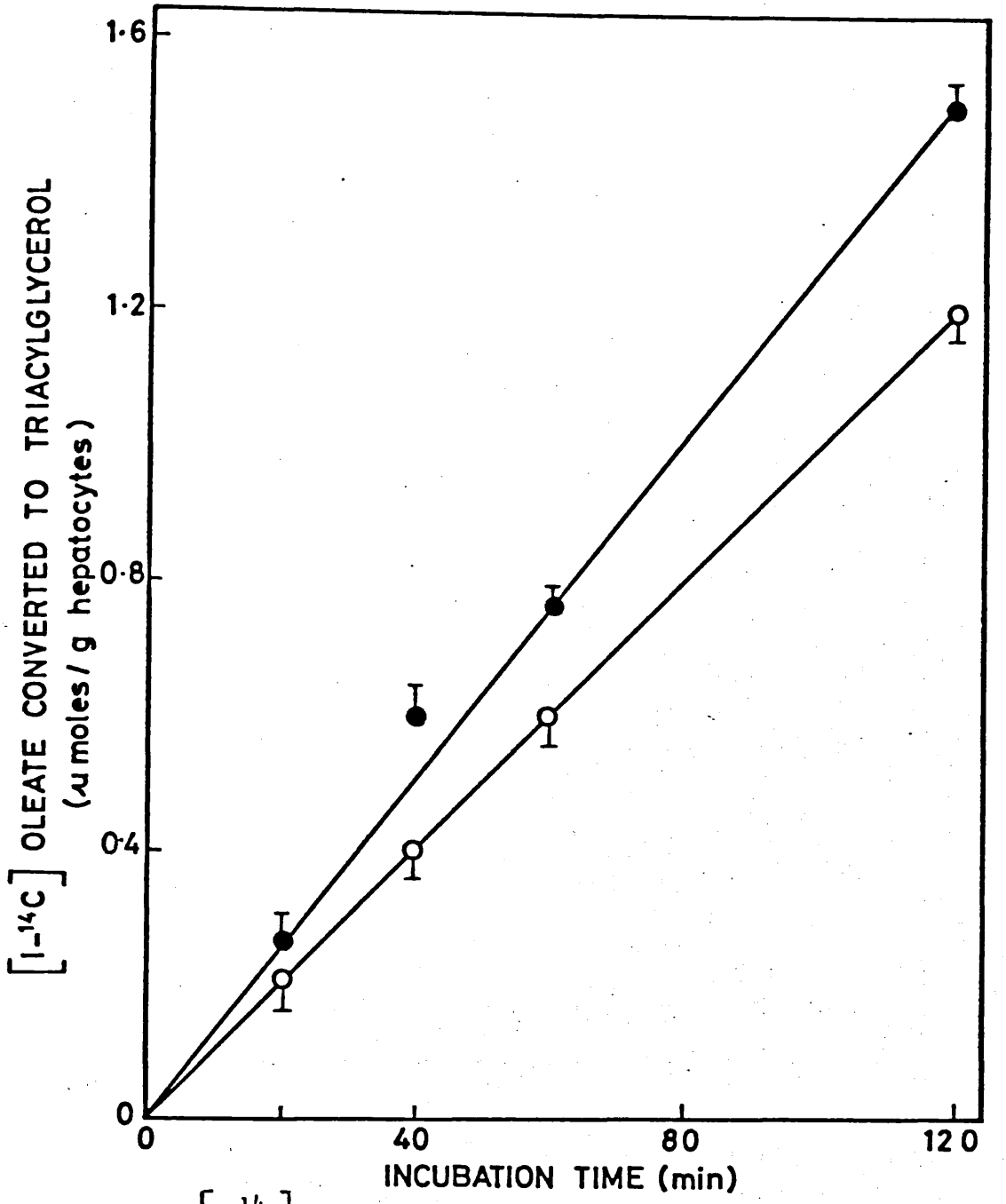


Fig. 23 Rate of $[1\text{-}^{14}C]$ oleate esterification by isolated hepatocytes. Isolated hepatocytes (4.0×10^6 cells/ml) from fed rats were preincubated and then incubated with $[1\text{-}^{14}C]$ oleate (0.5mM) in presence of either 9mM glucose (o) or 9mM fructose (•) for the appropriate time. Oleate utilization was determined as described in Fig. 22.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

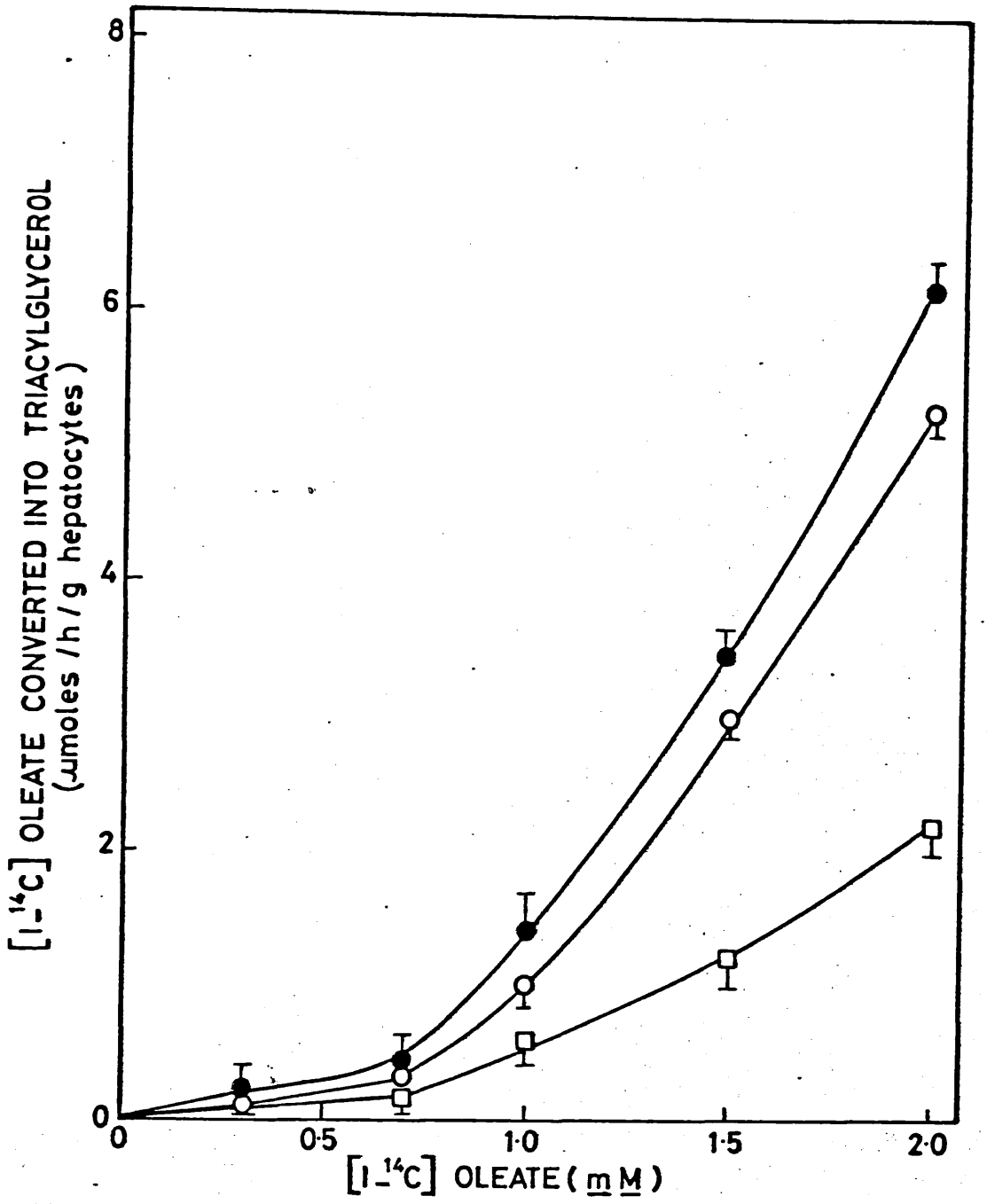


Fig. 24 Effect of various oleate concentrations on the esterification of the fatty acid by isolated hepatocytes from fed rats.

Hepatocytes (4.0×10^6 cells/ml) from fed rats were preincubated and then incubated for 1h with [1-^{14}C] oleate, in the absence of hexose (□), with 9mM glucose (o) or with 9mM fructose (●). Triacylglycerol was separated by TLC and estimated by liquid scintillation counting as described in Materials and Methods (Section VI.B.8).

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

Table VIII. Effect of fructose (9mM) and glucose (9mM) on the esterification of $[1\text{-}^{14}C]$ oleate into triacylglycerol.

Isolated rat hepatocytes (4×10^6 cells/ml., 1.0 ml) from fed rats were preincubated and then incubated for 1h without added hexoses in presence of $[1\text{-}^{14}C]$ oleate (2mM or 0.5mM) and with hexoses (9mM)

	µmole of $[1\text{-}^{14}C]$ oleate converted to triacylglycerol per h per g hepatocytes		
	control	fructose (9mM)	glucose (9mM)
2 mM $[1\text{-}^{14}C]$ oleate	0.93 ± 0.55 (n = 4)	3.14 ± 1.90 (n = 9)	1.52 ± 0.74 (n = 13)
0.5 mM $[1\text{-}^{14}C]$ oleate	0.33 ± 0.16 (n = 6)	0.96 ± 0.88 (n = 13)	0.50 ± 0.30 (n = 8)

The results are expressed as means ± S.D. with the number of animals used given in parenthesis.

A statistical analysis of the data is given in Table VIIIa.

Table VIIIa. Statistical analysis of the data in Table VIII

comparison		statistical significance P	
control (2mM oleate)	V fructose + oleate (2mM)	< 0.01	S
control (2mM oleate)	V glucose + oleate (2mM)	< 0.02	S
fructose + oleate (2mM)	V glucose + oleate (2mM)	< 0.02	S
control (0.5mM oleate)	V fructose + oleate (0.5mM)	< 0.02	S
control (0.5mM oleate)	V glucose + oleate (0.5mM)	> 0.1	N.S.
fructose + oleate (0.5mM)	V glucose + oleate (0.5mM)	= 0.1	N.S.

N.S. = Not significant

S = Significant

exogenous oleate 3.4 fold whereas glucose also exerts a smaller stimulatory effect (1.6 fold) on triacylglycerol synthesis. However, it is apparent that the effect of fructose is considerably greater than that of glucose. This trend is also apparent when a similar experiment was carried out in the presence of 0.5mM oleate but in this case the only statistically significant difference was between the control and fructose-treated hepatocytes. Fig. 25 shows the effect of fructose on esterification of low concentration of oleate (< 0.5mM), and here again a clear stimulation of oleate esterification by the ketose was observed.

It is clear from this data and that presented earlier (Section V B and C) that fructose and, to a lesser extent, glucose stimulate triacylglycerol synthesis from exogenous oleate and from endogenous fatty acid by isolated hepatocytes. This stimulation of oleate esterification, resulting in the formation of triacylglycerol, could be the result of a number of different factors. For example, the substrates required for triacylglycerol synthesis, acyl CoA, sn-glycerol 3-phosphate and/or dihydroxyacetone phosphate (DHAP) may be increased by the ketose. It is clear that sn-glycerol 3-phosphate levels (but not DHAP) are raised by the ketose (see Section V, A). However, there is no evidence in the literature which shows any increase in acyl CoA levels in response to fructose; indeed, Christiansen (1979) found that long-chain acyl CoA levels were decreased by fructose (10mM), an effect which was accentuated by addition of glucagon to the hepatocytes. Another possible way in which triacylglycerol synthesis could be increase is by changes in the activities of glycerol 3-phosphate acyltransferase or PPH. The changes observed in GPAT activity under various conditions such as diabetes (Bates and Saggerson, 1977), glucagon treatment (Soler-Argilaga et al., 1978b; Williamson et al., 1980) and sucrose feeding (Glenny et al., 1978b) are relatively small compared to the effect of fructose on triacylglycerol synthesis observed in this study. It is clear that both microsomal and mitochondrial GPAT are very sensitive to changes in sn-glycerol 3-phosphate concentration up to 1mM (Bates and Saggerson, 1977). Thus, the changes in sn-glycerol 3-phosphate content of hepatocytes incubated with fructose observed in the present study (see Section V, B) are consistent with the explanation that the stimulation of oleate esterification by the ketose is due to a change in GPAT activity as the result of a greater substrate

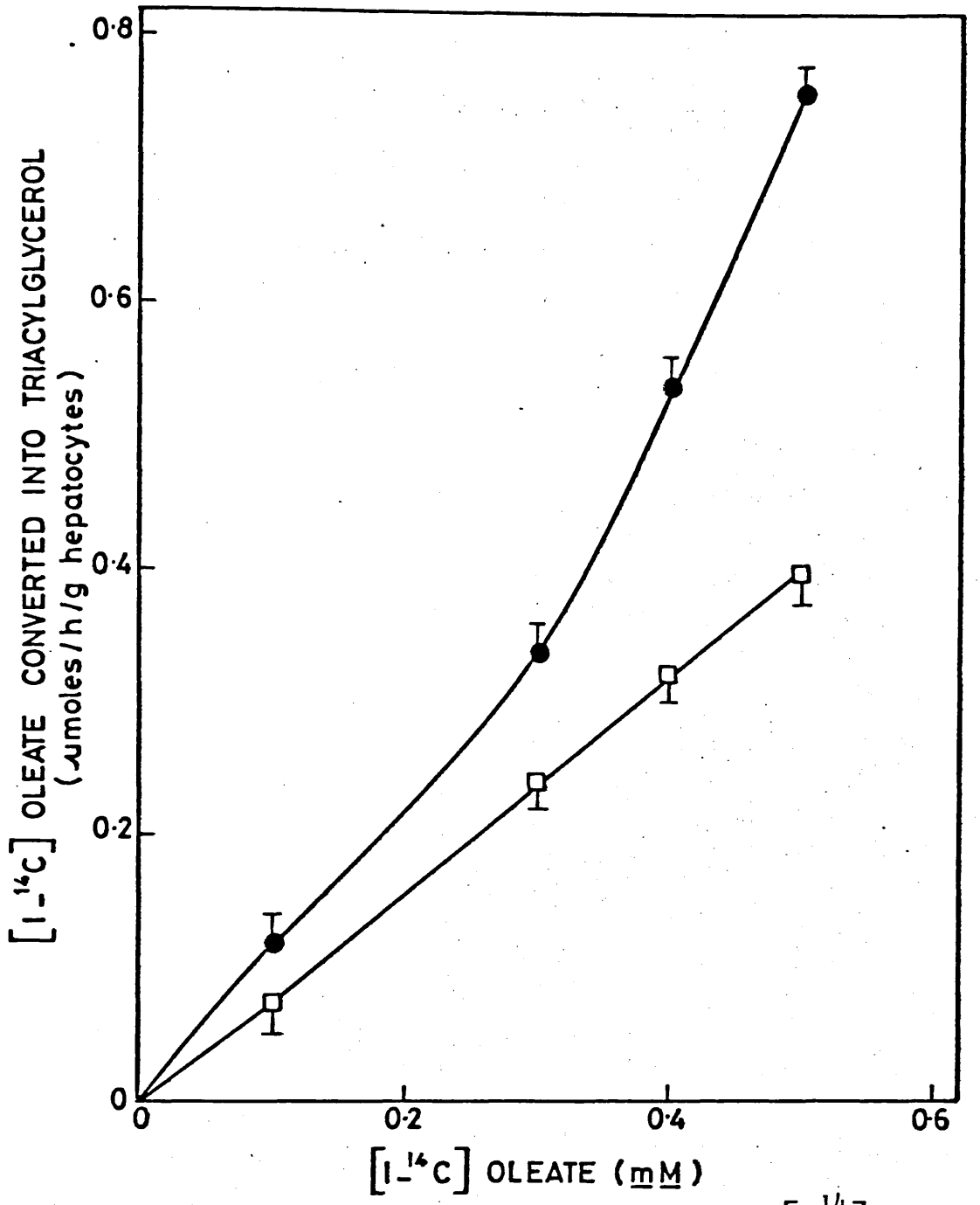


Fig. 25 Effect of fructose (9mM) on the esterification of [1-^{14}C] oleate at low concentrations of the fatty acid.

Hepatocytes (4.0 x 10^6 cells/ml) from fed rats were preincubated, then incubated with [1-^{14}C] oleate in presence (•) and absence (▫) of 9mM fructose for 1h.

