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ASPECTS OF THE HORMONAL REGULATION OF
HEPATIC CARBOHYDRATE AND LIPID METABOLISM

A thesis presented for the degree of

Doctor of Philosophy

by

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ABSTRACT

FACULTY OF MEDICINE

CHILD HEALTH

Doctor of Philosophy

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The liver is a major site in the rat for conversion of dietary carbo-
hydrate into glycogen and triglyceride. Hepatic rates of fatty acid and
glycogen synthesis were measured in vivo in response to meal-feeding
(2h/day) by the incorporation of $^3$H from $^3$H$_2$O. This technique has not
been applied previously to glycogen synthesis and was validated in control
and streptozotocin diabetic rats. Hepatic glycogen recycling was low in
fed adult rats but was apparently greater in foetal rats. The precursor
source for glycogen synthesis in vivo could not be determined from the
distribution pattern of $^3$H incorporation. Hepatic glycogen synthesis was
elevated in control rats for 5h after feeding. During this phase,
glycogen could not have been a net precursor for other synthetic pathways.

Hepatic fatty acid synthesis in control rats increased 20-fold 2h after
feeding. This response was impaired and delayed, but not abolished, by
streptozotocin diabetes (55mg/kg). Insulin pretreatment (3U P.Z.I.)
restored the low diabetic rate of lipogenesis to normal by 8h after
feeding. Streptozotocin reduced the hepatic Vmax activities of gluco-
kinase, ATP-citrate lyase and total acetyl CoA carboxylase. None of these
enzyme activities increased when hepatic fatty acid synthesis was stimu-
lated by feeding in control rats or by feeding and insulin in diabetic
rats. Feeding stimulated active acetyl CoA carboxylase in control, but
not diabetic, rats. The regulation of hepatic fatty acid synthesis by
both acetyl CoA carboxylase and increased substrate concentration is
discussed.

In control rats for the first 5h after feeding, hepatic glycogen could
not have been a net fatty acid precursor. Thus the inhibition of hepatic
fatty acid synthesis in this period by glucagon (1mg/kg) could not have
been directly due to depletion of glycogen. The glucagon inhibition of
lipogenesis was abolished by adrenalectomy but not potentiated by
corticotropin-treatment, suggesting a permissive role for glucocorticoid
hormones. Adrenalectomy also impaired the inhibition of hepatic pyruvate
kinase by glucagon but did not abolish the inactivation of pyruvate
kinase by 10 μM-cyclic AMP in vitro. The involvement of L-type pyruvate
kinase in the regulation of hepatic fatty acid synthesis is discussed.
The integrated regulation of the hepatic pathways of lipogenesis,
glycolysis, gluconeogenesis and ketogenesis is considered.

To Karen,

with love and thanks.

## ACKNOWLEDGEMENTS

Some of the material presented in this thesis is contained in the following publications :-

1. Johnston, D.G., Postle, A.D., Barnes, A.J. & Alberti, K.G.M.M. (1979) "The role of cortisol in the direction of substrate flow" in "Lipoprotein Metabolism and Endocrine Regulation" (ed. Hessel, L.W. & Krans, H.M.J.) p117, Elsevier, Amsterdam.

2. Postle, A.D. (1978) "The role of acetyl coenzymeA carboxylase in the response of hepatic lipogenesis to feeding in normal and streptozotocin diabetic rats" Diabetologia 15,262 (abstract).

3. Postle, A.D. & Bloxham,D.P. (1980) "The use of tritiated water to measure absolute rates of hepatic glycogen synthesis" Biochem.J. 190, in press.

4. Postle, A.D. & Bloxham, D.P. (1980) "The permissive action of glucocorticoid hormones on the inhibition of hepatic lipogenesis by glucagon in the rat" Biochem.Soc.Trans. 8,383.

5. Postle, A.D. & Bloxham, D.P. (1980) "Hepatic glycogen synthesis in tritiated water" Diabetologia, in press (abstract)

6. Postle, A.D. & Goodland, F.C. (1978) "A comparison of three automated methods of serum triglyceride analysis" Anal.Clin.Biochem. 15,18.

# CONTENTS

# ABBREVIATIONS

The following abbreviations were used in the schemes for metabolic pathways:-

| | |
|---|---|
| ADH | Alcohol dehydrogenase |
| ALD | Fructose-1,6-bisphosphate aldolase |
| FDPase | Fructose-6-phosphatase |
| FUM | Fumarase |
| GAK | Glycerol kinase |
| GK | Glucokinase |
| GPH | $\alpha$-Glycerophosphate dehydrogenase |
| G3PDH | Glyceraldehyde-3 Phosphate dehydrogenase |
| G6PDH | Glucose-6-phosphate dehydrogenae |
| G5 | Glycogen synthase |
| HK | Hexokinase |
| LDH | Lactate dehydrogenase |
| MDH | Malate dehydrogenase |
| ME | "Malic" enzyme |
| PCX | Pyruvate carboxylase |
| PEP | Phosphoenol pyruvate |
| PEPCK | Phosphoenol pyruvate carboxy kinase |
| PFK | Phosphofructokinase |
| PGI | Phosphoglucose isomerase |
| PGM | Phosphoglucomutase |
| PK | Pyruvate kinase |
| T | Tritium |
| TA | Transaldolase |
| TPI | Triose phosphate isomerase |
| $T_2O$ | Tritiated Water |
| UDPGT | UDP-glucosyl transferase |

Other abbreviations used are:-

| | |
|---|---|
| 3HMG | 3-hydroxy-3-methyl glutaryl |
| P.Z.I. | Protamine zinc insulin |
| $t_{\frac{1}{2}}$ | Half-life |

CHAPTER 1

INTRODUCTION

1.1.

Mammalian metabolism is well adapted to cope with intermittent feeding patterns where periods of high food consumption are often separated by prolonged intervals of fasting. Caloric intake during feeding in excess of immediate requirements is stored as the fuel reserves glycogen and triglyceride and then released slowly to maintain respiration during the subsequent fast. The liver is a major site for the regulation and integration of both anabolic and catabolic aspects of carbohydrate and lipid metabolism in the rat.

One significant restraint upon metabolic regulation is the need to maintain blood glucose concentration within a narrow range to support brain respiration. In the fed animal this glucose is provided directly from the diet but as fasting progresses the production of glucose by the liver becomes increasingly more important. Liver glucose release is due both to glycogenolysis and to gluconeogenesis from lactate and amino acids. In response to feeding, glycogen breakdown in liver is inhibited and glycogen synthesis initiated. The rate of gluconeogenesis is also decreased. For a comprehensive review of the regulation of blood glucose see Stalmans (1976).

Triglyceride is a more efficient energy reserve than glycogen. The respiratory quotient for fat oxidation is lower than for carbohydrate oxidation and triglyceride is stored in a compact anhydrous form not requiring water of crystallization. For these reasons, by far the greatest amount of energy stored in the body is deposited as triglyceride. It has been estimated that in fasted man the fat reserves are sufficient to maintain body functions for up to 2 months, while glycogen reserves are depleted after 24 to 48 hours (Owen and Reichard, 1971). The normal laboratory diet of the rat is low in fat and high in polysaccharide. Excess ingested carbohydrate is converted to fatty acid largely in the liver and exported to adipose tissue for storage as triglyceride.

In the fasted rat, adipose tissue triglyceride reserves are mobilized preferentially to carbohydrate reserves. Stimulated lipolysis releases fatty acids into the blood which can either be oxidized directly by muscle or modified by the liver. In contrast to other tissues, in the liver the major end product of fatty acid oxidation is not carbon dioxide but the ketone bodies, 3-hydroxybutyrate, acetoacetate and acetone. Under certain conditions, for instance in the neonatal period or during prolonged starvation, ketone bodies can partially support brain metabolism and thus reduce the demand upon the limited carbohydrate reserves. This mechanism has evolved because animals lacking an active glyoxylate cycle, such as mammals, cannot synthesize glucose from fatty acid.