Experimental details are described in Section VI.B.8.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

availability. However, the specific stimulation of triacylglycerol synthesis relative to phospholipid synthesis (see Fig. 16) indicates that there is also a regulatory enzyme which is subsequent to phosphatidic acid in the biosynthetic pathway, which is affected either directly or indirectly by fructose. PPH has been reported to regulate the rate of esterification (Brindley, 1978a). The activity of this enzyme is particularly affected by ethanol ingestion (Brindley, 1978a; Savolainen, 1977, Savolainen and Hassinen, 1980) and the effect has been attributed to the rise in hepatic sn-glycerol 3-phosphate levels as a result of changes in the redox state induced by ethanol. The effect of ethanol occurs after a significant time lag of 2h (Savolainen, 1977) and this probably does not explain the effects observed in this thesis. However, fructose does raise sn-glycerol 3-phosphate levels without affecting the redox state of the hepatocyte (see Section V, B) and this suggests that PPH may also be subject to short-term regulation by sn-glycerol 3-phosphate. Dietary fructose is known to cause an increase in PPH activity and a decrease in microsomal phosphatidate but, again, this is in the long-term (Lamb and Fallon, 1974b). This effect of the ketose has been ascribed to the elevation of plasma corticosterone without a concomitant stimulation of insulin secretion (Brindley et al., 1981). However, there is no evidence in the literature of a short-term regulation of PPH. Haagsman et al., (1981) have reported that triacylglycerol synthesis may be independently regulated via a short-term regulation of DGAT by glucagon, possibly by a phosphorylation/dephosphorylation mechanism.

F. Effect of glucagon on triacylglycerol synthesis

The effect of glucagon on triacylglycerol synthesis from oleate was further examined in the experiment shown in Fig. 26. Again, the rate of oleate incorporation into triacylglycerol was dependent on the fatty acid concentration and both fructose-and glucose-stimulated oleate esterification at high (2mM) oleate levels. In general, the stimulation observed at the lower concentrations of fatty acid was not statistically significant. Addition of glucagon (1μM) to the cells at the start of the incubation appears to result in an inhibition of oleate incorporation into triacylglycerol both in the presence of fructose and of glucose, but inhibition was not statistically significant except with 2mM oleate in the presence of glucagon. A statistical analysis of the data in Table IX indicated, as in a previous experiment (see Table VIII),

Fig. 26 Effect of glucagon (1×10^{-6} M) on the esterification of [1-^{14}C] oleate in the absence of added hexose (□), (b) with 9mM glucose (o) or (c) with 9mM fructose (●).

Isolated rat hepatocytes (4.0×10^{6} cells/ml) from fed rats were preincubated then incubated for 60 min with [1-^{14}C] oleate, in the absence of glucagon (○/□) or with 1 µM glucagon (■) added at the start of the incubation.

Experimental details are described in Section VI.B.8 and Section VI.B.10.

Each point represents the mean ± SD of the results with 4 hepatocyte preparations.

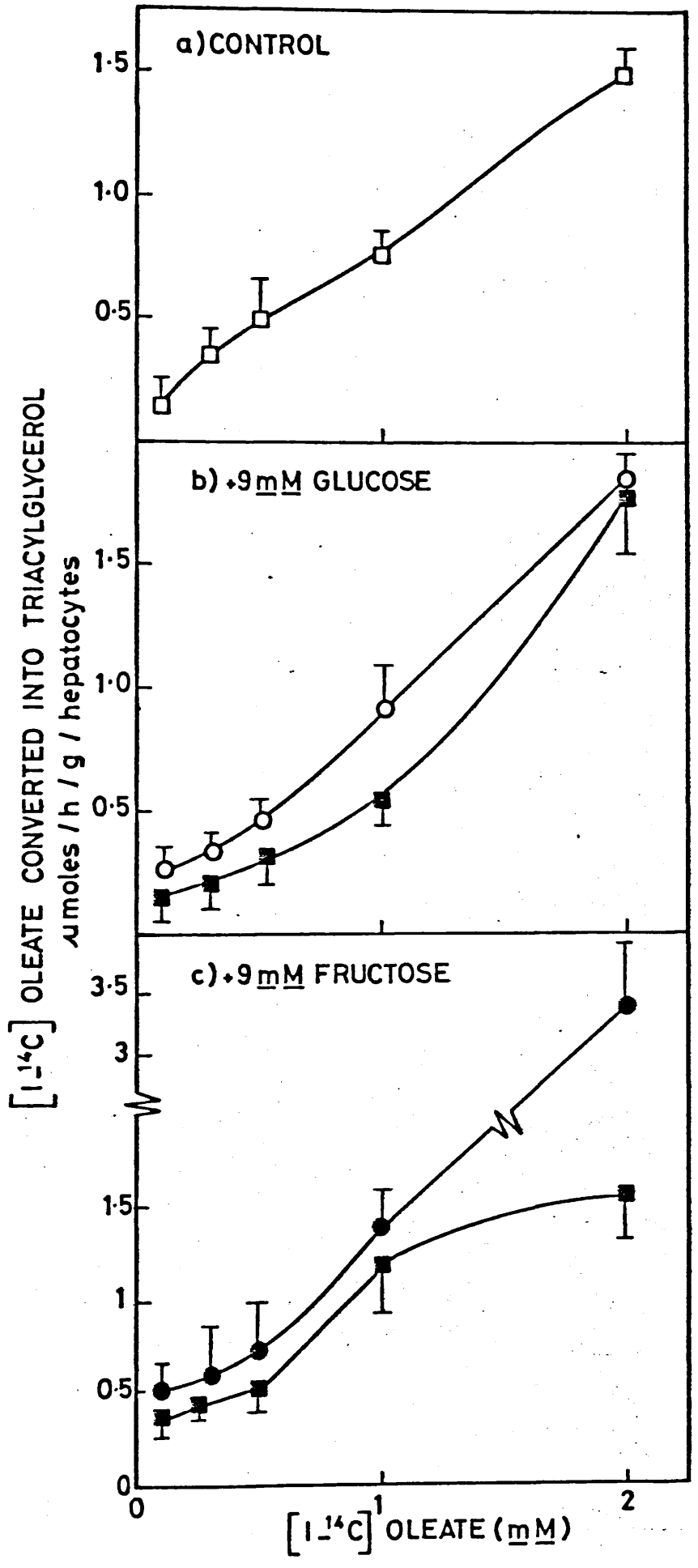

a) CONTROL
b) +9 mM GLUCOSE
c) +9 mM FRUCTOSE
[1-14C] OLEATE CONVERTED INTO TRIACYLGLYCEROL
μmoles / h / g / hepatocytes
[1-14C] OLEATE (mM)
1·5
1·0
0·5
3·5
3
1·5
1
0·5
0
0
1
2

Table IX. Effect of glucagon (10^{-6}M) on the esterification of [1-^{14}C] oleate into triacylglycerol in presence of fructose and glucose

Isolated rat hepatocytes (4×10^6 cells/ml., 1.0 ml) from fed rats were preincubated for 1h without substrate and then with [1-^{14}C] oleate (2mM) containing fructose (9mM) or glucose (9mM) in the presence or in the absence of glucagon (10^{-6}M).

	µmole [1-^{14}C] oleate converted to triacylglycerol per h per g liver		
	control	fructose (9mM)	glucose (9mM)
No hormone added	1.49 ± 0.02 (n = 3)	3.30 ± 0.53 (n = 3)	1.84 ± 0.30 (n = 3)
Glucagon (1µM)	NOT DETERMINED	1.50 ± 0.15 (n = 3)	1.80 ± 0.03 (n = 3)

The results are expressed as means ± SD with the number of animals used given in parenthesis. A statistical analysis of the data is given in Table IXa.

Table IXa. Statistical analysis of the data in Table IX

comparison		statistical analysis P
control	V glucose	N.S.
control	V fructose	= 0.005 S
fructose	V glucose	< 0.02 S
fructose (control)	V fructose + glucagon	< 0.01 S
glucose (control)	V glucose + glucagon	N.S.
fructose + glucagon	V glucose + glucagon	< 0.02 S

N.S. = not significant

S. = significant

that fructose and, to a lesser extent glucose, stimulated triacylglycerol synthesis from oleate compared to control. This effect of fructose was reversed by the addition of glucagon but this hormone was not effective with the glucose-treated cells. When the hormone-treated cells are compared, the stimulation by fructose is no longer found; indeed, the ketose caused a small inhibition compared to glucose. The inhibitory effect of glucagon in the presence of oleate (2mM) and fructose (9mM) is also apparent when D -[U-^{14}C] fructose incorporation into triacylglycerol was used as a measure of lipid synthesis (see Table VII).

The reason for this differential effect of glucagon in the presence of fructose and glucose is not immediately apparent. There is no evidence in the literature to suggest that the metabolism of fructose to sn-glycerol 3-phosphate is affected by glucagon but there is considerable evidence that glucagon affects glycolysis from glucose and gluconeogenesis from the triose phosphate (Hue, 1981). In addition, oleate is known to inhibit glycolysis and to stimulate gluconeogenesis. Williamson et al., (1980) found that added oleate completely inhibits lactate accumulation by isolated hepatocytes, and glucagon addition resulted in the utilization of lactate present at the start of the incubation. It is not clear at what stages of glycolysis and gluconeogenesis that these effects are mediated. However, Bremer et al., (1978), Sugden et al., (1980) and Declerq et al., (1982a) have provided evidence that glucagon lowers sn-glycerol 3-phosphate levels. This decrease is presumably brought about by inhibition of glycolysis to the triosephosphate and a stimulation of gluconeogenesis brought about by changes in fructose 2,6-bis-phosphate. If the stimulation of oleate esterification by fructose observed in this study is the result of the elevated sn-glycerol 3-phosphate level, then the effect of glucagon must be to lower sn-glycerol 3-phosphate levels, presumably by stimulating gluconeogenesis from the triosephosphate. There is evidence, however, that gluconeogenesis from fructose is not stimulated by glucagon at high concentrations of fructose but is stimulated at lower levelsof fructose (Venezaile, 1971; Zahlten et al., 1973). The results in the present study indicate that in the presence of oleate (2mM) glucagon may reverse the accumulation of sn-glycerol 3-phosphate caused by fructose. More recently Declerq et al. (1982a) have found that with DHA as a sn-glycerol 3-phosphate precursor, glucagon significantly

decreased the sn-glycerol 3-phosphate content of hepatocytes and the incorporation of palmitate into di- and tri- acylglycerol .

G. Effect of fructose on lipogenesis from L - [U-^{14}C] lactate

It was clear from preliminary results that of the [^{14}C] hexose incorporated into the triacylglycerol fraction by isolated hepatocytes, more of the D - [U-^{14}C] fructose (13%) was incorporated into the fatty acid moiety than of the D - [U-^{14}C] glucose (7%) and since there was a greater total incorporation from fructose, this indicated that the actual incorporation of carbon into fatty acid was greater in the case of D - [U-^{14}C] fructose. This is further shown in Fig. 27 which shows the relationship between the concentration of added hexose and the rate of carbon incorporation into the fatty acid fraction derived from a total lipid extract. The rates of fatty acid synthesis from both hexoses were found to be linear for up to 1h (data not shown). As expected, the ketose was shown to be a better precursor of fatty acid carbon than glucose in the concentration range studied. Clark et al., (1974) have found that 10mM fructose was a better precursor of fatty acid than 10mM glucose but they found that the reverse was true at higher concentrations. The preferential utilization of fructose carbon for lipogenesis was expected since the metabolism of fructose to fatty acid by-passes the first two regulatory enzymes of glycolysis, glucokinase and phosphofructokinase (PFK), whereas glycolysis from glucose does not. This finding is also in accordance with the data obtained by Salmon et al., (1974) who found that de novo fatty acid synthesis by perfused liver from fed mice was not stimulated by glucose at concentrations less than 17mM and that the sugar was not a good carbon source for lipogenesis. On the other hand, both glycogen and lactate contributed extensive carbon to fatty acid. This was also the conclusion of Clark et al., (1974) following their work on isolated hepatocytes. The evidence suggests that glucokinase is a rate-limiting step in glucose utilization for fatty acid synthesis. However, if the rate of carbon incorporation from D - [U-^{14}C] fructose is compared to published data concerning absolute rates of fatty acid synthesis from $^{3}H_2O$ by hepatocytes from fed rats, it is clear that rate of incorporation of carbon from fructose into fatty acid is very low, even at high concentration of the sugar. This is probably due to isotope dilution by glycogen, lactate and other non-radioactive lipogenic precursors.

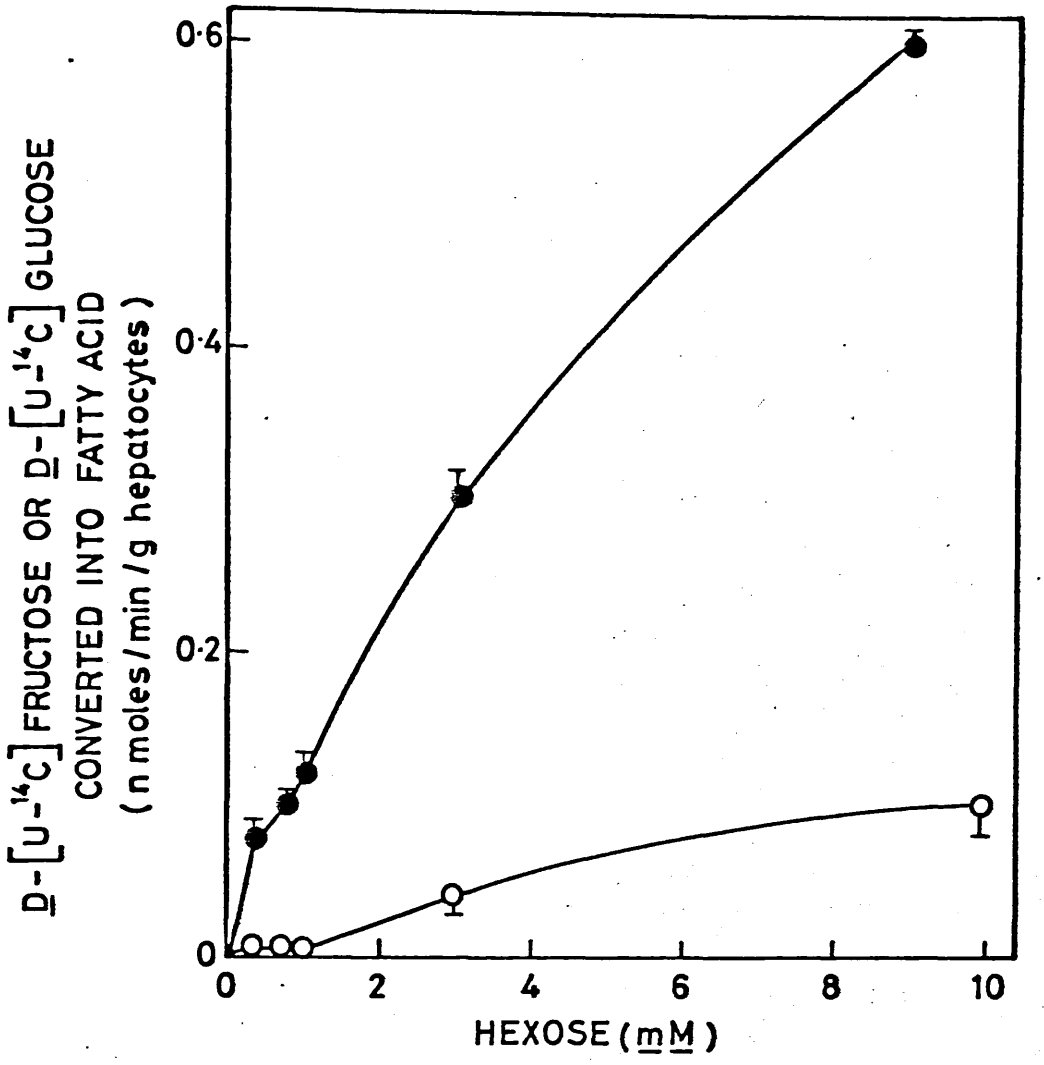


Fig. 27 Lipogenesis from D - [U-^{14}C] fructose or D - [U-^{14}C] glucose.

Hepatocytes were prepared from fed rats. Fructose (•) or glucose (o) was added to cells (4.0×10^6 cells/ml) which were preincubated at 37° for 1h in KRB + Ca^{++} + BSA (1.5%) and then incubated with hexoses for 1h.

Experimental details described in Section VI.B.5d.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

Lactate incorporation is generally regarded as being a good indicator of rates of lipogenesis (Clark et al., 1974; Salmon et al., 1974). The results obtained with this substrate as a carbon source are lower than those obtained using $^{3}H_2O$ but appear to accurately reflect changes in rates of lipogenesis (Hopkirk and Bloxham, 1979). Therefore, it was decided to examine the effect of fructose on the rates of incorporation of L - [U-^{14}C] lactate into fatty acid in order to determine whether or not fructose had an effect on lipogenesis, independent of its role as a carbon source.