As outlined above, in the fed rat hepatic metabolism is directed towards the conversion of dietary carbohydrate to glycogen and tri-glyceride. In the fasted rat these processes are reversed and the major products of hepatic metabolism are glucose and ketone bodies.

All these pathways in the liver are regulated in a co-ordinated manner and respond rapidly to changes in the environmental conditions. Such changes are signalled to the liver by variations in the circulating concentrations of hormones and metabolites. The anatomical position of the liver is ideally situated to be sensitive to such variations. The splanchnic bed is drained by the hepatic portal vein and the liver is exposed to a greater range of metabolite concentrations than other tissues. The pancreatic hormones insulin and glucagon are secreted into the portal circulation and about 50% of each hormone is degraded after a single passage through the liver (Sherwin et al, 1974). Thus the concentrations of these hormones acting on the liver are higher than those acting on peripheral organs. This position of the liver is made even more signifi-cant by the increased blood flow in the hepatic portal vein after feeding (Grim, 1963).

Insulin is the major anabolic hormone, characteristic of the fed state. Of the counter-regulatory hormones glucagon, adrenaline, vasopressin, corticosterone and growth hormone, glucagon is considered to cause the greatest effects on hepatic metabolism. In this context

it is thought that the absolute concentrations of insulin and glucagon
are less important than the relative molar concentrations of the hormones
(Unger, 1972). The insulin : glucagon ratio is elevated in the fed
state and decreased on fasting. Hormones such as cortisol and growth
hormone have more prolonged but less acute hepatic actions than glucagon.
One mechanism by which they act is to modify the responses of the liver
to insulin and glucagon (Section 1.4.4).

An important aspect of the study of the regulation of hepatic
metabolism is to elicit the mechanisms whereby changes of hormone con-
centrations cause their ultimate physiological responses. To construct
an overall view of hormone action requires a detailed knowledge of the
in vitro properties of the enzymology of the various metabolic pathways.
The majority of enzymes are near-equilibrium in vivo and are regulated
primarily by the relative concentrations of substrate and product. The
number of enzymes which are non-equilibrium in vivo and are regulated
by effectors other than substrate or product is relatively few. For a
general discussion of the distinctions between non-equilibrium and near-
equilibrium reactions see Newsholme and Start (1973). The discussion
below will concentrate mainly on regulation of enzyme activity by covalent
mechanisms and by changes in enzyme concentration. The nature of the
allosteric transition has been described by two models (Monod et al,
1965: Koshland et al, 1966).

## 1.2. Regulation of Enzyme Activity

### 1.2.1. Enzyme Concentration

Altering the concentration of an enzyme is one obvious mechanism
for regulation of enzyme activity. Induction and repression of enzyme
protein synthesis is the major mechanism whereby bacteria adapt to
changes in their external environment (Atkinson, 1970). In the rat this
mechanism, although important, is less universally applicable. Changes
in concentration of rat liver enzymes tend to relate to longer term
regulation of metabolic pathways and not to rapid responses to immediate
stimuli. For instance, the rate of hepatic fatty and biosynthesis in
the rat is elevated by feeding a low fat diet and depressed by diabetes,
fasting, or feeding a high fat diet. The associated and co-ordinated

changes in the hepatic concentrations of acetyl CoA carboxylase, fatty acid synthase, ATP:citrate lyase, NADP:malate dehydrogenase, glucokinase, glucose 6-phosphate dehydrogenase and pyruvate kinase are all due to changes in enzyme concentration (Numa and Yamashita, 1974). The half lives ($t\frac{1}{2}$) of these enzymes (section 4.1. , Table 16 ) are too long for enzyme induction and repression to cope with the demand for rapid changes of pathway flux.

Such a mechanism can operate where the $t\frac{1}{2}$ of an enzyme is unusually short. A good example is the regulation of hepatic cholesterol synthesis by 3hydroxy-3methyl glutaryl CoA reductase which has a $t\frac{1}{2}$ of between 30 min and 3.5 hours (Higgins et al, 1974; Bell et al, 1976). The pronounced circadian variation of enzyme activity, however, was not solely due to changes in enzyme protein concentration (Higgins and Rudney, 1972; Edwards et al, 1980) and recently a phosphorylation : dephosphorylation cycle for regulation of catalytic activity has been described (Ingebritsen et al, 1978). The hepatic activity of pyruvate kinase also varies in a circadian fashion, although less pronounced than for 3-hydroxy 3-methyl glutaryl CoA reductase (Hopkirk and Bloxham, 1979). This is possible despite a $t\frac{1}{2}$ of 75 hours due to a proposed 'nick' of the enzyme molecule into inactive fragments (Kohl and Cottam, 1977; Hopkirk and Bloxham, 1979). The major regulation of pyruvate kinase activity, however, seems to be due to cyclic AMP dependent phosphorylation (Engström, 1978).

1.2.2. Reversible Enzyme Phosphorylation

Reversible phosphorylation regulates the activities of many enzymes (for review see Krebs and Beavo, 1979). Phosphorylation induces a covalent conformational change of enzyme structure. A phosphorylation : dephosphorylation cycle requires the opposing activities of a protein kinase and a phosphoprotein phosphatase:-

$$\text{ENZYME} + n \begin{pmatrix} \text{ATP} \\ \text{GTP} \end{pmatrix} \xrightleftharpoons[]{\text{PROTEIN KINASE}} \text{ENZYME} - \text{Pn} + n \begin{pmatrix} \text{ADP} \\ \text{GDP} \end{pmatrix}$$

$$\text{ENZYME} - \text{Pn} + n\text{H}_2\text{O} \xrightleftharpoons[]{\text{PHOSPHOPROTEIN PHOSPHATASE}} \text{ENZYME} + n \text{ Pi}$$
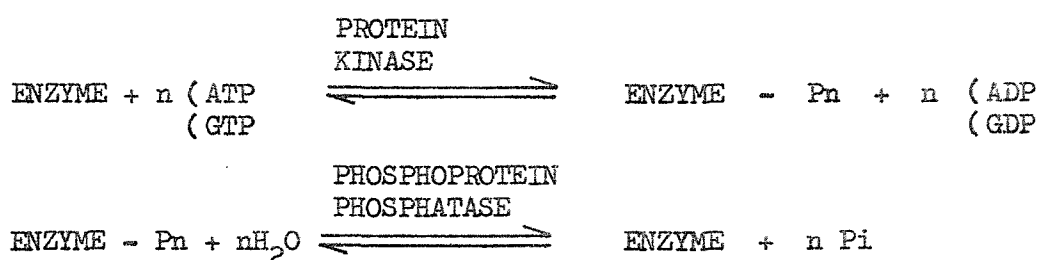
The phosphorylated residue is usually serine or threonine. Regulation of enzyme activity by this mechanism confers a number of significant advantages. Firstly, the phosphorylated and dephosphorylated enzyme forms possess different affinities for substrates, activators and inhibitors. This enables flux through the enzyme to be modified with little change in the concentrations of effector molecules in the tissue. Secondly, the covalent nature of the effect enables the response to be prolonged after removal of the stimulus. Thirdly, phosphorylation of more than one enzyme in a pathway can co-ordinate their activities. Fourthly, when part of a cascade system, such as cyclic AMP stimulation of glycogenolysis or lipolysis, phosphorylation provides a mechanism for amplifying a stimulus by up to several magnitudes.

## Cyclic AMP-dependent Protein Kinase

Binding of glucagon or $\alpha$-adrenergic agonists to plasma membrane receptors activates adenylate cyclase and increases the conversion of ATP to adenosine-3',5'monophosphate (cyclic AMP). Cyclic AMP binds to the regulatory subunit (R) of cyclic AMP dependent protein kinase and activates the catalytic apoenzyme (C) (Walsh et al, 1968):-

$$R_2 C_2 + 4 \text{ cyclic AMP} \rightleftharpoons R_2 (cAMP)_4 + 2C$$

(Inactive
holoenzyme)                                         (Active
                                                       apoenzyme)
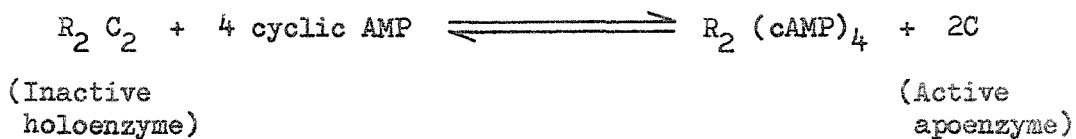
There are two cyclic AMP binding sites on each regulatory subunit (Corbin and Lincoln, 1977; Das and Fox, 1978) but possibly only one site need be occupied for full dissociation of subunits. There are two forms of cyclic AMP dependent protein kinase, which differ in the structure of their regulatory subunits (Corbin et al, 1975). Their substrate specificities are similar (Table 1 )

Table 1

Enzyme Substrates for Cyclic AMP Dependent Protein Kinase

ENZYME

Phosphorylase b kinase

Glycogen synthase

L-Pyruvate kinase

Acetyl CoA carboxylase

Hormone-sensitive lipase

Cholesterol ester hydrolase

3HMGCoA reductase

Glycerophosphate acyltransferase

(Ref: Krebs and Beavo, 1979)


A number of heat-stable protein inhibitors of cyclic AMP dependent
protein kinase can be isolated from skeletal muscle (Demaille et al,
1977), rat testis (Dedman et al, 1977) and brain (Szmigielski et al, 1977).
These inhibitors are present at low concentrations and may oppose the
actions of basal concentrations of cyclic AMP (Beavo et al, 1970).

Cyclic GMP-dependent Protein Kinase

Binding of cyclic GMP to cyclic GMP dependent protein kinase
exposes the catalytic site but does not dissociate the enzyme into
separate regulatory and catalytic subunits (Gill et al, 1973). Cyclic
GMP dependent protein has a similar substrate specificity to that of the
cyclic AMP dependent enzyme (Lincoln and Corbin, 1977; Khoo et al, 1977).
The functional significance of cyclic GMP dependent protein kinase is
still largely obscure and will not be discussed further. This enzyme
was recently reviewed (Gill and McCune, 1979).

Calcium-dependent Protein Kinases

Many metabolic responses are dependent upon and stimulated by calcium.
In muscle, phosphorylase b kinase and myosin light chain kinase are both
activated by calcium released from sarcoplasmic reticulum in response to
nervous stimulation (Meyer et al, 1964; Pires et al, 1974). In rat
liver, glycogenolysis and gluconeogenesis are both stimulated by

α-adrenergic agonists by a calcium mediated mechanism (Chan et al, 1979; Kneer et al, 1979). Calcium sensitivity resides in the calcium regulatory protein, calmodulin (Cohen et al, 1978; Yazawa and Yagi, 1977). The δ subunit of phosphorylase kinase is identical to calmodulin and has 50% homology with troponin C, the calcium binding protein of muscle actin. Calmodulin has interactions with cyclic nucleotide dependent protein kinases activating, for instance, cyclic GMP specific phosphodiesterase (Wang and Waisman, 1979).

## Messenger-independent Protein Kinases

A number of enzymes are phosphorylated in response to changes in the concentrations of intracellular metabolites. This mechanism was first described for the inactivation of pyruvate dehydrogenase by ATP (Lin, 1969). Analagous kinase reactions have since been described in the regulation of glycogen synthase (Itarte et al, 1977), acetyl CoA carboxylase (Hardie and Cohen, 1979; Yeh et al, 1980) and 3-hydroxy 3-methylglutaryl CoA reductase (Ingebritsen et al, 1978).

## Phosphoprotein Phosphatases

There is less information known about phosphatase reactions compared with the corresponding kinases. A multifunction protein phosphatase-1 has been isolated from liver (Brandt et al, 1975; Khandelwhal et al, 1976) and muscle (Antinow et al, 1977; Lee et al, 1978). These preparations are active against the β subunit of phosphorylase b kinase, site 2 of glycogen synthase, phosphorylase a and L-pyruvate kinase. Protein phosphatase-1 is isolated from tissue in a latent less active form of molecular weight 250,000. The active species of 35,000 molecular weight is generated during purification by exposure to ethanol or heat. Other, specific, phosphatases in muscle include phosphatase-2 (molecular weight 170,000) which acts on the α subunit of phosphorylase b kinase and phosphatase-3 (molecular weight 300,000) which acts on phosphorylated histones (Antinow et al, 1977). Phosphatase-2 is stimulated by insulin (Foulkes et al, 1980). The mitochondrial phosphatase is specific for pyruvate dehydrogenase (Linn et al, 1969).

Hers and his colleagues have purified from the liver glycogen pellet

Fig. 1    Hormonal Regulation of Glycogen Metabolism

⊕ activation        ⊖ inhibition
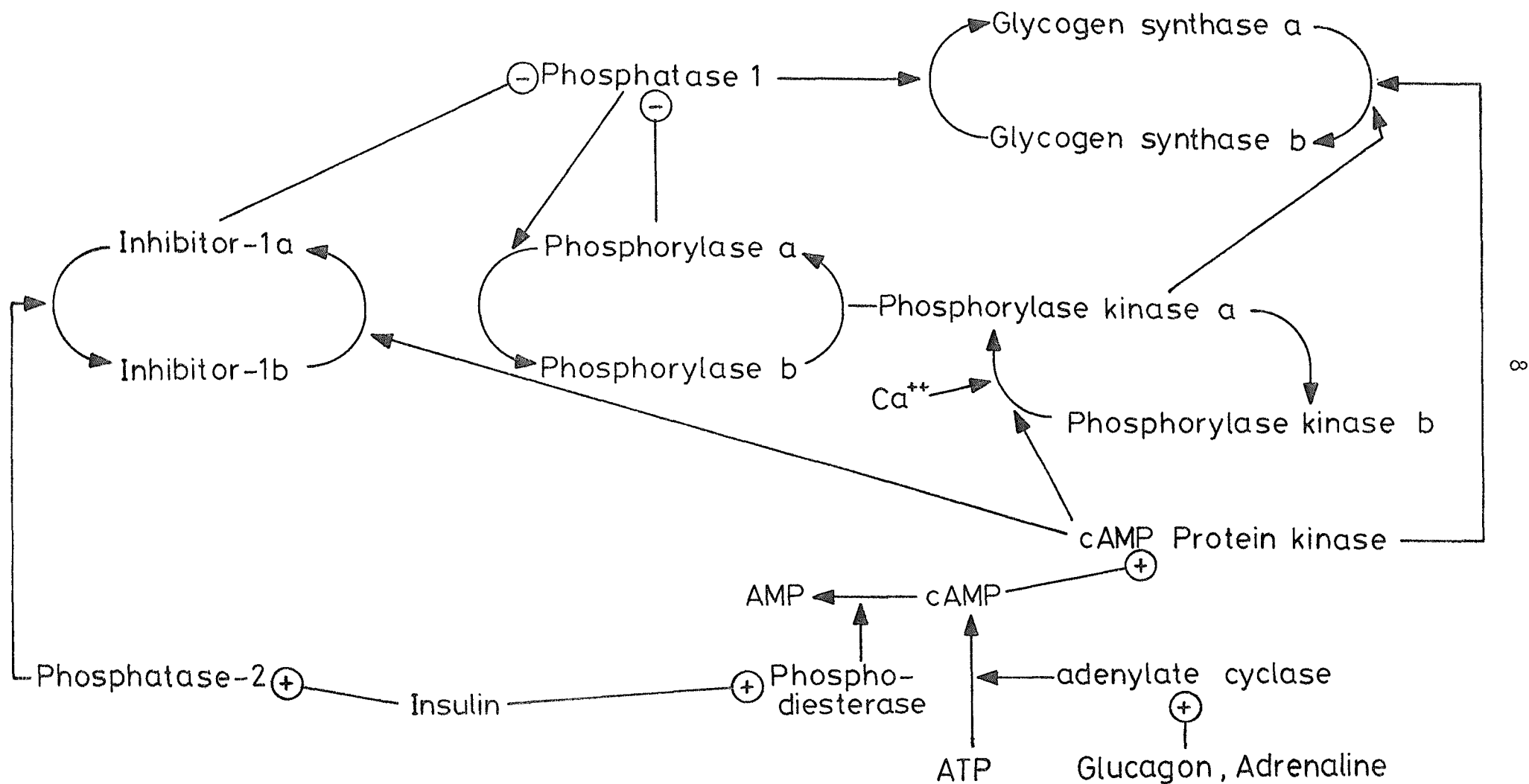
a "native" glycogen synthase b phosphatase that is separate from phosphory-
lase a phosphatase (Doperé et al, 1980). They question the relevance of
phosphatase-1 activity in liver because of the treatment with heat or
organic solvents required for full expression of activity. The opposing
opinion is that dissociation of an active apo-enzyme from a larger
holoenzyme might be one regulatory mechanism of phosphatase-1 activity
(Khandelwhal et al, 1976).