Fig. 28 shows the results of a typical experiment showing a time course for the incorporation of L - [U-^{14}C] lactate (3mM) into fatty acid in the presence of fructose and glucose. Lipogenesis from lactate was linear over a 60 min period in the presence of 1mM glucose. At 9mM glucose there was a stimulation of lipogenesis after a short time lag.

However, the rates of lipogenesis in the presence of fructose were not linear. In the case of 1mM fructose there was a short initial lag followed by a marked stimulation of lipogenesis between 15 min and 60 min. At 9mM fructose, however, there was a considerable inhibition of lipogenesis from lactate which was apparent for about 30 min, then the hepatocytes showed some recovery and were capable of lipogenesis after an initial lag period. The inhibition of lipogenesis from lactate by high concentration of fructose is also apparent in Fig. 29, which shows the effect of fructose and glucose concentrations on fatty acid synthesis. The results indicate a small stimulatory effect on lipogenesis by glucose but a marked stimulation at low concentrations of fructose. A rate of lipogenesis from lactate of 0.69 ± 0.19 S.D. (n = 4) nmoles/min/g hepatocytes was observed with 3mM glucose added, whereas with 3mM fructose this value was increased ($P < 0.05$) to 1.50 ± 0.65 nmoles/min/g. These values are considerably lower than rates of lipogenesis observed in vivo (Lowenstein, 1971) but of the same order as those reported by Hopkirk and Bloxham (1979) with isolated hepatocytes, especially if the considerable diurnal variation in lipogenesis by hepatocytes observed by these workers is taken into account. It is also clear that the diet and sex of the animal have a considerable effect on the level of hepatic lipogenic enzymes (Pridham and Davies, 1979). Lipogenic rates could be optimized by feeding high carbohydrate diets to female rats.

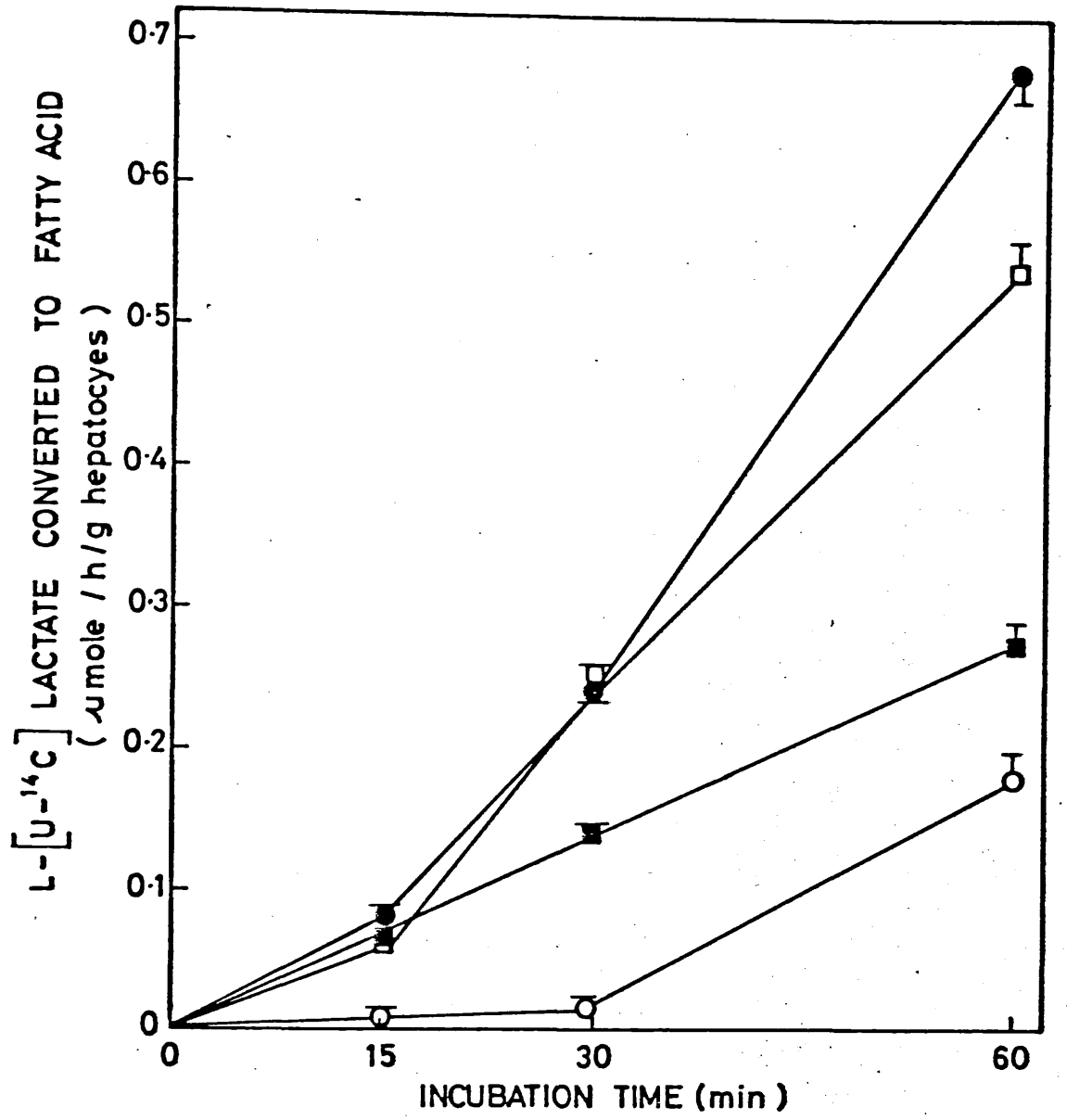


Fig. 28 Time course of fatty acid synthesis from L - [U-^{14}C] lactate.

Isolated hepatocytes (4.0×10^6 cells/ml) from fed rats were preincubated for 1h and then incubated with L - [U-^{14}C] lactate (3mM) in presence of 1mM glucose (■), 9mM glucose (□), 1mM fructose (●) or 9mM fructose (o). The reaction was stopped with $HClO_4$ the total lipid extracted and the fatty acid saponified as described in Materials and Methods Section VI.B.5d.

Each point represents the mean ± SEM of values obtained with one hepatocyte preparation.

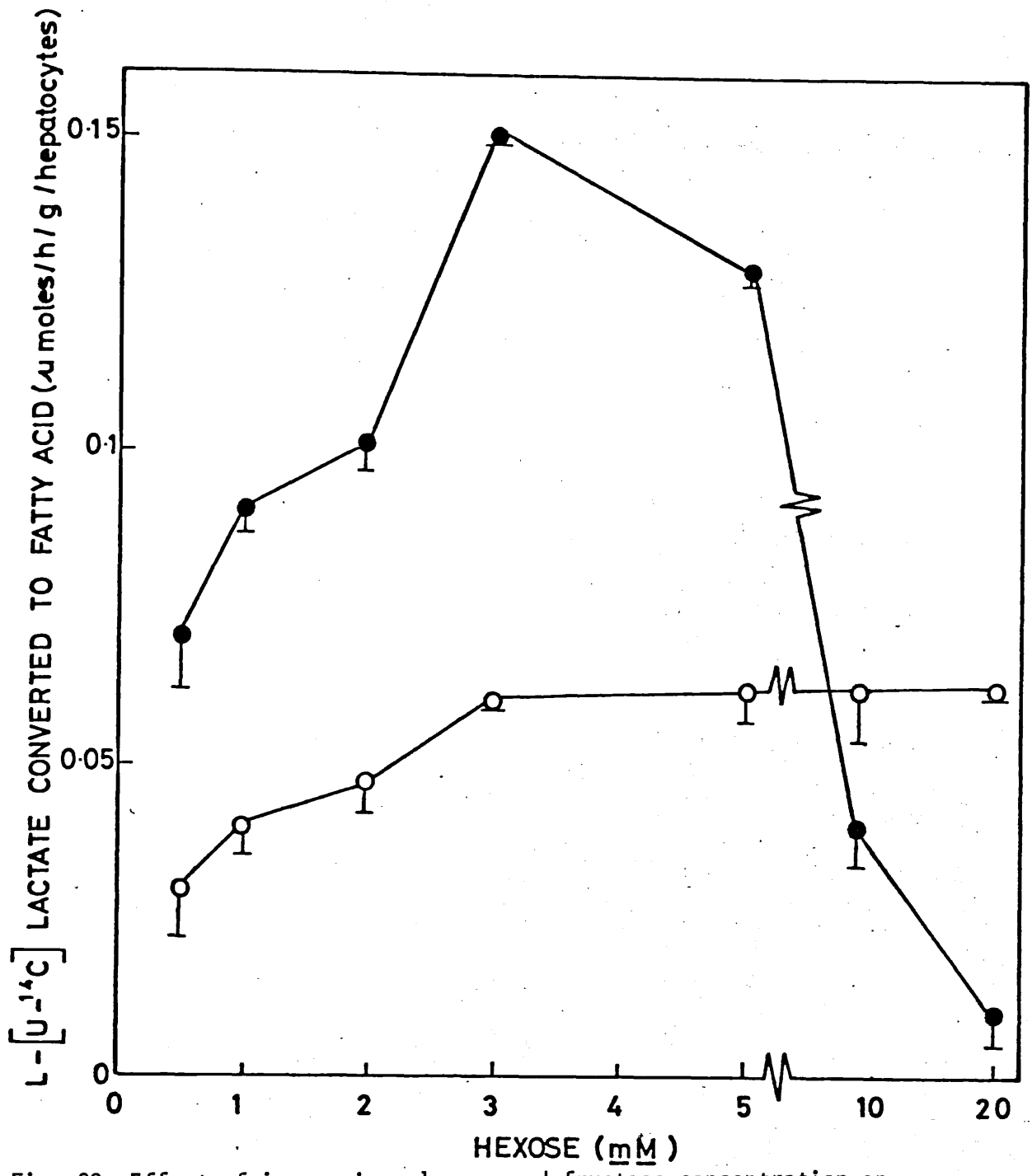


Fig. 29 Effect of increasing glucose and fructose concentration on lipogenesis from lactate (3mM).

Hepatocytes (4.0×10^6 cells/ml) from fed rats were pre-incubated for 1h and then incubated with L - [U-^{14}C] lactate (3mM) in presence of various concentrations of fructose (•) and glucose (o) for 1h.

Experimental details are described in Section VI.B.9a.

Each point represents the mean ± SEM of triplicate values obtained with one hepatocyte preparation.

The inhibition of lipogenesis by fructose at 9mM and 20mM (Fig. 29) has also been described by others (Berry and Friend, 1969; Clark et al., 1974; Selmer and Grunnet, 1976) but, to my knowledge, there is no report in the literature which shows that lipogenesis is restored after an initial period of inhibition (see Fig. 28). This inhibition of lipogenesis may be due to the rapid fall in adenine nucleotide content, especially ATP, which is known to occur when hepatocytes are incubated with fructose (Clark et al., 1979; Van den Berghe et al., 1980). The latter workers claim that ATP levels are not restored following incubation of the cells from fasted animals for up to 1h but Mapungwana (1982) in this laboratory has shown that ATP levels are at least partially restored over a 1h incubation period following a profound depletion in the first 10 min after addition of fructose (10mM). This restoration of ATP level could explain the increase in the rate of lactate-lipogenesis observed in the present study after 30 min in the presence of 9mM fructose.

There are at least three possible sites for the utilization of ATP in lipogenesis; ATP citrate lyase, acetyl CoA synthase and acetyl CoA carboxylase. Selmer and Grunnet (1976) have discounted a fructose-induced inhibition of the former two enzymes on the grounds that the ratio of incorporation from $^{3}H_2O$ and $[1\text{-}^{14}C]$ acetate is unchanged, but they also state that the depletion of ATP is unlikely to lead to an inhibition of acetyl CoA carboxylase because of the high affinity of the enzyme for ATP. An alternative explanation for the change in lipogenic rate with time in hepatocytes incubated with fructose is the possibility that the accumulated FIP may inhibit acetyl CoA carboxylase but it seems that the effect of this metabolite on the acetyl CoA carboxylase is stimulatory rather than inhibitory (see Section V, 1).

A further possibility is that the reversal of the inhibition is due to the accumulation of lactate, a known stimulator of lipogenesis (Harris, 1975). Mapungwana (1982) has clearly shown that lactate plus pyruvate output by isolated hepatocytes is stimulated by fructose at all concentrations of the ketose. She has shown that the lactate concentration of isolated hepatocytes can change by about 1mM in 1h in the absence of hexose or with glucose (10mM) and by 4mM in presence of 10mM fructose. The effect of lactate concentration on the rate of

lipogenesis is shown in Fig. 30. It is clear that the assay of lipogenesis from 3mM lactate underestimates the maximal rate of lipogenesis and it is possible that the stimulatory effects of fructose observed in Fig. 28 and Fig. 29 could be explained by a change in the lactate pool. However, it is clear from the results in Fig. 30 that the stimulatory effect of fructose is increased with increasing lactate concentration. This is incompatible with the view that fructose may stimulate lipogenesis via an increase in the level of lactate and suggests that fructose is exerting an effect on lipogenesis independent of its effect on the lactate content of hepatocyte.

In an attempt to clarify this problem the fate of D - $[U\text{-}^{14}C]$ fructose in various metabolites in the isolated hepatocytes was examined. It is well-known that the major products of fructose metabolism are glucose and lactate (Exton and Park, 1967). The method of Hue et al. (1978) utilizing ion-exchange resins to separate glucose, lactate and fructose was used. The rates of gluconeogenesis from fructose 1mM (Fig. 31a) and 9mM (Fig. 32a) were found to be linear for up to 30 min. L - $[U\text{-}^{14}C]$ lactate accumulation was not linear with time in either case (Fig. 31a and Fig. 32a). A further examination by thin-layer chromatography of the 'lactate' fraction formed after 30 min incubation with both 1mM and 9mM fructose revealed that the 'lactate' fraction was, in fact, contaminated with a substantial amount of fructose 1-phosphate which had accumulated in the cells and was eluted off Dowex AG1-Cl^- with 1M NaCl (see Fig. 33). In further experiments the lactate and FIP were further resolved using the TLC step and the results in Fig. 31b and Fig. 32b show that the rate of lactate accumulation is linear for up to 30 min and that FIP accumulates up to 30 min, then there is a decline in the level of this metabolite. This was borne out by observation in the laboratory by Mapungwana (1982) who found that hepatocyte FIP levels, as measured by a linked FIP aldolase/glycerol 3-phosphate dehydrogenase enzymic determination, peak after 30 min of incubation with fructose then gradually decline.

The next experiment (Fig. 34) shows the accumulation of radioactive metabolites after incubation for 15 min with various concentrations of fructose. It is clear that the accumulation of each metabolite is dependent on the concentration of the ketose, but the nature of the relationship is different in each case. At low concentrations of

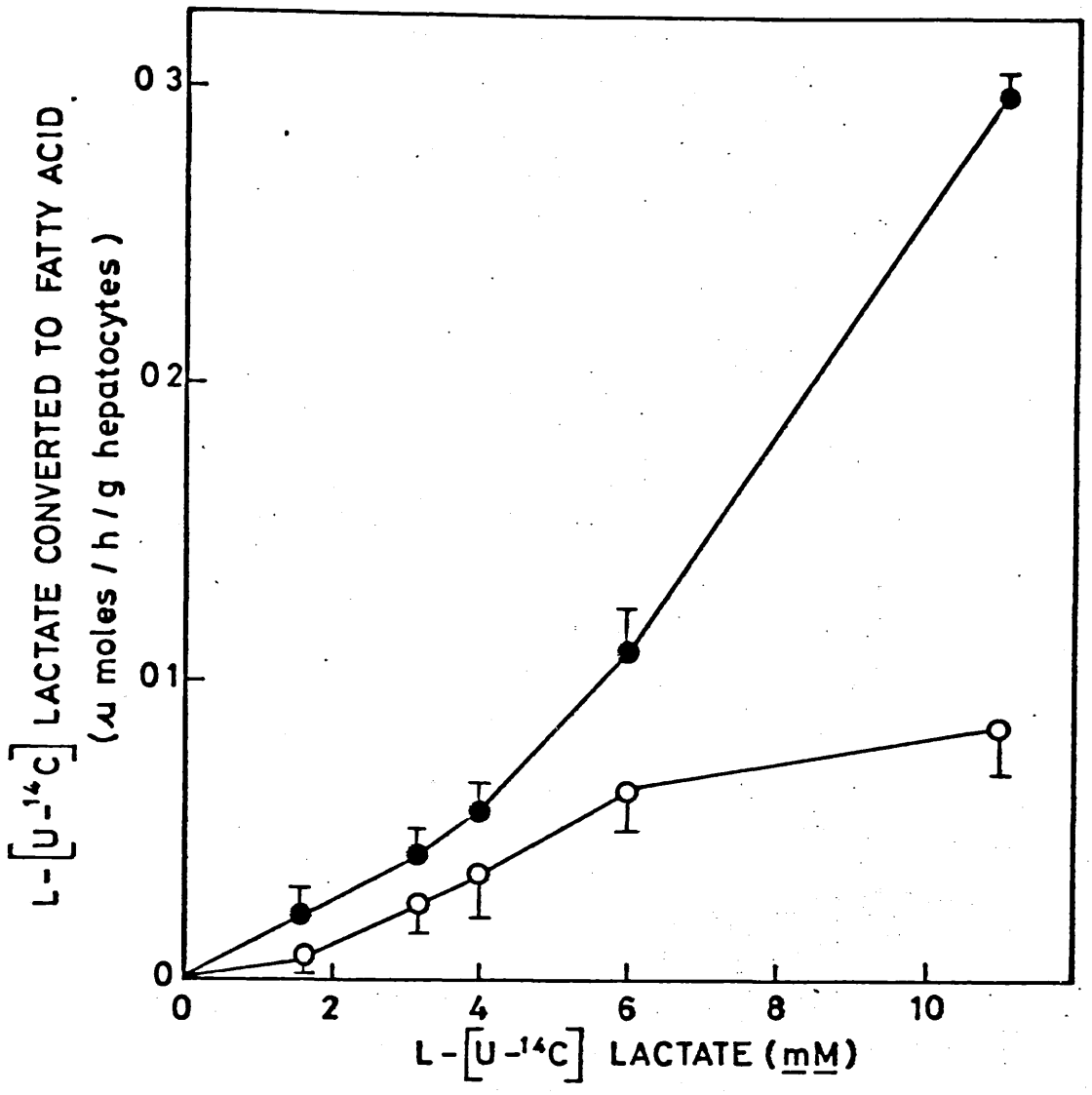


Fig. 30 Effect of 3mM fructose or 3mM glucose on the incorporation of various concentrations of L - [U-^{14}C] lactate into fatty acid by hepatocytes.