Muscle and liver contain two proteins that are heat-stable and
trypsin-labile and which inhibit phosphoprotein phosphatase-1 (Brandt
et al, 1974; Huang and Glinsmann, 1976). Inhibitor-1 is activated by
cyclic AMP dependent phosphorylation of a threonine residue (Cohen et al,
1977) and is inactivated by phosphatase-2 (Foulkes et al, 1980). The
proposed role for inhibitor-1 in the regulation of glycogen metabolism
is discussed below (Section 1.3.1, Fig. 1    ). Inhibitor-2 is at
lower concentration than inhibitor-1 in muscle and has an uncertain role
in the regulation of enzyme phosphorylation (Nimmo and Cohen, 1978).

## 1.3. Properties of Selected Liver Enzymes

### 1.3.1. Hepatic Glycogen Metabolism

This review will concentrate on the properties and regulation of
glycogen synthase and phosphorylase, the rate-limiting enzymes respectively
of glycogen synthesis and degradation (Fig. 1). Both glycogen synthase
and phosphorylase are subject to reversible phosphorylation; the phos-
phorylated form of glycogen synthase is inactive while that of phos-
phorylase is active.

Phosphorylase ( E.C.2.4.1.1 )

Rat liver phosphorylase has a molecular weight of 185,000 (Stalmans
and Hers, 1975). The dephosphorylated b form requires high AMP concentration
for activity to be measured and is considered to be inactive in vivo
(Stalmans, 1976). The active a form is generated by phosphorylation of
serine 14 by phosphorylase b kinase (Titani et al, 1975). Dephosphory-
lation of phosphorylase a has been described both by the latent multi-
functional phosphoprotein phosphatase-1 (Brandt et al, 1975) and by the
"native" phosphatase associated with the glycogen pellet (Goris et al, 1974).

Binding of glucose converts phosphorylase a to a better phosphatase substrate and this mechanism has been proposed to explain the inhibition of glycogenolysis by elevated blood glucose concentration (Hers, 1976; Fig 1.)

### Phosphorylase b Kinase (E.C.2.7.1.38)

Phosphorylase b kinase from muscle is a hexadecamer of 4 subunits with the probable subunit structure ( $\alpha \beta \gamma \delta$ )4 (Cohen, 1978). Isolated and purified $\gamma$ subunit is fully active (Skuster and Graves, 1977) while the other three subunits are regulatory. Phosphorylation of the $\beta$ subunit by cyclic AMP dependent protein kinase converts phosphorylase b kinase from the inactive b form to the active a form (Cohen, 1973). Phosphorylation of the $\alpha$ subunit inhibits dephosphorylation of the $\beta$ subunit by phosphatase-1 and effectively locks phosphorylase b kinase in the active form (Cohen, 1978). The calcium sensitivity of phosphorylase b kinase is conferred by the $\delta$ subunit which appears identical to calmodulin (Cohen et al, 1978). Nervous stimulation of muscle increases the cytosolic calcium concentration with no change in cyclic AMP concentration. Activation of phosphorylase b kinase by calcium is accompanied by multiple autophosphorylation mainly on the $\alpha$ subunit (Wang et al, 1976).

Phosphorylase b kinase has only been partially purified from liver, where it is present at a lower concentration than in muscle. A number of preparations are calcium sensitive but do not respond to the catalytic subunit of cyclic AMP-dependent protein kinase (Vandenheede et al, 1977; Sakai et al, 1979). Recently, phosphorylase b kinase sensitive to cyclic AMP dependent protein kinase has been prepared from the soluble fraction (Vandenheede et al, 1979) or the particulate glycogen fraction (Chrisman and Exton, 1980) of rat liver. Enzyme activation by cyclic AMP and ATP: $Mg^{2+}$ is accompanied by incorporation of $^{32}P$ from [ $\gamma$ $^{32}P$] ATP into two subunits that have identical mobility on SDS gel electrophoresis to $\alpha$ and $\beta$ subunits of phosphorylase b kinase purified from skeletal muscle. There is no available information about any $\gamma$ or $\delta$ subunits but the molecular weight of the complex, about $1.3 \times 10^6$, is similar to that of the muscle enzyme (Vandenheede et al, 1979). $\alpha$-adrenergic agonists activate hepatic phosphorylase b kinase by a calcium mediated mechanism analagous to nervous stimulation of phosphorylase b kinase (Blackmore et al, 1979).

## Glycogen Synthase (UDPG: α-1,4-Glucan α -4-Glucosyltransferase, EC 2.4.1.11)

Glycogen synthase is a tetramer of subunit molecular weight 77,000 to 84,000 (Lin and Segal, 1973; McVerry and Kim, 1974). The enzyme is a substrate for three protein kinases and up to six different phosphorylation sites per subunit have been described (Proud et al, 1977; Itarte et al, 1977; Salavert et al, 1979; Soderling et al, 1979). The kinases active on glycogen synthase are cyclic AMP dependent protein kinase, calcium dependent glycogen synthase kinase-2 and cyclic AMP independent glycogen synthase kinase-3. Glycogen synthase kinase-2 and phosphorylase b kinase in muscle are the same enzyme (Embi et al, 1979; Soderling et al, 1979). Phosphorylated glycogen synthase b is inactive in vivo. The activation of glycogen synthase b by 10 mM glucose-6-phosphate in vitro (Villar-Palasi and Larner, 1960) is probably not significant in vivo. Activation of hepatic glycogen synthase by a glucose load is accompanied by a 60% decreased hepatic concentration of glucose-6-phosphate (De Wulf and Hers, 1967).