Isolated hepatocytes (4.0×10^6 cells/ml) from fed rats were preincubated and then incubated with L- [U-^{14}C] lactate for 1h in the presence of 3mM glucose (o) or with 3mM fructose (•).

Experimental methods are described in Section VI.B.9a.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

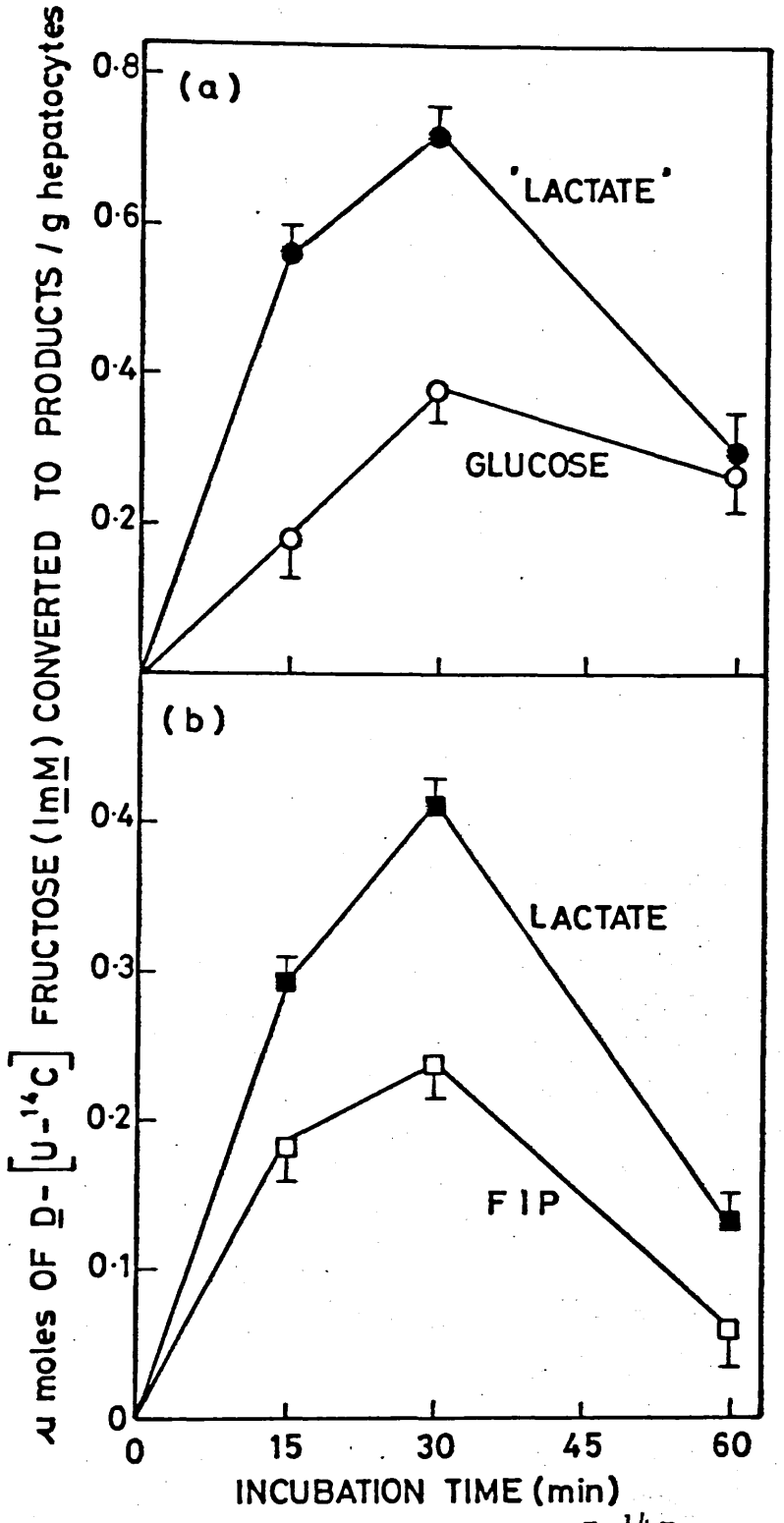


Fig. 31 Time course of incorporation of D - [U-^{14}C] fructose (1mM) into glucose, lactate and FIP.

Isolated hepatocytes (4 x 10^6 cells/ml) from fed rats were preincubated for 1h and then with D - [U-^{14}C] fructose (1mM) for various times. The products were separated as described in Materials and Methods Section VI.B.6b. Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

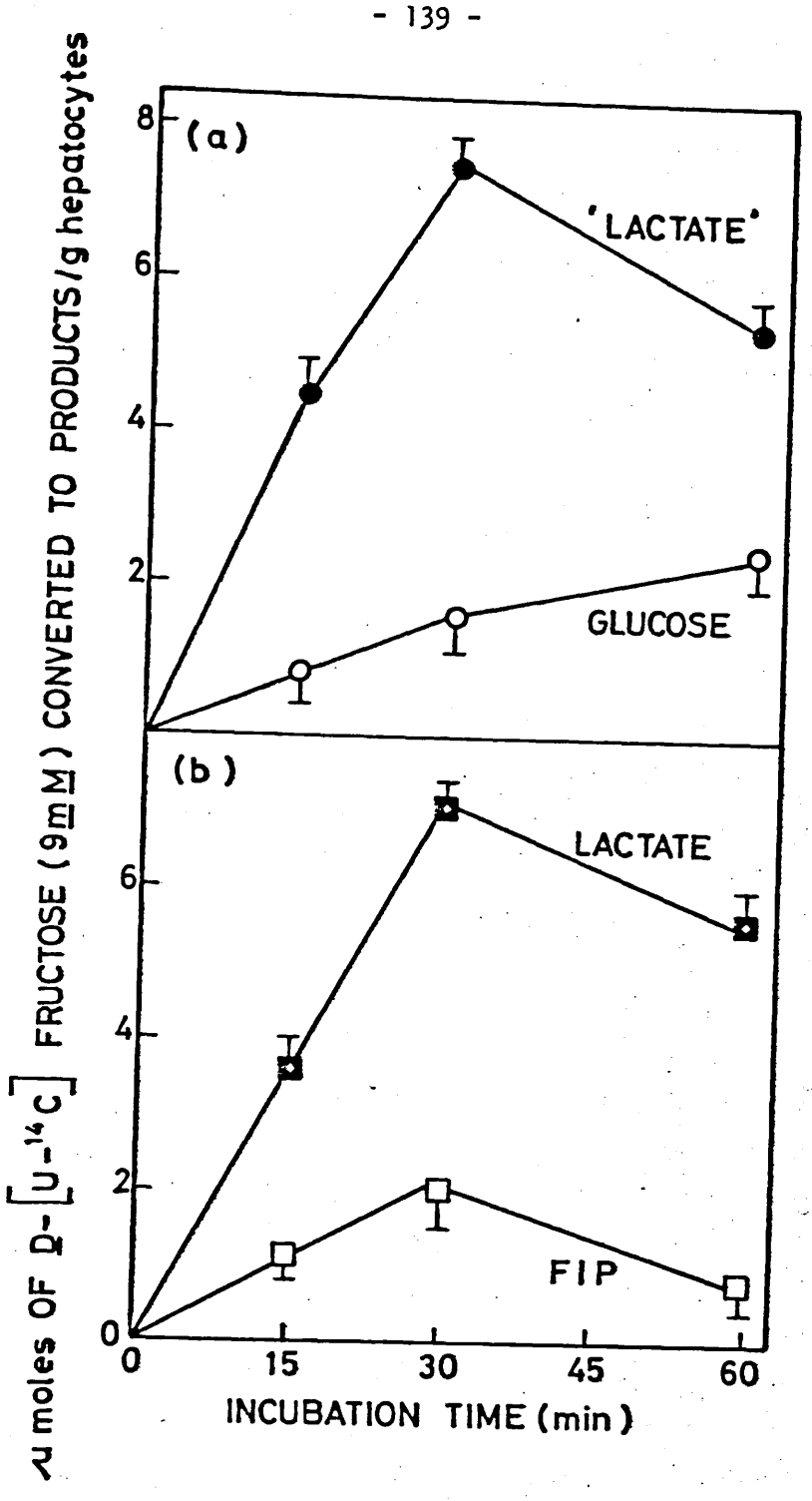


Fig. 32 Time course of incorporation of D - [U-^{14}C] fructose (9mM) into glucose, lactate and FIP.

Hepatocytes (4 x 10^6 cells/ml) from fed rats were preincubated for 1h and then with D -[U-^{14}C] fructose (9mM) for various times. The products were separated as described in Materials and Methods Section VI.B.6b.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

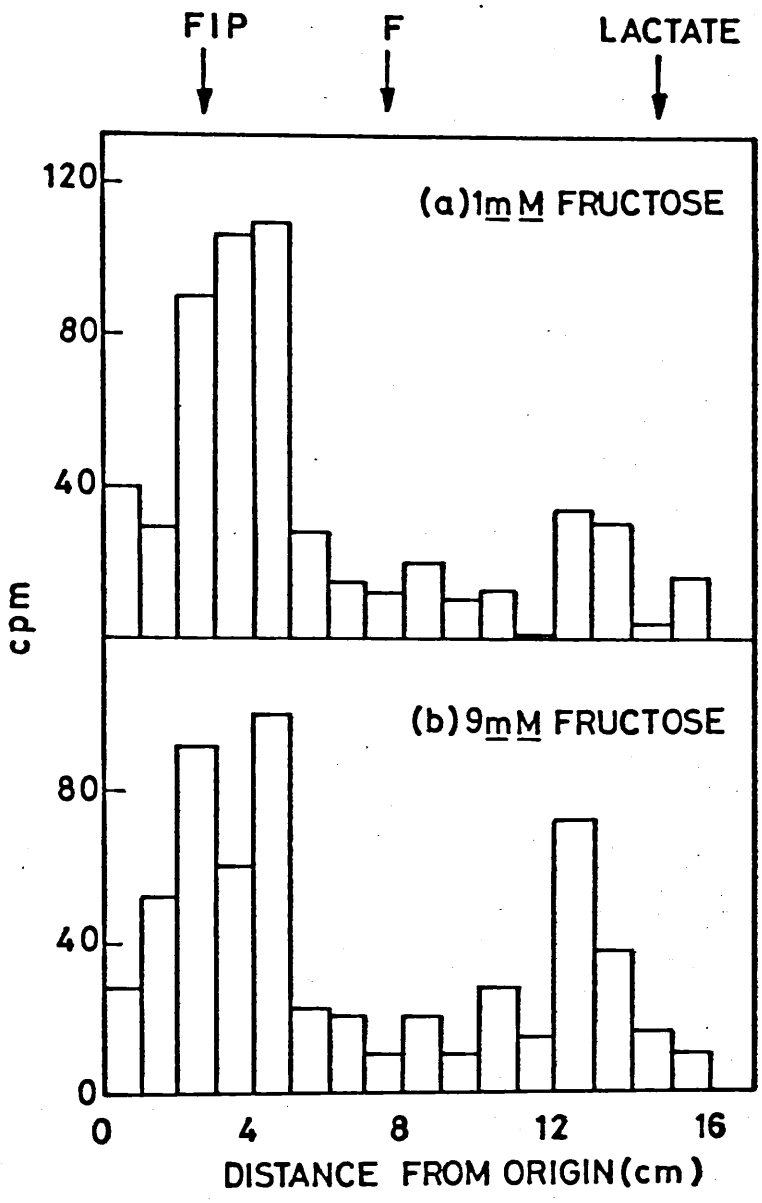


Fig. 33 Thin-layer chromatographic fractionation of the 'lactate' fraction obtained by elution of Dowex AG1 Cl^- with 1 M NaCl.

Hepatocytes were incubated with (a) 1mM or (b) 9mM fructose for 30 min. The cells were extracted with $HClO_4$ and the neutralised extract applied to the ion-exchange column and the neutral sugars eluted with water as described in Materials and Methods Section VI.B.6c.

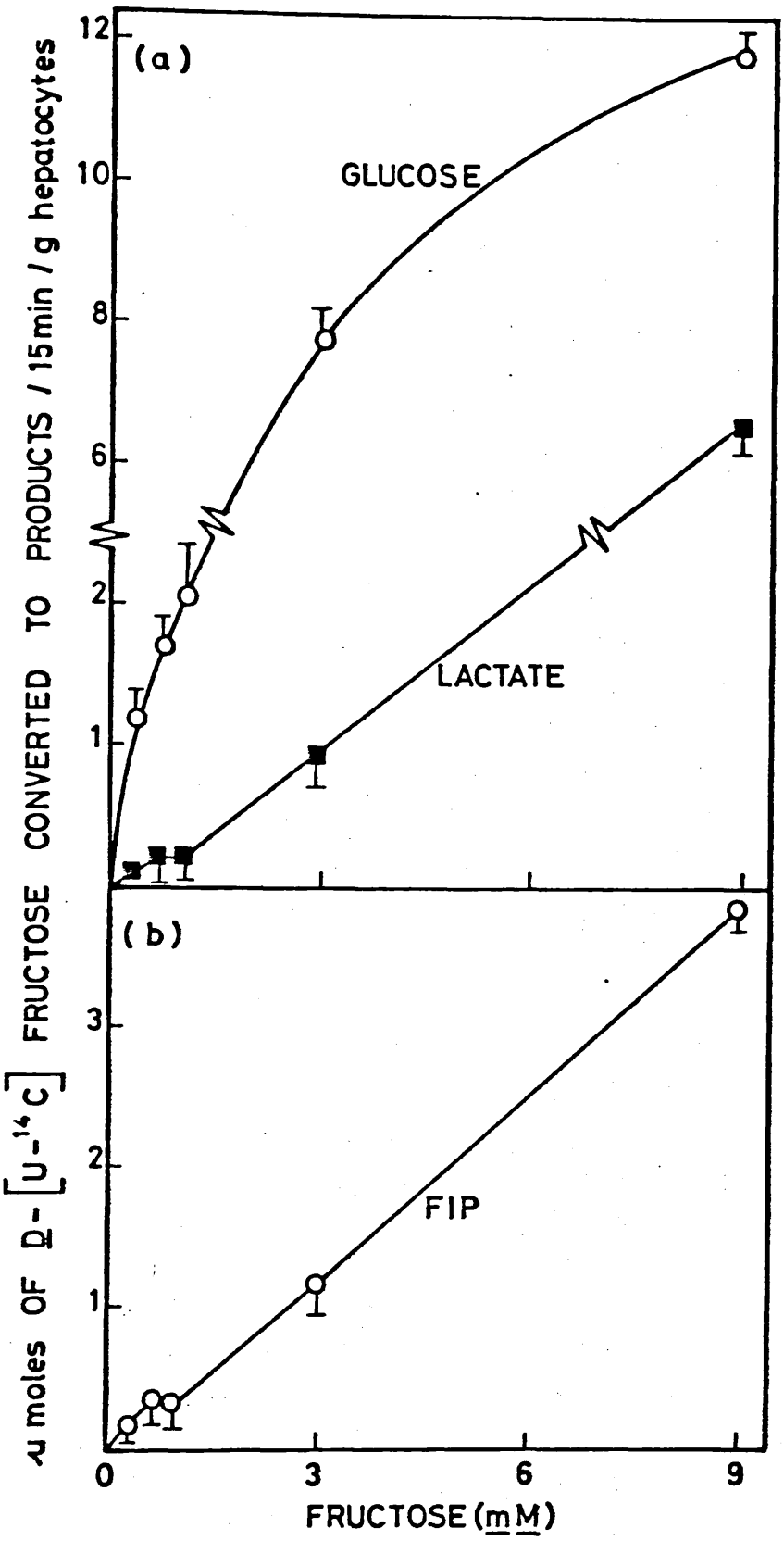


Fig. 34 Effect of fructose concentration on the incorporation of radioactivity into glucose, lactate and FIP.