Inactive glycogen synthase can be dephosphorylated by phosphatase-1 or by a phosphatase bound to particulate glycogen (Stalmans et al, 1974). Phosphorylase a, but not phosphorylase b, is an inhibitor of glycogen synthase phosphatase in liver. Glycogen synthase is not activated in response to feeding until phosphorylase a has been inactivated below a threshold value. Dephosphorylation of phosphorylase a is potentiated by feeding induced hyperglycaemia. Enzyme bound glucose renders phosphorylase a a better substrate for phosphatase-1 and would thus stimulate activation of glycogen synthase (for reviews see Hers, 1976; Stalmans, 1976). An alternative mechanism to co-ordinate activation of glycogen synthase with inactivation of phosphorylase is the phosphorylation state of inhibitor-1. Activation of phosphatase-2 by insulin (Foulkes et al, 1980) would dephosphorylate inhibitor-1 and activate phosphatase-1. The resulting dephosphorylations would activate glycogen synthase and inactivate phosphorylase.

### 1.3.2. Hepatic Gluconeogenesis and Glycolysis

Rat liver possesses the complete enzyme complement required for

Fig 2    Substrate Cycles in Hepatic Carbohydrate Metabolism

GK

Glucose → glucose-6- $\xrightarrow{\text{PGM}}$ glucose-1-
phosphate ⇌ phosphate

G6Pase

PGI

fructose-6-
phosphate                    UDP-glucose

FDPase / PFK                  GS

fructose-1,6-                 glycogen
bisphosphate

ALD

glyceraldehyde-3-        dihydroxy-
phosphate ⇌ acetone
TPI           phosphate

phosphoenol
pyruvate

PK                          PEPCK

LDH
Lactate ⇌ pyruvate                    oxaloacetate

cytosol

mitochondria

AAT                    PCX
Alanine ⇌ pyruvate ————→ oxaloacetate
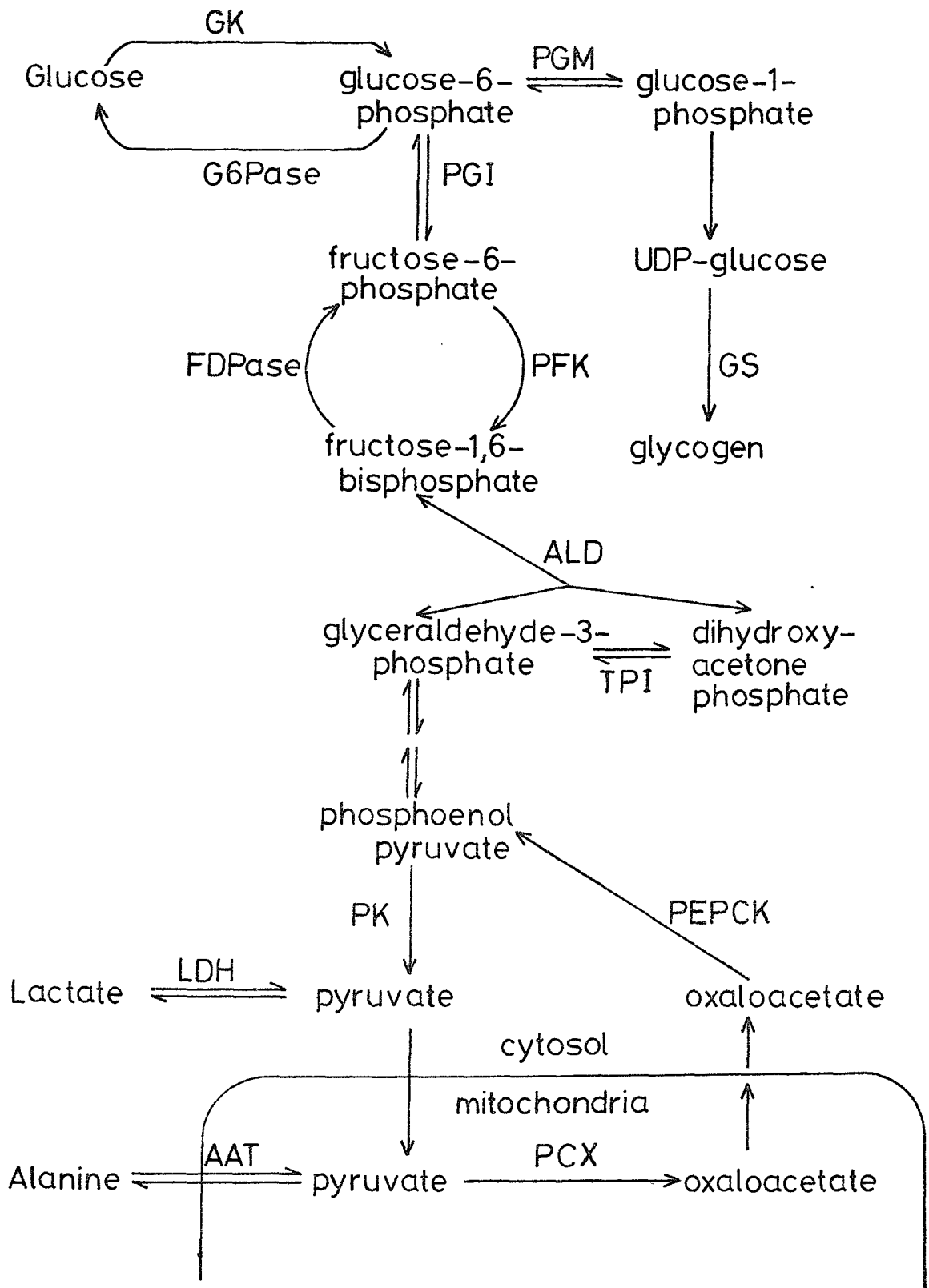
The substrate cycles are catalysed by:-

1) glucokinase (GK) and glucose-6-phosphatase (G6 Pase)
2) phosphofructokinase (PFK) and fructose-1,6-bisphosphatase (FD Pase)
3) L-pyruvate kinase (PK), pyruvate carboxylase (PCX) and
   phosphoenol pyruvate carboxykinase (PEPCK)

both gluconeogenesis and glycolysis. Most of these enzymes are near-equilibrium in vivo and are common to both directions of pathway flux. Three irreversible reactions are catalysed by different enzymes in the gluconeogenic and glycolytic directions (Fig. 2). These reactions are the inter-conversions between glucose and glucose-6-phosphate, between fructose-6-phosphate and fructose-1,6-biphosphate and between phosphoenol pyruvate and pyruvate (for review see Katz and Rognstad, 1976). Substrate cycling catalyzed by opposing enzymes of comparable activity has been proposed as a sensitive mechanism for regulation of metabolism (Newsholme and Underwood, 1966; Newsholme and Crabtree, 1977). Enzyme activity of 100 arbitrary units in the forward reactions and 90 units in the reverse reaction will result in a net activity of 10 units. If the forward reaction is stimulated and the reverse reaction inhibited by 10% each the net resulting flux would increase to 29 units, an increase of 190%. Such magnification of small changes in enzyme activity enables the overall cycle flux to be rapidly and accurately regulated, at the expense, however, of ATP hydrolysis.

The liver is primarily a gluconeogenic organ and [$^{14}$C] lactate is incorporated into glucose in fed rats in vivo or in perfused liver preparations (Clark et al, 1974; Schimassek et al, 1974). These results suggest that the Cori cycle is active in fed as well as in fasted rats. Rats feed nocturnally during their active phase. Peripheral lactate production by increased muscular activity and intestinal metabolism is greatest under these conditions. It is in the fed state that the liver is presented with the greatest lactate accumulation to be metabolized. Continued hepatic gluconeogesis from lactate in fed rats thus makes good physiological sense. The stimulated gluconeogenesis of fasting is fueled largely by circulating amino acids derived from protein breakdown (Owen et al, 1969). An important corrollary of this hypothesis is that hepatic uptake of glucose is negligible in fed rats. This view is supported by arterial : venous and portal vein : hepatic vein concentration differences across the liver (Rémésey et al, 1978). Net uptake of glucose by liver is only detectable when rats are fed a diet high in soluble carbohydrate. The absence of hepatic glucose uptake could explain why blood glucose is a poor precursor for the hepatic synthesis of both fatty acid (Salmon et al, 1973; Clark et al, 1974) and glycogen (Katz et al, 1979).

## Glucose : Glucose-6-Phosphate Substrate Cycle

This substrate cycle is catalyzed by glucokinase (E.C.2.7.1.2) and glucose-6-phosphatase (E.C.3.1.3.9) (For enzyme reviews see Weinhouse, 1976; Nordlie, 1974). Both enzymes are non-equilibrium $\underline{\text{in vivo}}$ and are regulated by substrate concentration. The high $K_m$ of glucokinase for glucose (10 mM) enables the rate of glucose phosphorylation to increase with portal glucose concentration within the physiological range. Activity of this substrate cycle provides a molecular basis for the autoregulation of hepatic glucose metabolism by glucose concentration according to the Soskin concept (Section 3.1.1) (Newsholme and Gevers, 1967). There is net hepatic glucose uptake when rats are given a large glucose load (Stalmans, 1976) but in more physiological conditions this substrate cycling probably serves to limit glucose output by the liver. Limitation of hepatic glucose production in fed rats will tend to divert hexose monophosphates derived from gluconeogenesis towards glycogen synthesis. The extent of substrate cycling has been estimated $\underline{\text{in vivo}}$ following injection of $[2\text{-}^{3}\text{H},\text{U}^{14}\text{C}]$ glucose. Comparison of decay curves of $^{3}\text{H}$ and $^{14}\text{C}$ in sequential blood samples provides a measure of the rate of recycling between blood glucose and the hexose monophosphate pool in the liver (for review see Katz and Rognstad, 1976). This rate is increased after feeding and by glucocorticoid treatment in dogs (Issekutz, 1977).

## Fructose-6-Phosphate : Fructose-1,6-Bisphosphate Substrate Cycle

This substrate cycle is catalyzed by phosphofructokinase (ATP:D-fructose-6-P-1 phosphotransferase E.C.2.7.1.11) and fructose-1,6-bisphosphatase (E.C.3.1.3.11). The properties of these enzymes have been extensively reviewed (Newsholme and Gevers, 1967; Bloxham and Lardy, 1973; Goldhammer and Paradies, 1979). Briefly, phosphofructokinase is inhibited by ATP, citrate and phosphorylation and activated by fructose-6-phosphate, fructose-1,6-bisphosphate, AMP and dephosphorylation. Fructose-1,6-bisphosphatase is inhibited by AMP and activated by phosphorylation.

Limitation of gluconeogenesis requires phosphofructokinase to be activated. Stimulation of phosphofructokinase by decreased hepatic

concentrations of ATP and citrate is not a likely mechanism in vivo as
the fed state is characterized by increased concentrations of these
metabolites (Greenbaum et al, 1970; Bücher et al, 1964; Start and
Newsholme, 1968). Reversible enzyme phosphorylation provides an attrac-
tive and plausible mechanism for the reciprocal regulation of the
activities of phosphofructokinase and fructose-1,6-bisphosphatase.
Phosphofructokinase is inactivated by cyclic AMP independent phos-
phorylation (Soling et al, 1977; Nieto and Castaño, 1980) while fructose-
1,6-bisphosphatase can be activated by cyclic AMP dependent phosphory-
lation (Riou et al, 1977; Pilkis et al, 1980). However, the physio-
logical significance of phosphofructokinase phosphorylation is not fully
established as the responsible kinase and phosphatase are not characterized.

The rate of this substrate cycle has been measured by comparison of
the rates of detritiation of $[2-^3H]$ glucose and $[5-^3H]$ glucose.
(Bloxham et al, 1973 a,b). Results from this method, when corrected for
transaldolase activity (Hue and Hers, 1974; Section 3.1.3.5),suggest that
substrate cycling is low in fasted rats when gluconeogenesis is stimulated
but becomes more appreciable after feeding (Clark et al, 1974; Katz and
Rognstad, 1976).

## Phosphoenolpyruvate : Pyruvate Substrate Cycle

The hepatic interconversion of phosphoenolpyruvate and pyruvate is
more complex than the substrate cycles discussed above. L-pyruvate kinase
(ATP : pyruvate phosphotransferase, EC 2.7.1.40) catalyses the formation
of pyruvate in the cytosol from phosphoenolpyruvate (for review see
Engstrom, 1978). The reverse reaction requires transport of pyruvate into
the mitochondria, conversion to oxaloacetate by pyruvate carboxylase
(pyruvate : carbon dioxide ligase (ADP), EC 6.4.1.1; Barritt et al, 1976),
export of oxaloacetate from the mitochondria as malate or aspartate and
finally decarboxylation by phosphoenolpyruvate carboxykinase (GTP :
oxaloacetate carboxylase (transphosphorylating), EC 4.1.1.32; Utter
and Kolenbrander, 1972; Longshaw et al, 1972). The Vmax activity of
L-pyruvate kinase in liver (50 μ moles/min / g) is much higher than the
Vmax activities of the opposing enzyme pair (6.7 μ moles/min/g; Scrutton
and Utter, 1968). L-pyruvate kinase activity must be suppressed in vivo
for significant rates of substrate cycling or gluconeogenesis to occur.

Rat liver L-pyruvate kinase is a tetrameric enzyme of subunit molecular weight 56,000 (Engström, 1978; Hopkirk and Bloxham, 1979). The reaction velocity-substrate kinetics display positive co-operativity with respect to phosphoenolpyruvate. At in vivo substrate concentrations of 0.2 to 0.8mM (Flory et al, 1974; Siess et al, 1974) the activity of L-pyruvate kinase is about 10% of Vmax, a rate comparable with the activities of pyruvate carboxylase and phosphoenolpyruvate carboxykinase. Regulation of the in vivo activity of L-pyruvate kinase can therefore determine the rate of substrate cycling. A number of factors do not alter the Vmax activity of L-pyruvate kinase but change the degree of co-operativity. Increased co-operativity represents effectively enzyme inhibition in vivo at constant substrate concentration. L-pyruvate kinase is inhibited by cyclic AMP dependent phosphorylation and is dephosphorylated and activated by phosphatase-1. Four moles of $^{32}P$ can be incorporated from [ $\gamma$ $^{32}P$]ATP per mole of tetrameric enzyme. L-pyruvate kinase is a major substrate for glucagon induced phosphorylation (Van den Berg et al, 1979). Allosteric effects are superimposed upon this covalent phosphorylation regulation. Fructose-1,6-biphosphate at 0.5mM activates L-pyruvate kinase and abolishes all effects of phosphorylation (Taylor and Bailey, 1967; Engström, 1978).

Substrate co-operativity is increased by ATP and alanine. The interaction of allosteric and phosphorylation mechanisms in the regulation of enzyme activity was recently discussed (El-Maghrabi et al, 1980). Feeding results in activation of L-pyruvate kinase by decreasing the enzyme phosphorylation and increasing the hepatic concentrations of fructose-1,6-bisphosphate and substrate phosphoenolpyruvate (Newsholme and Gevers, 1967).

There are other possible regulatory mechanisms of the phosphoenolpyruvate : pyruvate substrate cycle. Pyruvate carboxylase is activated by acetyl CoA (Barritt et al, 1976). This mechanism has been proposed to account for the stimulation of gluconeogenesis by fatty acid oxidation (Williamson et al, 1968) but discounted in vivo because of the high fatty acid concentrations used (Exton and Park, 1967). Nevertheless, stimulation of gluconeogenesis by glucagon in isolated hepatocytes is accompanied by increased mitochondrial concentrations of acetyl CoA (Siess et al, 1975).