The labelled substrate was added after a 1h preincubation and the incubation was terminated with $HClO_4$ after a 15 min incubation period. The products were separated as described in Materials and Methods Section VI.B.6c. Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

fructose (up to 1mM) similar to those which are thought to occur in vivo (Topping and Mayes, 1971). The relative proportion of D - [U-^{14}C] glucose to L-[U-^{14}C] lactate formed is high (>10:1). The ratio decreases at higher fructose levels. This probably reflects a saturation of the gluconeogenic pathway from triose phosphate at the high concentrations of the ketose whereas the glycolytic pathway is stimulated at these high concentrations, possibly by the accumulation of FIP (Fig. 34b) and Fru-1, 6-P_2 (Mapungwana and Davies, 1982) which are known to stimulate pyruvate kinase (Eggleston and Woods, 1970) and the depletion of ATP (Mapungwana and Davies, 1982) a potent inhibitor of the enzyme. It is of interest in this context that there appears to be a direct relationship between FIP levels and lactate accumulation. The findings in Fig. 34b contrast somewhat with data for FIP levels in hepatocytes obtained by Mapungwana and Davies (1982) who have observed substantial accumulation of FIP as assayed by an enzymic method even at lower concentration of fructose. For example, they found levels of FIP of > 2 µmole/g after incubation at 1mM fructose for 15 min. The reason for this discrepancy is not clear but the preliminary results of Davies and Phillips (unpublished) in this department also reveal a discrepancy between FIP accumulation assayed by the radiochemical technique and by the enzymatic procedure.

The results for glucose and lactate accumulation compare with those of Foster and Blair (1978) using hepatocytes from fed and fasted rats incubated with fructose (10mM) or DHA (10mM). They also found a greater rate of gluconeogenesis than glycolysis from the triose phosphate in hepatocytes from both fed and fasted animals but they could not distinguish between the glucose arising from glycogen and that arising by gluconeogenesis from triose phosphate by that technique. Hepatocytes from fed animals exhibit a high rate of endogenous glucose output which is stimulated by fructose (see Fig. 11 and Fig. 12).

The data shown in Fig. 31, Fig. 32 and Fig. 34 suggest that D - [U-^{14}C] fructose contributes only a relatively small proportion of the total lactate produced by isolated hepatocytes from fed rats. If the contribution from endogenous metabolites is ignored then the maximum increase in L - [U-^{14}C] lactate levels as a result of the incubation of hepatocytes with fructose is about 0.5mM after 30 min which would be unlikely to stimulate the apparent rate of lipogenesis

significantly, particularly as there is a considerable level of unlabelled lactate arising from another source, probably glycogen.

It is clear from this work that the interpretation of the stimulation of lipogenesis from lactate by fructose is subject to a number of criticisms which arise from a consideration of the utilization of endogenous precursors. It was apparent, however, that fructose was exerting a stimulatory effect on lipogenesis. This was tested further using an alternative substrate ([1-^{14}C]acetate) to measure lipogenesis.

H. Effect of fructose on lipogenesis from [1-^{14}C] acetate

In a further series of experiments rates of lipogenesis from [1-^{14}C] acetate were examined. The method of choice would have been to use $^{3}H_2O$ in order to determine absolute lipogenic rates, but this procedure requires the use of high levels of labelled material and could not be used because facilities were not available. However, [1-^{14}C] acetate incorporation into fatty acid is also regarded as a good indication of lipogenic rates (Capuzzi et al., 1974) which reflect only the flux through acetyl CoA synthase, acetyl CoA carboxylase and fatty acid synthase.

Fig. 35 shows that the rate of incorporation of [1-^{14}C] acetate (1mM or 5mM) into total saponifiable lipid is linear with time for up to 60 min in the presence of 3mM added glucose. Addition of fructose (3mM) resulted in a stimulation of lipogenesis from [1-^{14}C]acetate at both concentrations of the substrate. There was no evidence of a time-lag in the stimulation of lipogenesis as was found with L - [U-^{14}C] lactate (Fig. 28). Indeed, the time course for acetate incorporation is linear for only 30 min with fructose added. The explanation for this phenomenon is not clear. Fig. 36 shows the relationship between lipogenesis and acetate concentration. It was evident that 5mM acetate produced maximal rates of lipogenesis and hence this concentration of substrate was normally used in subsequent studies together with an incubation time of 30 min.

Acetate is probably a physiological substrate for fatty acid synthesis since it is known to be present in rat blood. Concentrations of 800 µM acetate have been found in hepatic portal vein of fed animals

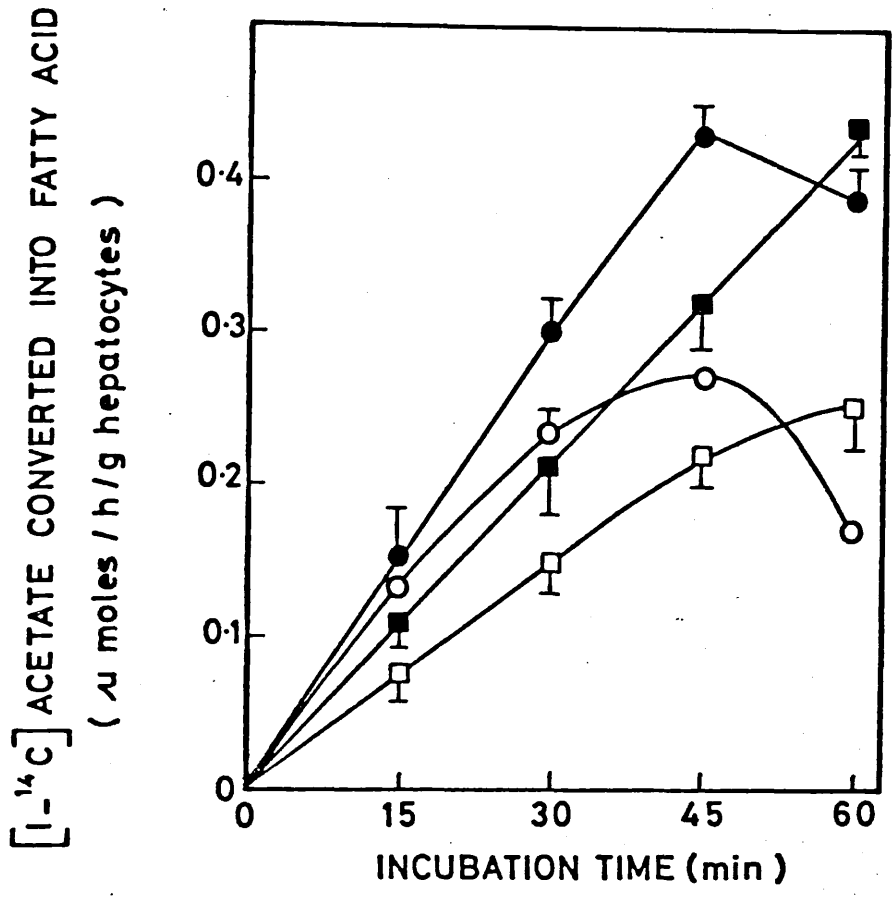


Fig. 35 Time course of incorporation of [1-^{14}C] acetate (5 or 1mM) into fatty acid in the presence of glucose (3mM) or fructose (3mM).

Isolated hepatocytes (4 x 10^6 cells/ml) from fed rats were preincubated for 1h and then incubated with [1-^{14}C] acetate (1mM open symbols or 5mM closed symbols) in presence of 3mM fructose (○, ●) or 3mM glucose (□, ■) for 1h. The reaction was terminated with $HClO_4$ and the total lipid extracted and saponified as described in Section VI.B.5d.

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

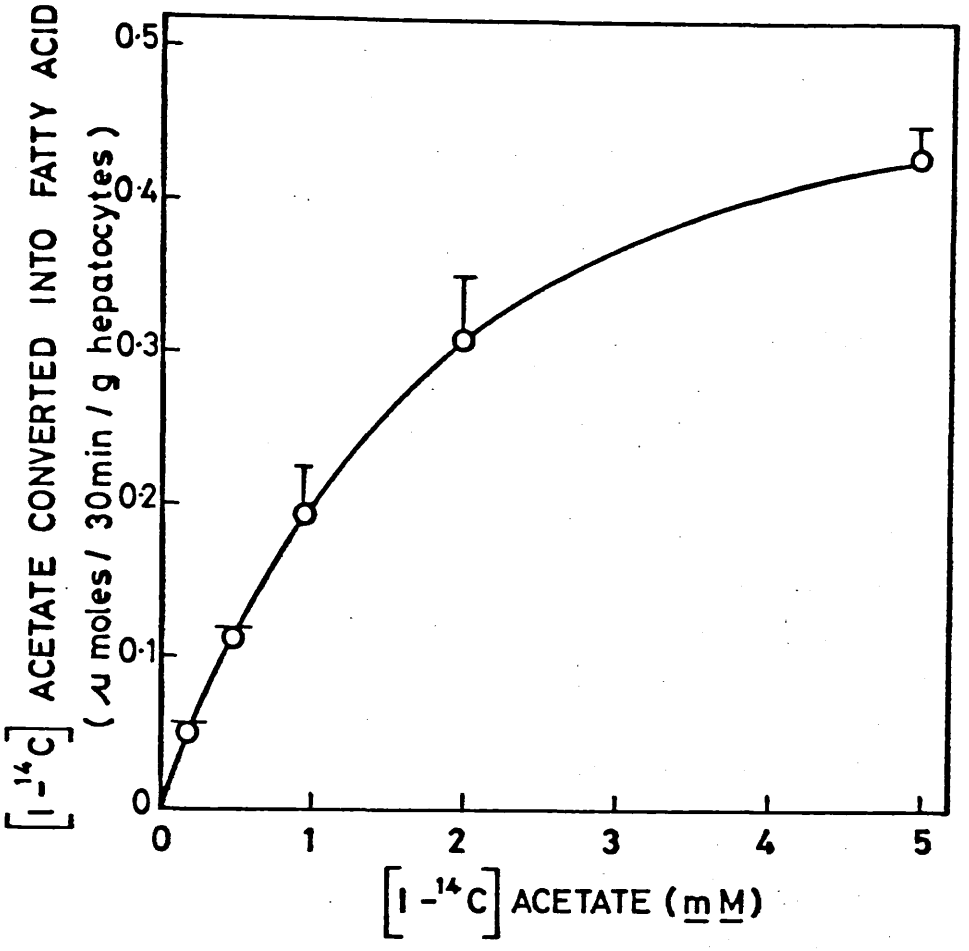


Fig. 36 Lipogenesis from [1-^{14}C] acetate at various concentrations of substrate.

Isolated hepatocytes (4×10^6 cells/ml) from fed rats were preincubated for 1h and then incubated with [1-^{14}C] acetate in presence of glucose (3mM) for 30 min.

Experimental method is described in Section VI.B.9b.

Each point represents the mean ± SEM of results with 3 hepatocyte preparations.

(Buckley and Williamson, 1977). Acetate is converted to acetyl CoA by acetyl CoA synthase which has a Km of approximately 0.03mM for acetate (Knowles et al., 1974) and a V_{max} of 2.55 µmole/min/g. If one compares the relatively low V_{max} and high Km for acetate utilization in lipogenesis (Fig. 36) it is clear that the enzyme is not rate-limiting in fatty acid synthesis from acetate.

Fig. 37 shows the effect of various concentrations of added hexose on rates of lipogenesis from [1-^{14}C] acetate. At low concentration of fructose there was a stimulation of lipogenesis by fructose compared to glucose but this increase was not statistically significant in this experiment. At 9mM and 20mM glucose there was a significant ($P < 0.005$) stimulation of lipogenesis compared to 2mM glucose and below. At high concentrations of fructose (9mM and 20mM) however, there was a significant ($P < 0.005$) inhibition of lipogenesis compared to low concentration of fructose and to high concentration of glucose (9mM and 20mM). This finding is in agreement with the earlier observations (Fig. 29) with L - [U-^{14}C] lactate as a precursor.

Clark et al., (1974) and Selmer and Grunnet (1976) have shown that high concentrations of fructose inhibit fatty acid synthesis from $^{3}H_2O$ by isolated rat hepatocytes but Goodridge et al., (1974) and Clarke et al., (1979) found a stimulation of lipogenesis from [1-^{14}C] acetate by chick hepatocytes incubated with 11mM and 25mM fructose respectively. The mechanism of the inhibition of lipogenesis from [1-^{14}C] acetate observed in the present study is not obvious. The relatively low Km for ATP and high V_{max} of acetyl CoA synthase (Farstad et al., 1967) suggest that it is unlikely that this enzyme is rate-limiting. Thus the depletion of hepatocyte ATP levels would have little effect on the conversion of acetate to acetyl CoA. In addition, the inhibition must be the result of acetyl CoA carboxylase or fatty acid synthesis inhibition. According to Selmer and Grunnet (1976) the depletion of ATP is unlikely to affect acetyl CoA synthase activity but they suggest that fructose increases acyl CoA levels and thus inhibits acetyl CoA carboxylase. However, there is little other evidence in the literature to support this view.

The results in Fig. 35 and Fig. 37 suggest that fructose at low concentration stimulates lipogenesis. The results shown in Table X

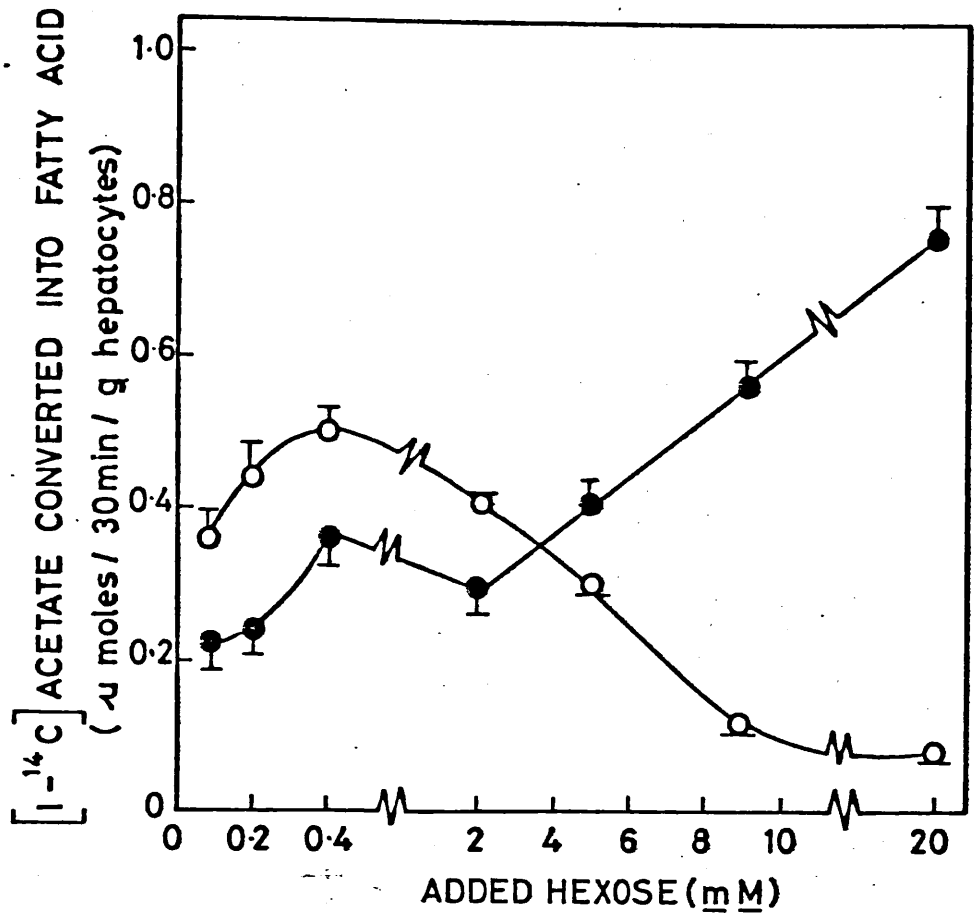


Fig. 37 Effect of various concentrations of hexose on incorporation of [1-^{14}C] acetate into total saponifiable fatty acid.

Isolated hepatocytes (4 x 10^6 cells/ml) from fed rat were preincubated for 1h and then incubated with [1-^{14}C] acetate (5mM) in presence of various concentrations of fructose (○) or glucose (●) for 30 min.

Experimental method is described in Section VI.B.9b.

Each point represents the mean ± SEM of the results with 4 hepatocyte preparations.

Table X. Effects of fructose, glucose, dihydroxyacetone and pyruvate on the incorporation of [1-^{14}C] acetate (5mM) into fatty acid.

Isolated hepatocytes (4 x 10^6 cells/ml., 1.0 ml) from fed rats were preincubated for 1h without substrate and then incubated for 30 min with glucose (2mM or 5mM) or fructose (2mM or 5mM) in presence or absence of pyruvate (1mM) or with dihydroxyacetone (5 or 10mM). Lipogenesis from [1-^{14}C] acetate was measured by following the incorporation of label into the total saponifiable fraction as described in Materials and Methods (Section VI, 5d).

µmoles of [1-^{14}C] acetate incorporation into fatty acid/30 min/g hepatocytes									
glucose (2mM)	fructose (2mM)	glucose(2mM) + pyruvate (1mM)	fructose(2mM) + pyruvate (1mM)	glucose (5mM)	fructose (5mM)	glucose(5mM) + pyruvate (1mM)	fructose(5mM) + pyruvate (1mM)	DHA (10mM)	DHA (5mM)
0.316±0.12 (n = 16	0.44±0.17 (n = 16)	0.40±0.155 (n = 8)	0.71±0.31 (n = 8)	0.392±0.147 (n = 20)	0.31±0.088 (n = 20)	0.44±0.13 (n = 8)	0.394±0.21 (n = 8)	0.66±0.21 (n = 8)	0.617±0.17 (n = 12)

The results are expressed as µmoles utilized per 30 min per g liver ± S.D. Number of animals used are given in parenthesis.

A statistical analysis of the data is given in Table Xa.

Table Xa. Statistical analysis of the data in Table X.

comparison	statistical analysis P	
glucose (2mM) V fructose (2mM)	< 0.05	S
glucose (2mM) + pyruvate (1mM) V fructose (2mM) + pyruvate (1mM)	< 0.05	S
glucose (5mM) V fructose (5mM)	< 0.05	S
glucose (5mM) + pyruvate (1mM) V fructose (5mM) + pyruvate (1mM)	N.S.	
DHA (10mM) V fructose (5mM)	< 0.01	S
DHA (10mM) V glucose (5mM)	< 0.01	S
DHA (5mM) V glucose (2mM)	< 0.005	S
DHA (10mM) V glucose (2mM)	< 0.005	S
DHA (5mM) V glucose (5mM)	< 0.005	S

N.S. = Not significant

S = Significant

shows that fructose (2mM) does in fact stimulate ($P < 0.05$) acetate lipogenesis compared to glucose 2mM but that 5mM fructose produces an inhibition ($P < 0.05$) as suggested by the data in Fig. 37. Thus the concentration of the ketose is very important in determining whether or not activation or inhibition of lipogenesis occurs.

Table X also shows the effect of a number of other substrates on lipogenesis from [1-^{14}C] acetate; maximal rates of lipogenesis from $^{3}H_2O$ are reported to be dependent on the presence of various substrates, for example pyruvate (Harris, 1975). The effect of addition of pyruvate (1mM) on lipogenesis from [1-^{14}C] acetate by hepatocytes was examined. There was a stimulation of lipogenesis by pyruvate observed at each level of fructose and glucose examined. Again, fructose (2mM) in the presence of pyruvate, stimulated lipogenesis compared to the appropriate glucose control ($P < 0.05$) but no significant effect was observed at 5mM hexose in the presence of pyruvate. The addition of pyruvate had no effect on the linearity of the time course of the incorporation of [1-^{14}C] acetate into fatty acid. The highest rate of lipogenesis was observed with 2mM fructose and 1mM pyruvate.

In this series of experiments the effect of DHA on lipogenesis was also examined. Williamson and Whitelaw, (1977) have shown that this triose stimulates lipogenesis from $^{3}H_2O$. Table X shows that the incorporation of [1-^{14}C] acetate into fatty acid is stimulated ($P < 0.01$) by DHA compared to glucose (2mM or 5mM). Again the rate of incorporation was found to be linear in the presence of this substrate (result not shown).

Fructose and DHA are metabolised by similar routes via triose phosphates except that the initial phosphorylation of DHA is catalysed by the triokinase reaction (Hill *et al.*, 1951; Leuthardt *et al.*, 1953; Hers, 1955; Dahlquist, 1962; Frandsen and Grunnet, 1971) yielding dihydroxyacetone phosphate whereas the metabolism of fructose involves ketohexokinase and aldolase. Hepatocyte glycolysis to lactate from fructose is however more rapid than from DHA (Foster and Blair, 1978; Mapungwana, 1982) and DHA does not deplete ATP in perfused liver (Williamson et al., 1969; Woods and Krebs, 1973). However, Mapungwana (1982) has shown some depletion of hepatocyte ATP at 10mM DHA but not at 4mM substrate. However, the depletion observed at 10mM DHA is minor

compared to that with 10mM fructose and is further evidence to suggest that inhibition of lipogenesis is due to lack of ATP.

The stimulatory effect of fructose on lipogenesis may be related to a similar effect of DHA. An attempt to show a correlation between the effects of DHA and fructose in this laboratory (Mapungwana, 1982) has shown that at high substrate levels FIP and to a lesser extent Fru-1, 6-P_2 levels are raised by fructose whereas DHA raises the level of Fru-1, 6-P_2 only.

I. Effect of Fru-1, 6-P_2 and FIP on hepatocyte ACC activity

Since both DHA and fructose stimulate lipogenesis and they both elevate Fru-1,6-P_2 it was decided to examine the effect of this metabolite on ACC activity. This enzyme is thought to be a key regulatory enzyme in lipogenesis (Halestrap & Denton 1973; Geelen et al., 1978b; Witters et al., 1979b). In addition, it is a regulatory enzyme which is common to both lipogenesis from [1-^{14}C] acetate and from L - [U-^{14}C] lactate and since stimulation of the incorporation of both substrates was observed ACC was considered the most likely target for regulation. Other possible regulatory enzymes such as pyruvate dehydrogenase or ATP citrate lyase are directly involved in the utilization of lactate but not of acetate.

Isolated hepatocytes were used as a source of ACC in these experiments. The cells were prepared from fed male animals, the cell pellet was homogenised and a particle-free supernatant used for enzyme assay. The activity of the enzyme is low in crude extracts and pre-incubation with citrate and Mg^{2+} has been used routinely to maximise the enzyme activity. When the method was used in the present study values of approximately 400 nmoles/min/g hepatocytes were found. However, several independent authors (Geelen et al., 1978b; Witters et al., 1979b, Allred and Roehrig, 1978; Assimacopoulos-Jeannet et al., 1981; Ly and Kim, 1981) have claimed that crude liver preparations can be used for the assay of ACC and that indeed the citrate activation step abolishes the hormonal effects on enzyme activity in isolated hepatocytes. For this reason it was decided to assay the enzyme without the citrate preincubation step but to include citrate in the extraction and incubation media since this is considered

essential by some authors (Allred and Roehrig 1978; Witters et al., 1979b). Fig. 38 shows that the rate of acetyl CoA carboxylase incorporation of $H^{14}CO_3$ into acid-stable material is linear with time for up to 3 min under various conditions and preliminary experiments indicated that both FIP and Fru-1,6-P_2 stimulated enzyme activity. The linearity of the reaction(s) suggests that there is no citrate activation during the course of the incubation. The extent of the stimulation was confirmed using a number of different enzyme preparations obtained from different hepatocyte preparations (Table XI). A stimulation by FIP (10mM) was evident at both concentrations of citrate used. Stimulation by Fru-1,6-P_2 (0.1mM) was only observed in the presence of 10mM citrate. These concentrations of the ketose phosphates are similar to those which may occur in vivo under certain conditions, e.g. following fructose or DHA loading of liver. However, the enzyme activity is generally low compared to the activity found when ACC is fully activated by preincubation with citrate. Incubation of the enzyme with 10mM citrate, rather than 5mM citrate, resulted in a higher level of enzyme activity and the stimulation by both ketose phosphates was observed. Preincubation of the crude preparation with citrate abolished the stimulatory effect of FIP and Fru-1,6-P_2 (data not shown). This observation is in agreement with those of other workers (Witters et al., 1979b; Geelen et al., 1978b). Citrate is a known allosteric regulator of ACC but there is no evidence in the literature concerning the stimulation of the enzyme by either FIP or Fru-1,6-P_2. No inhibition of ACC activity by FIP was observed, suggesting that the inhibition of lipogenesis observed at high levels of fructose (see Fig. 29 and Fig. 37) is not the result of FIP accumulation in the hepatocytes.

Recent work by Davies et al. (1982) casts some doubt on the observed stimulation of ACC by ketose phosphates. Crude liver extracts contain both ACC and pyruvate carboxylase. The former enzyme is responsible for the carboxylation of acetyl CoA in the presence of ATP; the latter for the ATP dependent carboxylation of pyruvate, a reaction stimulated by acetyl CoA. In addition, pyruvate is present in crude liver extracts and accumulates during the incubation. The product of pyruvate carboxylase is oxaloacetate which is rapidly transformed to either malate or aspartate. The major product in crude extracts appears to be the amino acid and this casts some doubt on the use of crude extracts to assay ACC activity. Indeed, Inoue and

Lowenstein (1975) have drawn attention to this problem. In this context Davies (unpublished results) has shown an apparent stimulation of ACC by Fru. 2,6-P_2 which was shown to be attributed to a stimulation of glycolysis and the accumulation of low concentration of pyruvate and, thus, of pyruvate carboxylation products in the incubation medium. The regulation of ACC by metabolites in vivo remains a mysterious problem but it is clear that many reports on the regulation of the enzyme in crude extracts need to be reinvestigated.

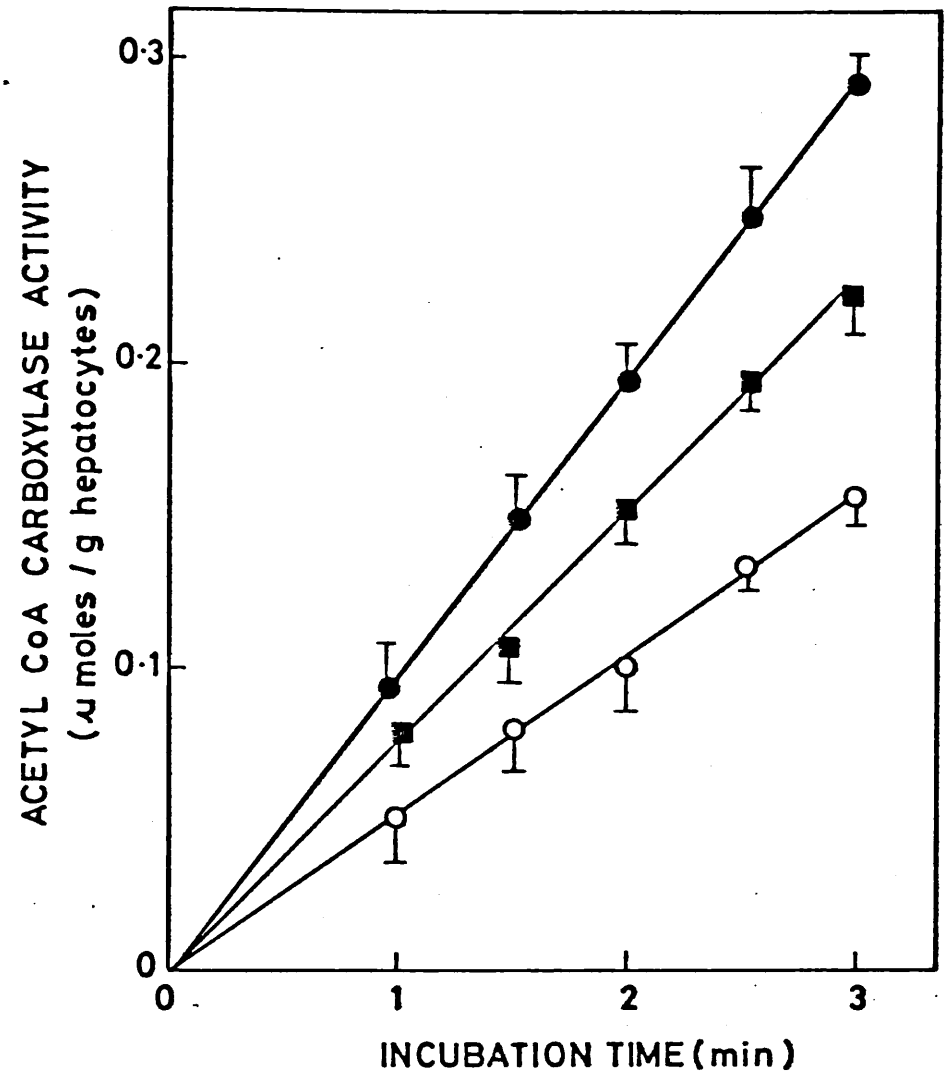


Fig. 38 Time course for the acetyl CoA carboxylase incorporation of $H^{14}CO_3$ into acid-stable products.

Hepatocytes (3.9g) from fed rats were homogenised in homogenising buffer (11.7 ml) and the extract was centrifuged at 100,000 x g for 30 min and used immediately for enzyme assay. The reaction was started by the addition of enzyme and terminated by the addition of 0.8M HCl. The reaction mixture was dried under N_2 and assayed for radioactivity as described in Materials and Methods Section VI.B.11a. The enzyme was incubated in the presence of Fru-1, $6P_2$ (0.1mM) (■), or of FIP (10mM) (●) or in the absence of ketose phosphates (O).

Each point represents the mean ± SEM of the results with 3 hepatocyte preparations.

Table XI The effect of FIP and Fru-1,6-P_2 on acetyl CoA carboxylase activity.

Hepatocytes (3.9g) from fed rats were homogenised in homogenising buffer (11.7 ml) and the extract was centrifuged at 100,000 x g for 30 min and used immediately for enzyme assay. The reaction was started by the addition of enzyme and terminated by the addition of 0.8M HCl. The reaction mixture was dried under nitrogen and assayed for radioactivity as described in Materials and Methods. The enzyme was incubated in the presence of FIP or Fru 1,6-P_2 or in the absence of ketose phosphate.

acetyl CoA carboxylase activity

	nmoles/3 min/g hepatocytes		
	control	FIP (10mM)	Fru 1,6-P_2 (0.1mM)
citrate (5mM)	106 ± 10 (n=18)	167 ± 44 (n=6)	110 ± 20 (n=6)
citrate (10mM)	145 ± 20 (n=14)	220 ± 36 (n=14)	189 ± 22 (n=14)

Each point represents the mean ± SEM of results. Number of preparations is given in parenthesis.

A statistical analysis of the data is given in Table XIa

Table X1a. Statistical analysis of the data in Table XI

comparison		Statistical analysis P
control (5mM citrate)	V FIP + 5mM citrate	< 0.005 S
control (5mM citrate)	V Fru 1,6 P_2 + 5mM citrate	N.S.
FIP + 5mM citrate	V Fru 1,6 P_2 + 5mM citrate	< 0.02 S
control (10mM citrate)	V FIP + 10mM citrate	< 0.005 S
control (10mM citrate)	V Fru 1,6 P_2 + 10mM citrate	< 0.005 S
control (10mM citrate)	V control (5mM citrate)	< 0.005 S
FIP + 5mM citrate	V FIP + 10mM citrate	< 0.02 S
Fru 1,6 P_2 + 5mM citrate	V Fru 1,6 P_2 + 10mM citrate	< 0.005 S

N.S. = Not significant

S = Significant

CONCLUSIONS

The fate of fatty acids in the liver is dependent on a number of different factors which determine the rate of synthesis of glycerolipids. For example, in the starved state there is an increase in the activity of CAT and a decrease in the activity of GPAT which directs the metabolism of fatty acid towards β-oxidation rather than esterification. These effects are presumably due to hormonal, principally insulin and glucagon, changes which occur between the fed and the fasted state. In the fed state the liver is the major site for the synthesis and secretion of triacylglycerol so that excess fatty acid in the diet may be deposited in the adipose tissue. In the fed state carbohydrate can also be converted into fatty acids and exported as triacylglycerol by the liver. Sucrose and fructose have been reported to cause hypertriglyceridaemia an effect which can be attributed in part to the induction of high levels of key regulatory enzymes of lipid metabolism which direct the metabolism of carbohydrate towards fatty acid and triacylglycerol synthesis. It was of interest to discover if fructose, as a result of its unique hepatic metabolism could also exert short-term regulatory effects on these processes.