Phosphoenolpyruvate carboxykinase is regulated by changes in protein synthesis (Tilghman et al, 1976). This enzyme has a circadian variation in rat liver with an activity maximum in fasted state.

The phosphoenolpyruvate : pyruvate substrate cycle has been demonstrated in isolated rat hepatocytes incubated with $[^{14}C]$-bicarbonate, pyruvate and lactate (Katz and Rognstad, 1976). $[4-^{14}C]$ oxaloacetate formed by pyruvate carboxylation equilibrates by action of malate dehydrogenase and fumanase to give $[1,4-^{14}C]$ oxaloacetate. Completion of the substrate cycle generates $[1-^{14}C]$ pyruvate. Incorporation of $[^{14}C]$-bicarbonate into pyruvate is an estimate of L-pyruvate kinase flux in the intact cell. This flux is inversely related to the gluconeogenic rate. Nevertheless substrate cycling was measurable even under gluconeogenic conditions. Measurement of metabolite concentrations in perfused rat liver have demonstrated a crossover between pyruvate and phosphoenolpyruvate in response to glucagon (Exton et al, 1972). These studies indicate that this substrate cycle is an important site in the regulation of gluconeogenesis from lactate.

## 1.3.3. *Precursors for Hepatic Fatty Acid Synthesis*

Incorporation of tritium from tritiated water into fatty acid during lipogenesis is independent of the precursor source (Section 3.1.3.2). This property has been used to quantify the relative significances of a variety of metabolites as potential substrates for fatty acid biosynthesis. Isolated liver is perfused, or hepatocytes incubated, with tritiated water and $[^{14}C]$ substrate. A high $^{14}C:^{3}H$ ratio indicates that metabolite could be a reasonable potential fatty acid substrate. Studies have consistently shown lactate and pyruvate to be good fatty acid precursors while blood glucose accounts for less than 10% of the lipogenic rate (Clark et al, 1974; Salmon et al, 1974; Bloxham et al, 1977). Hepatic glycogen can support lipogenesis in isolated hepatocytes in the absence of added substrate (Clark et al, 1974). Fatty acid synthesis in perfused liver from post-prandial rats correlates with glycogen depleted (Woods and Krebs, 1971).

Pyruvate is a common metabolite of fatty acid synthesis from both lactate and glycogen. Regulation of the phosphoenolpyruvate : pyruvate

Fig. 3    Pathway of Fatty Acid Synthesis from Pyruvate
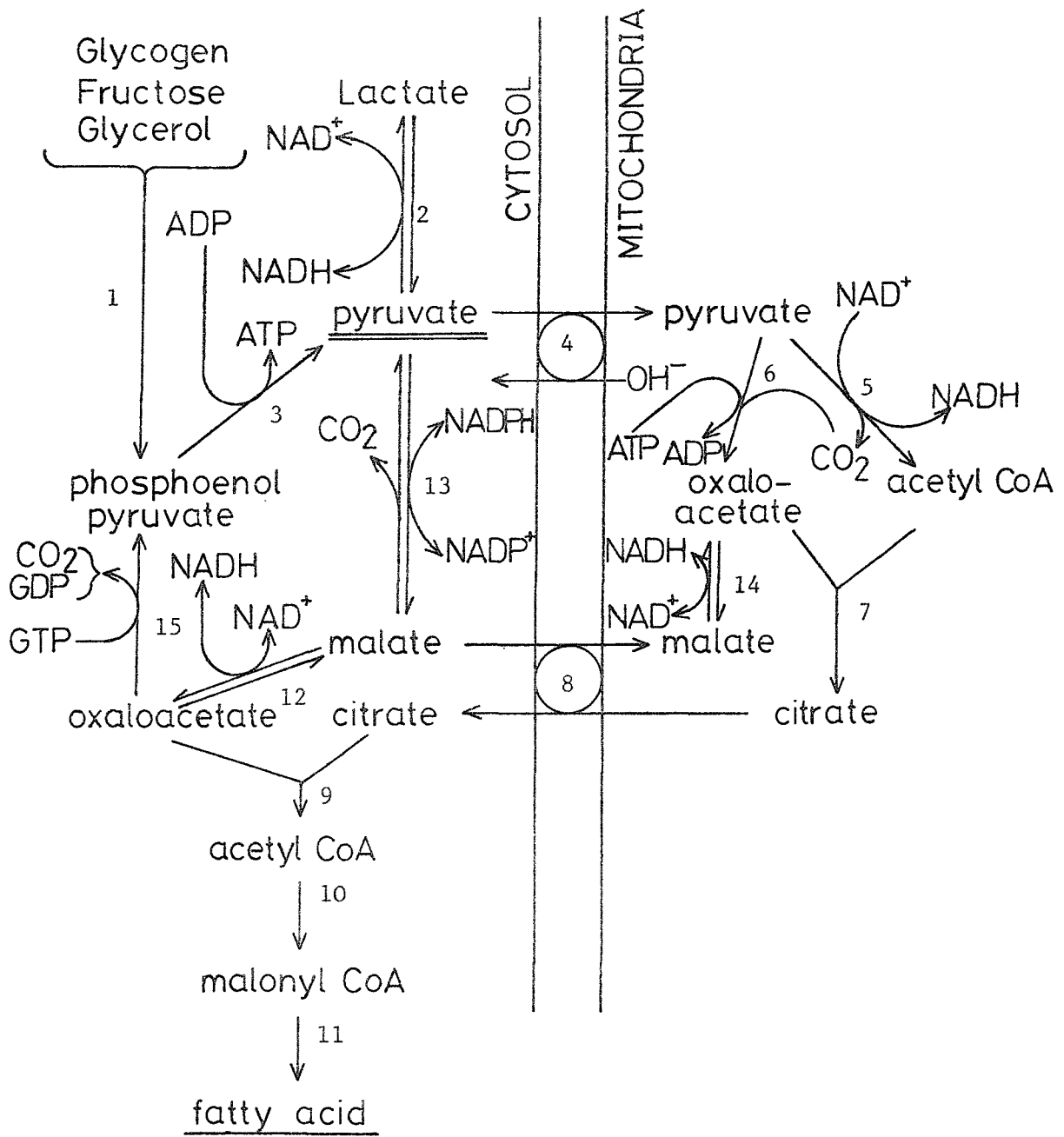


The enzymes in this scheme are:  1, glycolysis;  2, lactate dehydrogenase; 3, L-pyruvate kinase;  4, mitochondrial pyruvate transport;  5, pyruvate dehydrogenase;  6, pyruvate carboxylase;  7, citrate synthase; 8, mitochondrial tricarboxylate transport;  9, ATP: citrate lyase; 10, acetyl CoA carboxylase; 11, fatty acid synthase;  12, malate dehydrogenase (cyt.);  13, NADP: malate dehydrogenase;  14, malate dehydrogenase (mit);  15, phosphoenolcarboxykinase.

substrate cycle influences the rate of conversion of pyruvate to cyto-
solic acetyl CoA as well as the rate of gluconeogenesis. L-pyruvate
kinase is activated both by dephosphorylation and enzyme induction in
conditions of enhanced hepatic lipgenesis such as carbohydrate re-feeding
and weaning. (Engström, 1978; Hopkirk and Bloxham, 1979).