It is clear from the work presented in this thesis that fructose is a better carbon source than glucose for the synthesis of glyceride-glycerol and of glyceride-fatty acid in isolated hepatocytes. This is in agreement with previous work using liver slices (Pereira and Jangaard, 1971) which could be criticised because of the adverse changes in the redox state and the adenine nucleotide content of this type of liver preparation. The results presented in this thesis suggest that the well-oxygenated, isolated hepatocyte preparation, when the lactate/pyruvate ratio is maintained at approximately 10:1 and the ATP content remains high, is a good model system for the investigation of lipid metablism.

It is proposed that the effects of fructose on triacylglycerol synthesis may be mediated, at least in part, via changes in the sn-glycerol 3 - phosphate content of the hepatocyte which occur without a change in the cytoplasmic $NADH/NAD^+$ ratio. Increasing fructose concentration resulted in the greater utilization of the ketose carbon for triglyceride-glycerol synthesis, an effect which was observed in the presence and in the absence of added oleate. In the latter case, fructose stimulated the esterification

to give maximal rates of triacylglycerol synthesis. Topping and Mayes (1972) were the first to show that the ketose enhances triacylglycerol secretion by the perfused liver in the short-term and that fructose causes increased esterification and decreased ketogenesis. Ontko (1972) and Prager and Ontko (1976) have concluded that these reciprocal changes are mediated primarily via the inhibitory effects of fructose on long-chain fatty acid oxidation. They suggest that fructose metabolism generates an intermediate which retards β-oxidation at an early stage in the sequence. However, recently evidence has been accumulating that the reciprocal relationship between fatty acid oxidation and esterification in the fed and fasted states is governed by the intracellular concentration of sn-glycerol 3-phosphate (Debeer et al., 1981; Declerq et al., 1982a). Glucagon is thought to lower hepatocyte sn-glycerol 3-phosphate content and, as a result, to inhibit triacylglycerol synthesis. Declerq et al. (1982b) have provided evidence that the level of sn-glycerol 3-phosphate may limit hepatic triacylglycerol synthesis in both fed and starved animals. sn-glycerol 3-phosphate has not previously been considered to be an important metabolic regulator. One of the problems with this metabolite is that the levels change considerably according to the redox state of the tissue preparation and these changes may mask regulatory changes which occur in vivo. In this context recent reports that sn-glycerol 3-phosphate is also involved in the inhibition of PFK1 and PFK2 (Claus et al., 1982) and in the activation of fructose 2, 6-bisphosphatase (Van Schaftingen et al., 1982). There is evidence that the triose phosphate pool, especially sn-glycerol 3-phosphate, is closely regulated in vivo. Thus one may expect other substrates (e.g. DHA) which affect sn-glycerol 3-phosphate levels, without altering the cytoplasmic redox state, to affect triacylglycerol synthesis. This possibility needs to be tested.

In the present study glucose was also shown to stimulate oleate esterification to a lesser extent but in this case the major product was phospholipid rather than triacylglycerol. This suggests a specific stimulation of either PPH or DGAT activity by the ketose. There is no report in the literature to suggest that the increased sn-glycerol 3-phosphate concentration or any of the other consequences of fructose metabolism are involved in the regulation of these enzymes but this possibility needs to be tested. In the present study the magnitude of the effect of fructose on triacylglycerol synthesis was dependent on fructose concentration but was observed at both physiological and higher concentrations of the ketose.

However, the nature of the effect on lipogenesis was concentration dependent. Stimulation occurred at low fructose concentrations and inhibition at high fructose levels. There are a number of effects of fructose on the liver which are concentration dependent. For example, high fructose concentrations activate pyruvate dehydrogenase in the perfused liver but physiological fructose levels are without effect (Topping and Mayes, 1977). Pyruvate kinase is stimulated at low concentration of the ketose but is inhibited at concentrations $>$ 3mM (Mapungwana and Davies, 1982). Under the conditions used in this study hepatocyte ATP content has been shown to be depleted at concentrations greater than 2mM fructose (Mapungwana, 1982). Many of the effects of fructose on enzyme have been attributed to the depletion of ATP which results in a general disruption of cellular metabolism. Thus it is likely that only the effects observed with physiological concentrations of fructose have a significance for the *in vivo* effects of the sugar. Thus it appears that in the hepatocyte from fed rats incubated with low concentrations of fructose, there are ideal conditions for the synthesis of fatty acid from endogenous substrates, including fructose, and for the esterification of the fatty acid produced *de novo* and of exogenous fatty acid. The stimulation of esterification may be attributed to a change in the *sn*-glycerol 3-phosphate content, although much more work needs to be done to confirm this hypothesis. The stimulation of lipogenesis remains to be explained in terms of the known effects of fructose, although one possibility is that the supply of endogenous precursor, notably lactate, may be enhanced by the stimulatory effect of fructose on glycolysis.

MATERIALS AND METHODS

VI. MATERIALS AND METHODS

A. Materials

1. Chemicals

All the chemicals used in the project were of Analar grade and all aqueous solutions were prepared using glass distilled, deionized water. Substrates and enzymes were purchased from Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K., unless otherwise stated. Collagenase from clostridium histolyticum lypophilized, clostridiopeptidase A, EC 3.4.24.3 was used for the preparation of hepatocytes. All radioactive materials were obtained from the Radiochemical Centre, Amersham, Bucks.

2. Animals

The rats used in this study were of the Wistar strain and were bred in the laboratory animal house.

3. Diets

The animals were fed on a commercial diet Dixon. 86 obtained from E. Dixon and Sons (Ware) Ltd., Crane Mead Mills, Ware, Herts. The composition of this diet is shown in Table XII and the calculated percentage composition of this diet and its calorific values are presented in Table XIII.

Table XII

Composition of Dixon. 86 diet*

Dixon. 86	g/kg
Wheat	500
Barley	50
Concentrated meat meal	75
White fish meal	70
Grass	50
Yeast	50
Molasses	50
Salt	0.2
Vitamin 706	0.022

*Values for approximate quantities and composition were obtained from the manufacturer; E. Dixon & Sons (Ware) Ltd., Crane Mead Mills, Ware, Herts, U.K.

Table XIII

Calculated percentage composition of Dixon. 86 diet*

	Dixon. 86 (%)
Crude Oil	2.03
Crude protein	19.29
Crude fibre	3.01
Digestible crude oil	1.44
Digestible crude protein	15.73
Digestible crude fibre	1.90
Digestible carbohydrate	50.83
Saturated fatty acids	0.42
Linoleic acid	0.72
Other unsaturated fatty acids	0.88
Cals/Kg	
Gross energy	3,942
Metabolizable energy	3,548

*Values obtained from manufacturer E. Dixon and Sons (Ware) Ltd., Mead Mills, Ware, Herts.

4. Liver perfusion cabinet

The perfusion cabinet consisted of a metal cabinet with a glass front door and a fan heater connected to a thermostat for the control of temperature inside the cabinet and a glass 'lung' was made to specification (Miller Smith 1973). A Watson-Marlow H.R. flow inducer (Watson - Marlow, Ltd., Marlow, Bucks, U.K.) was used to circulate the perfusion media and Swinnex - 13 filter units (Buc. France) were used to filter the circulating perfusate.

B. Methods

1. Animals

All experiments were performed on male Wistar rats weighing between 300 and 350g unless otherwise stated. The animals were bred in the laboratory animal house and were fed ad libitum on Dixon. 86 diet from weaning. For part of the study the rats were fasted for 24h or 48h from 9.00h. The temperature of the animal house was kept at $22^{o} \pm 2^{o}$ and the animals were subjected to a 12h light/12h dark regime with the light on between 0.800 and 20.00h.

2. Preparation of hepatocytes

Hepatocytes were prepared between 09.00 and 10.00h according to the method originally described by Berry and Friend (1969) and later modified by Krebs et al., (1973) and Wagle and Ingebretsen (1975). Rats were anaesthetized with Nembutal sodium pentobarbitone (May and Baker Ltd., Dagenham, Essex, U.K.) 60 mg/ml, 0.1ml/100g body weight . The peritoneal cavity was opened by two lateral incisions and the portal vein and the inferior vena cava were exposed. Two ligatures were placed loosely around the portal vein. A third ligature was then placed loosely around the inferior vena cava just anterior to the right renal vein. A sterile leur cannula (Braunla sterile leur; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) was inserted into the hepatic portal vein and the two ligatures were firmly tied. The liver was then perfused with Krebs-Ringer bicarbonate (KRB) without Ca^{2+} (Table XIV, Krebs and Henseleit, 1932) which had been preincubated at 37^{o} and gassed with 95% O_2 and 5% CO_2. The inferior vena cava was immediately severed below the right kidney to prevent

Table XIV. Composition of Krebs-Ringer bicarbonate.

Solutions required	Krebs-Henseleit original Ringer bicarbonate (parts by vol.)	Final concentration mM
0.90% NaCl (0.154M)	100	118
1.15% KCl (0.154M)	4	3.5
1.22% $CaCl_2$ (0.11M)	3	2.5
2.11% KH_2PO_4 (0.154M)	1	1.2
3.82% $MgSO_4.7\ H_2O$ (0.154M)	1	1.2
1.3% $NaHCO_3$ (0.154M)	21	24.8

The above solutions (except $CaCl_2$) were mixed together and gassed with CO_2 for 1h and stored at 4°. $CaCl_2$ solution was added and the buffer gassed with O_2:CO_2 (95 : 5 V/V) before the start of each experiment.

the liver from swelling and the perfusate allowed to flow freely through the liver. Immediately after this step the rib cage was cut open, exposing the thoracic cavity. The inferior vena cava was cannulated just anterior to the diaphragm via the right atrium of the heart. This cannula was firmly held in place by a tight ligature and the ligature around the inferior vena cava in the peritoneal cavity was then tied. The liver was perfused with KRB (without Ca^{2+}) at a rate of approximately 25ml/min.

The liver is then placed in the perfusion apparatus at constant temperature of 37^{o} and when the liver appeared pale and the perfusate was free from blood (usually after 2-5 min) it was then perfused with collagenase (30 mg suspended in 100 ml of KRB with Ca^{2+}) until the liver was smooth and swollen and the leakage of medium from the liver was rapid (usually after 15-20 min). The liver was then removed and placed in a plastic beaker (250 ml capacity) containing 50ml of KRB buffer (without Ca^{2+}) which was gassed with 95% O_2 and 5% CO_2 and the tissue was minced using scissors for 1-2 min. A plastic funnel with a nylon mesh (Nybolt, No. 10. 132 micron, J. Staniar and Co., Manchester, U.K.) was used to filter the cell suspension into centrifuge tubes (MSE 50 ml capacity). The cells were sedimented at 50xg (50 sec.) in a bench centrifuge (MSE Minor). The supernatant was aspirated and the pellet was resuspended in 20 ml of KRB (with Ca^{2+}) containing bovine serum albumin fraction V, (fatty acid-free) Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K.). Small aliquots of the hepatocytes were diluted 1:1 V/V with trypan blue (0.25% trypan blue in 0.9% NaCl) and examined under a microscope using a haematocrit chamber. The concentrations of cells in the suspensions were adjusted as required. The hepatocytes were preincubated for 1h in KRB (with Ca^{2+}) containing 1.5% BSA and gassed continuously with 95% O_2 and 5% CO_2 in order to restore the ATP/ADP ratio to normal (Claus et al., 1975).

All glassware used in the preparation of hepatocytes was siliconized by rinsing with a solution of dimethyl chlorosilane (BDH Chemicals Ltd., Poole, England) in 1,1,1-trichloroethane (2% w/v) and air-dried overnight. Finally, the glassware was rinsed with distilled water to remove HCl formed in the treatment and the glassware was then oven-dried.

3. Methods for determination of hepatocyte viability

a. Trypan blue

Structual viability of isolated liver cells was judged by the percentage of cells which excluded trypan blue. This value was normally greater than 80%. Cell preparations with low viability were discarded. This method gives an indication of the integrity of the plasma membrane.

b. Estimation of ATP level in isolated hepatocytes

Isolated rat hepatocytes (4.0×10^6 cells/ml; 1ml) were incubated in KRB (with Ca^{2+} + 1.5% BSA) in the presence of various substrates and hormones. After the required time interval the reaction was stopped by addition of 20% perchloric acid (100 µl). The precipitated protein was removed by centrifugation and the supernatant was transferred into a microcentrifuge tube and neutralized using 5M K_2CO_3 (25 µl). The resulting $KClO_3$ was removed by centrifugation and the supernatant assayed for ATP. ATP levels were assayed in the extract by an enzymic method involving a coupled enzyme reaction.

The principle of the test was the ATP-dependent conversion of glycerate 3-phosphate to sn-glycerol 3-phosphate in a sequence of reactions catalysed by glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase with the utilization of ATP resulting in NADH oxidation. The incubation mixture (total Vol. 1.2ml) contained 0.41M triethanolamine, pH 7.6, 3.3mM $MgSO_4$, 5mM glycerate 3-phosphate, 0.21 mM NADH and the deproteinized supernatant. The decrease in absorbance at 340 nm was followed after the addition of an enzyme mixture (10 µl) containing glyceraldehyde 3-phosphate 350 u/ml; phosphoglycerate kinase 450 u/ml; triose phosphate isomerase 800 u/ml; glycerol 3-phosphate dehydrogenase 60 u/ml.

c. Gluconeogenesis from L - [U-^{14}C] lactate

After preincubation the cell suspension (4×10^6 cells/ml; 1 ml) was added to L - [U-^{14}C] lactate (0.025µci/µmol, 5µl) in polycarbonate tubes. The cells were incubated for various time intervals in presence and absence of glucagon (10µM) in a shaking water bath and gassed continuously with 95% O_2 and 5% CO_2 at 37° (200 cycles/min). The reaction was stopped by the addition of 0.15M $ZnSO_4$ (0.5 ml), 0.3M Ba $(OH)_2$ (0.5 ml) and water

(2ml) was then added and the precipitate was removed by centrifugation. The protein-free supernatant was shaken for 1h with moist Dowex 50W - X8 (H^+ form, 200 - 400 mesh; 200 mg) and moist Duolite ES561 (Dia-prosium, Vitry, Chauny, France; 800 mg) to separate lactate and glucose. A second treatment with ion-exchange resin resulted in a complete removal of lactate (Exton and Park, 1967; Claus et al., 1975). D - [U-^{14}C] glucose remaining was assayed in Triton X - 100/toluene scintillant (1:2 v/v) using a Packard - Tri-Carb liquid scintillation spectrometer.

4. Metabolite assays

a. Preparation of perchloric acid extract

Hepatocytes were incubated under various conditions as described in the Results and Discussion section and the reactions were stopped by addition of 0.1 vol of ice-cold perchloric acid (20% w/v). The precipitated protein was removed by centrifugation at 6,000 x g in a microcentrifuge. The supernatant was removed and neutralised with 5M K_2CO_3 (0.025 vol.) and was recentrifuged for further 10 min to remove $KClO_3$.

b. Glucose

Glucose was assayed in the deproteinised sample by the glucose oxidase method (Fleming and Pegler, 1963; Catley, 1967). The glucose oxidase reagent 75 mg glucose oxidase Boehringer EGAC (Grade II) - 15424, 7.5 mg horse radish peroxidase, Boehringer 15302 EPAB and 25 mg o-dianisidine hydrochloride (Sigma) dissolved in 0.3M Tris-HCl buffer containing 40% glycerol (250 ml; pH 7.0) was added to the sample (1ml) in a stoppered glass test tube. The solution was thoroughly mixed and incubated at 37^{0}C for 1h. The reaction mixture was acidified by addition of 9M sulphuric acid (2ml) and the absorbance measured at 540nm. A number of glucose standards(0-50 µg) were used to construct a calibration curve for the glucose oxidase test.

c. sn-glycerol 3-phosphate

The formation of NADH as measured by the increase in optical density at 340 nm was used as a measure of sn-glycerol 3-phosphate (Michal and Lang, 1974). The reaction mixture (total volume 1.05 ml) contained hydrazine-buffer pH 9.5 (i.e.; 0.189M hydrazine, 0.47M glycine and 2.7mM EDTA);

2.3 mM NAD^+ and the deproteinized sample (0.5ml). The reaction was started by adding 10 µl of sn-glycerol 3-phosphate dehydrogenase (10 mg/ml) and the formation of NADH was followed using a recording spectrophotometer.

d. Lactate

The method of Wieland (1974) was used to measure lactate. The reaction mixture contained hydrazine-glycine (0.2M glycine, 1M hydrazine, 2mM $MgCl_2$) buffer, pH 9.8; (1.40 ml), ATP (50 mM, 0.05 ml), NAD^+ (20 mM, 0.05 ml) and deproteinised sample, pH 9-9.5 (see Section VI, 4a) (0.5 ml). The increase in absorbance at 340 nm was followed using a recording spectrophotometer after addition of 20 µl lactate dehydrogenase (10.6 units, from rabbit muscle, Boehringer Manheim). Lactate dehydrogenase catalyses the conversion of lactate to pyruvate and the latter is removed by trapping with hydrazine.

e. Pyruvate

Pyruvate was assayed by method of Michal and Beutler (1974). Triethanolamine buffer, pH 7.6 (0.4M, 1.25 ml), NADH (5mM, 0.02 ml) and deproteinised sample (see Section VI, 4a) (1.0ml) were placed in a quartz cuvette (1 cm light path). The decrease in absorbance at 340 nm was followed using a recording spectrophotometer following the addition of 10 µl lactate dehydrogenase (5.3 units).

5. Incorporation of hexose into lipid

a. Incubation of hepatocytes

Hepatocytes were preincubated for 1h at 37°. The cell suspension (8×10^6 cells/ml; 0.5 ml) was added to 0.5 ml of either fructose or glucose dissolved in KRB (with Ca^{2+}) containing 1.5% bovine serum albumin (Fraction V, Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K.). In addition, the incubation mixture contained D - [U-^{14}C] fructose (9×10^5 dpm) or D - [U-^{14}C] glucose (9×10^6 dpm). The incubations were carried out in siliconized glass test tubes in a shaking water bath (200 cycles/min) for the appropriate time. The cells were gassed continuously with 95% O_2 and 5% CO_2 for the time of the incubation. The reaction was stopped by the addition of 1 ml of ice-cold perchloric acid (6% w/v).

b. Total lipid extraction

Lipids were extracted by a simplified modification of the method of Folch et al. (1957). Methanol : Chloroform (2 : 1, v/v) (6ml) was added to the perchloric acid extracted samples and the mixture ultrasonicated for 15 sec (MSE ultrasonic disintegrator, maximum amplitude). The mixture was left overnight and then transferred to plastic 50 ml centrifuge tubes and 7.6 ml of methanol : chloroform : water (2 : 1 : 0.8) was added to each tube and this mixture was sonicated for a further 15 sec. To this mixture 4 ml of chloroform and 4 ml of water were added and the two phases were separated by centrifugation in a bench centrifuge (MSE Minor) at 2000 x g for 10 min. The aqueous phase was removed by aspiration, deionised water (25 ml) was added to the chloroform layer and the two phases were separated as described previously. The aqueous phase was then removed by aspiration and the lower chloroform phase was filtered into scintillation vial through phase separating paper (Whatman 1PS) to remove protein and water. The chloroform extract was evaporated to dryness under a stream of nitrogen. The radioactivity was then assayed in 10 ml of a toluene scintillant (5 g PPO/L toluene) using a Packard Tri-Carb liquid scintillation spectrometer. Quenching was estimated using the E.S.R. method.

c. Separation and analysis of lipids

The total lipid was extracted as previously described. The chloroform extracts were then evaporated to dryness under a stream of nitrogen, dissolved in a small volume of dry diethylether and applied to activated silica gel G (Kieselgel G nach Stahl (Type 60) MERCK) thin layer chromatography (TLC) plates prepared in laboratory by the method of Smith and Seakins (1976). Authentic standards were also chromatographed. The lipid fractions were separated by TLC in a solvent system containing hexane : dry diethylether : glacial acetic acid (80 : 20 : 1 v/v/v) (Clark et al., 1974). After evaporation of the solvent the thin layer plates were placed into a tank containing a small trough filled with iodine crystals. The lipids appeared as brown bands on a pale background. The contours of the bands were marked and the plates were left in an oven at 50^{o} until no visible sign of iodine stain was left on the plates. The bands were scraped into a centrifuge tube and extracted with diethylether (2 x 10 ml). The ether extract was transferred directly into a counting vial and was evaporated to dryness under a stream of nitogen. The radioactivity was then assayed in toluene scintillant (5 g PPO/L toluene) (10 ml) using a Packard Tri-Carb liquid scintillation spectrometer.

d. Estimation of fatty acid and glycerol

The total lipid was extracted as previously described. After evaporation of chloroform extract to dryness under a stream of nitrogen, lipid was dissolved in a small volume of dry diethylether and was spotted on to the activated silica gel TLC plates (see Section VI, 5C above). The lipids were located with iodine vapour; bleached at 50° until no visible trace of iodine is left and the areas corresponding to triacylglycerol (R_f ∿ 0.5) were scraped into centrifuge tubes and extracted with diethylether (2 x 10 ml). The ether extract was decanted and evaporated to dryness under a stream of nitrogen and radioactivity in the triacylglycerol fraction was assayed in toluene scintillant (5g PPO/L toluene) (10ml) using a Packard Tri-Carb liquid scintillation spectrometer. The determination of label in the fatty acid and glycerol moieties was carried out following saponification with alcoholic potassium hydroxide in the manner described by Rodbell (1964). Two ml of ethanolic KOH (1 ml of saturated aqueous KOH per 100 ml of 95% ethanol) was added to the lipid extract and the mixture heated for 1h in a water bath at 70°. The test tubes then cooled to room temperature by placing the tubes in cold water. Deionised water (2ml) was added together with 2 drops of methyl orange (BDH, 0.1g/250 ml distilled water) and the mixture was acidified with 5NHCl (70 µl) until a colour change from red to orange (pH 3.1 - 4.1) was observed. The fatty acids were then extracted with two portions (3 ml) each) of hexane (60 - 80°). The two phases were separated by filtering through phase separating paper (Whatman 1PS) to remove the aqueous phase. The hexane extract was transferred to a scintillation vial and the solvent was evaporated to dryness under a stream of nitrogen. The radioactivity corresponding to fatty acid was counted in toluene scintillant (5g PPO/L toluene) (10 ml). The difference in radioactivity was assumed to represent the radioactivity present in glycerol moiety and is referred to as glyceride-glycerol (Rodbell, 1964).

6. Measurement of [^{14}C] lactate and [^{14}C] glucose synthesis from D - [U-^{14}C] fructose

a. Incubation of hepatocytes

The hepatocytes suspension (total vol 1 ml) was incubated with D - [U-^{14}C] fructose (9 mM or 1 mM; 9 x 10^6 dpm/assay) as described in

Section VI, 5a above. The reaction was stopped by the addition of 20% perchloric acid (0.5 ml). After neutralization with 5M K_2CO_3 (125 µl) the precipitate was removed by centrifugation at 2000 x g for 10 min.

b. Ion-exchange fractionation of labelled products

Labelled glucose and labelled lactate were separated from labelled fructose by the method of Hue et al., (1978). A 1 ml portion of the deproteinized extract was passed through a column (0.5 cm x 4 cm) of Dowex AG1 (X8; Cl^- form) (Sigma) to remove free sugars from anions (mainly lactate). The hexose fraction was eluted with deionised water (3 ml), [^{14}C] lactate was eluted with 1M NaCl (3 ml). A portion (100 µl) of the hexose fraction was dried on paper disc (Whatman No. 1 2.4 cm) and counted in toluene scintillant (5g PPO/L toluene) (10 ml). The remainder of this fraction was incubated with glucose oxidase (1 mg/1 ml) (Sigma) in the presence of 25 mM Tris/HCl buffer pH 8.0 for 3h at 37° with gentle shaking and then passed again through a column (0.5 cm x 4 cm) of Dowex (Cl^- form). The [^{14}C] fructose was washed out with deionised water (8 ml) and the [^{14}C] gluconate formed from glucose was retained on the column and could be eluted with 1M NaCl (3 ml). A sample of each fraction (100 µl) was dried on to paper disc (Whatman 2.4 cm) and radioactivity was assayed in toluene/ PPO (5 g PPO/1 toluene) (10 ml) and counted using a Packard Tri-Carb liquid scintillation spectrometer.

c. Separation of [^{14}C] fructose 1 - phosphate from [^{14}C] lactate

Fructose 1 - phosphate (FIP) was eluted from a column (0.5 cm x 4 cm) of Dowex AG1 (X8; Cl^- form) (Sigma) with 0.01M HCl, followed by 1M HCl (Rintoul, 1976), but very little count was eluted. Therefore, samples of [^{14}C] lactate (100 µl) were applied to cellulose TLC plates (TLC ready plastic sheets without binder Schleicher and Schüll) together with standard 0.2M fructose 1-phosphate (Sigma) (3 µl) and standard [^{14}C] lactate. The TLC plates were placed in a developing tank containing the solvent system 95% ethanol : ammonium acetate (1M, pH 3.8) (7.5 : 3.0 v/v). After the first run, plates were dried and developed again in the same solvent system. The section of the plate containing the standard FIP was dipped in PABA reagent (10% paraaminobenzoic acid in methanol), dried and placed in an oven at 110° for 5 min. The fructose 1-phosphate spot was visualised under fluorescent light. The [^{14}C] lactate standard was located by scraping off 0.5 cm bands from the origin to the solvent front. Similarly the areas corresponding to the samples were scraped into vials and the radioactivity

was assayed using either Instagel : water (10 : 3.5 v/v) (Packard) or PCS^{TM} : H_2O (6 : 4 v/v) (Amersham, Bucks). A quench correction was performed on cell samples by adding an internal standard, [^{14}C] hexadecane. The percentage recovery of the standard [^{14}C] lactate preparation by this method was 92%.

7. Preparation of oleic acid (sodium salt) - albumin complex

The oleic acid - albumin complex was prepared as described in the literature (Heimberg et al., 1969; Van Harken et al., 1969). Sodium oleate (Sigma) was dissolved in a minimum amount of KRB (with Ca^{2+}) containing 1.5% bovine serum albumin (fatty acid free, fraction V, Sigma), by heating to 40 - 50°. Then ice-cold KRB (with Ca^{2+}) containing bovine serum albumin (1.5%) was added to the required volume. In some experiments sodium [^{14}C] oleate (220,000 dpm/assay) was added to the preparation.

8. Triacylglycerol synthesis from [1-^{14}C] oleate

Hepatocytes were prepared as described in Section VI, 2.B and pre-incubated in a shaking water bath at 37° for 1h. The cell suspension (8×10^6 cells/ml; 0.5 ml) was added to 0.5 ml of [1-^{14}C] oleate (220,000 dpm) dissolved in KRB (with Ca^{2+} and 1.5% BSA) in presence or in the absence of hexoses and incubated in shaking water bath (200 cycles/min) at 37° for appropriate times. The reactions were then stopped with ice-cold 6% perchloric acid (w/v; 1 ml). The total lipid was extracted using the method of Folch et al., (1957) and various classes of lipid were fractionated by TLC as described in Section VI, 5.C. The areas corresponding to triacylglycerol and unesterified fatty acid were scraped into scintillation vials and the radioactivity measured in Instagel : water (10 : 3.5 v/v). The mixture was shaken vigorously and counted as a gel using a Packard Tri-Carb liquid scintillation spectrometer.

9. Lipogenesis

a. Lipogenesis from L - [U-^{14}C] lactate

Hepatocytes from fed rats were prepared as described in Section VI. B.2. and preincubated in a shaking water bath at 37° for 1h. The cell suspension (8×10^6 cells/ml; 0.5 ml) was added to 0.5 ml of 6mM

L - [U-^{14}C] lactate (1.1×10^6 dpm) dissolved in KRB (with Ca^{2+} + 1.5% BSA) in presence of either fructose or glucose (0-20 mM). The cells were incubated in a shaking water bath (200 cycles/min) at 37° for appropriate times. The reaction was stopped with perchloric acid (6% w/v; 1 ml). The lipids were extracted by method of Folch et al., (1957) and the total lipid was saponified with ethanolic KOH as described in Section VI. 5.d. Fatty acids were extracted with hexane (60 - 80°) (2 x 3 ml) and evaporated to dryness under a stream of nitrogen. The radioactivity was measured in 10 ml toluene/PPO (5g PPO/L toluene) using a Packard Tri-Carb liquid scintillation spectrometer.

b. Lipogenesis from [1-^{14}C] acetate

The method is as described above for L - [U-^{14}C] lactate except that the lactate in the hepatocyte incubation mixture was replaced by a final concentration of 5mM sodium [1-^{14}C] acetate (880,000 dpm/assay).

10. Hormone studies with isolated hepatocytes

In certain experiments (see Fig. 26, Table VI, VII, IX), the effects of insulin (Sigma) and glucagon (SERVA, Feinbiochemica, Heidelberg) on lipid synthesis were examined. In these experiments stock solutions of 1mM insulin and 1mM glucagon were made in 0.9% NaCl under slightly acidic (pH 4.0) and slightly alkaline (pH 9.0) conditions, respectively. The appropriate volume of solutions were added to KRB (with Ca^{2+} + BSA) containing the required substrate and the mixture was preincubated for a short time before the addition of hepatocyte suspension.

11. Acetyl CoA carboxylase

a. Enzyme preparation

Isolated liver cells are prepared as described in Section VI, B.2. Cells were counted in KRB (with Ca^{2+} + 1.5% BSA) and were then centrifuged at 50 g for 50 sec. The supernatant was aspirated off and pellet was dispersed in ice-cold homogenising buffer (1 : 3 w/v) containing trisodium citrate (20mM), Tris-HCl buffer pH 7.5 (0.05M), EDTA (0.5mM) and 2-mercaptoethanol (5mM) and homogenised using a loose-fitting (clearance 0.4mm) teflon pestle. The homogenate was centrifuged at 1000 x g for 30 min and then the supernatant was recentrifuged at 100,000 x g in an

MSE superspeed 65 centrifuge for 1h. The supernatant was carefully separated to avoid any contamination with either the floating lipid particles or the pellet and used as the source of enzyme. All enzyme preparation steps were carried out at 0 - 4°.

b. Enzyme assay

The radioisotopic method of Inoue and Lowenstein (1975) was followed. This is based on the incorporation of [^{14}C] bicarbonate into the carboxyl group of malonyl CoA (Alberts and Vagelos, 1972). The reaction involves the conversion of an acid-volatile compound into an acid-stable compound. Addition of acid (HCl) stops the reaction and the unreacted [^{14}C] bicarbonate escapes as $^{14}CO_2$ during the drying of the mixture under a stream of nitrogen. Citrate and Mg^{2+} (Inoue and Lowenstein, 1975) were used to activate the enzyme in some experiments but in most cases this preincubation step was omitted as recommended by Witters et al., (1979a).

The reaction was started by addition of 0.1 ml of crude enzyme to the incubation mixture containing 100 mM Tris-HCl buffer pH 7.5, 1 mM DTT, 0.2 mM acetyl CoA, 20 mM $NaH^{14}CO_3$ (0.25 µci/ µmole), 5 mM ATP, the specified concentration of sodium citrate, 20 mM $MgCl_2$ and 0.5 mg/ml BSA final concentration and reaction was carried out in a shaking water bath at 37° for up to 3 min. The final volume of assay mixture was 0.4 ml and the reaction was stopped by the addition of 0.1 ml of 4M HCl. The mixture was dried under a stream of nitrogen in a fume cupboard to remove volatile $^{14}CO_2$ and the residue was dissolved in 1.0 ml of deionized water and then 9 ml of the scintillant added. The scintillant consisted of a mixture of PPO in toluene (5g/litre) and Triton X-100 (1:3 v/v). The radioactivity was assayed in a Packard liquid scintillation counter with the appropriate correction for chemical quenching. The blank consisted of the complete reaction mixture except that acetyl CoA was omitted.

12. Statistical analysis

The treatment of statistics were obtained from Zivin and Bartko (1976). Standard deviation was calculated from the formula:

$$SD = \sqrt{\frac{\Sigma (x^2) - [(\Sigma x)^2 / N]}{N - 1}}$$

where Σ = "sum of"

$\bar{x}$ = mean of arithmetic average $\frac{\Sigma x}{N}$

X = observed values

N = number of observations

SD = standard deviation

df = degrees of freedom (df = n - 2)

t-test for comparison of results used the formula

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{(SD_1)^2}{N_1} + \frac{(SD_2)^2}{N_2}}}$$

$$S.E.M. = \frac{S.D.}{\sqrt{N}}$$

where SEM is the standard error of mean

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