The pathway of fatty and biosynthesis from pyruvate is shown in
Fig. 3. Non-equilibrium reactions in this scheme are the lipogenic
enzymes acetyl CoA carboxylase and fatty acid synthase (Section 1.3.4)
and the mitochondrial enzymes pyruvate dehydrogenase and citrate synthase.
These latter two enzymes are regulated largely by changes in metabolite
concentrations within the mitochondria. Hepatic mitochondria from fasted
rats have high concentrations of ATP, acetyl CoA and NADH and      low
concentrations of ADP, $NAD^+$ and oxaloacetate. These concentration ratios
are reversed on feeding, when lipogenesis is stimulated. They are largely
secondary to the rate of fatty acid oxidation (Guynn et al,1972)

Pyruvate dehydrogenase from rat liver is a multi-enzyme complex of
molecular weight approaching $10^7$ consisting of seven subunits (for review
of properties see Denton et al, 1975). Within this complex there are a
specific cyclic AMP independent protein kinase and a specific phos-
phatase. Phosphorylated pyruvate dehydrogenase b is inactive and is
activated by dephosphorylation to pyruvate dehydrogenase a. The enzyme
phosphorylation state is increased in vitro by elevated metabolite ratios
of ATP:ADP, NADH:$NAD^+$ and acetyl CoA:coenzyme A. Pyruvate dehydrogenase
activity in isolated hepatocytes correlates inversely with the mito-
chondrial ATP:ADP ratio (Siess et al, 1975). The ATP concentration in
hepatic mitochondria from fasted rats effectively inactivates pyruvate
dehydrogenase and limits the mitochondrial conversion of pyruvate to
acetyl CoA (Wieland et al, 1972). The changes in metabolite ratios in
response to feeding are sufficient to activate pyruvate dehydrogenase
and allow fatty acid synthesis to proceed.

Inactivation of pyruvate dehydrogenase in the fasted rat prevents
the irreversible conversion of pyruvate to acetyl CoA. Nevertheless,
mitochondrial acetyl CoA is elevated in livers of fasted rats due to the
stimulated fatty acid oxidation (Guynn etal,1972; Siess et al 1977)

Conversion of this acetyl CoA to fatty acid is precluded by two possible
mechanisms. Firstly, acetyl CoA carboxylase can be inhibited by the
elevated hepatic content of fatty acyl CoA or enzyme phosphorylation
(Section 1.3.4). Secondly, the mitochondrial membrane is impermeable
to acetyl CoA which must be exported to the cytosol for lipogenesis by
the combined actions of citrate synthase, mitochondrial tricarboxylate
transporter and ATP:citrate lyase (Fig. 3, Watson and Lowenstein, 1970;
Lowenstein, 1971). In the fasted state citrate synthase activity is
inhibited by the decreased mitochondrial oxaloacetate concentration and
increased ATP concentration (for review see Newsholme and Start, 1973).
Mitochondrial citrate transport is inhibited by increased fatty acyl CoA
concentration (Halperin et al, 1972). These effects combine to prevent
acetyl CoA derived from fatty acid oxidation being recycled back to
fatty acid.

The metabolite and hormonal changes associated with feeding activate
L-pyruvate kinase in the liver and inhibit adipose tissue lipolysis.
Decreased fatty acid oxidation and fatty acyl CoA concentration effectively
relieve the inhibitions of pyruvate dehydrogenase, citrate synthase and
mitochondrial citrate transport. Continued fatty acid biosynthesis
requires replenishment of mitochondrial oxaloacetate (Fig. 3). The
activation of L-pyruvate kinase is important in this context as well as
in the inhibition of gluconeogenesis. Oxaloacetate formed in the cytosol
by ATP:citrate lyase can be converted to malate, aspartate or phos-
phoenolpyruvate. Malate is transported into the mitochondria either
directly or as pyruvate formed by NADP : malate dehydrogenase. Transport
of aspartate into the mitochondria is precluded by the electrochemical
gradient across the mitochondrial membrane (Williamson, 1976). Formation
of phosphoenolpyruvate would divert oxaloacetate to gluconeogenesis in
the absence of activation of L-pyruvate kinase. Thus elevated hepatic
L-pyruvate kinase activity is required to enable hepatic fatty acid bio-
synthesis to proceed from either glycogen or lactate.

### 1.3.4.  Fatty Acid Synthesis from Acetyl CoA

De novo fatty acid synthesis from cytosolic acetyl CoA is catalyzed
by the sequential actions of acetyl CoA carboxylase (acetyl CoA : carbon
dioxide ligase EC.6.4.1.2) and the multi-enzyme complex fatty acid

synthase (Fig.17). The concentrations of these two enzymes are regulated
by changes in protein synthesis or degradation in concert with the rate
of fatty acid biosynthesis (Section 1.2.1; Majerus and Kilburn, 1969;
Nakanishi and Numa, 1970). Quantitative precipitin analysis indicates
that specific enzyme activity is constant in rats that were fed, fasted
or diabetic. Although hepatic fatty acid biosynthesis is regulated by
variation of enzyme concentration over the longer term, such a mechanism
is unlikely to control short term fluctuations. The half lives of acetyl
CoA carboxylase and fatty acid synthase are too long to provide a sufficiently
rapid response (Section 4.1.1). Possible mechanisms for regulating
catalytic activities of these enzymes are discussed below.

### Acetyl CoA Carboxylase

Rat liver acetyl CoA carboxylase is a cytosolic enzyme of molecular
weight 220,000-250,000 (Inoue and Lowenstein, 1972; Carlson and Kim,
1974; Tanake et al, 1975). The enzyme in this protomeric form is in-
active but can be activated by aggregation to a polymer of molecular
weight in excess of $8 \times 10^6$ (Bortz and Lynen, 1963). The purified enzyme
protomer has not been dissociated into active subunits and the partial
reactions have been deduced by analogy with the enzyme from E. Coli.
The three functional subunits isolated from the bacterial enzyme have
described as biotin carboxylase, biotin carboxyl carrier protein and
transcarboxylase (Fig.17), (Dimroth et al, 1970; Alberts et al, 1971;
Fall and Vagelos, 1972). The interconversion of protomeric and polymeric
forms of the hepatic enzyme is reversible and possible regulatory mechanisms
include phosphorylation : dephosphorylation (Carlson and Kim, 1972) and
the effectors citrate and fatty acyl CoA (Numa et al, 1965). Purified
acetyl CoA carboxylase contains 2.1 moles of phosphate per 215,000 dalton
subunit (Inoue and Lowenstein, 1972) and incubation of partially purified
enzyme with [ $\gamma$ $^{32}$P] ATP incorporates $^{32}$P into the enzyme (Carlson and Kim,
1973). A cyclic AMP independent protein kinase can be isolated from
partially purified enzyme by ammonium sulphate fractionation (Carlson and
Kim, 1974a; Lent et al, 1978). Purified acetyl CoA carboxylase can
also be phosphorylated by cyclic AMP dependent protein kinase (Hardie
and Cohen, 1978; Lent et al, 1978). It has been suggested recently that
the phosphorylation state of hepatic acetyl CoA carboxylase is regulated
by the relative cytosolic concentrations of ATP, ADP and AMP (Yeh et al, 1980).

Fatty acid synthesis from acetate in liver homogenates is stimulated by the addition of citrate (Brady and Gurin, 1952). Citrate activates and increases the polymerization of acetyl CoA carboxylase from adipose tissue (Vagelos et al, 1963) and liver (Numa, 1964). Inactivation and depolymerization of acetyl CoA carboxylase by long chain fatty acyl CoA is competitive with citrate activation (Numa et al, 1965). The apparent Ki for fatty acyl CoA in the presence of physiological concentrations of binding protein is 20-25 μM (Lunzer et al, 1977). This suggests that potentially fatty acyl CoA inhibition could be regulatory in vivo, as estimated cytoplasmic fatty acyl CoA concentration range from 1.5 to 15 μM (Greenbaum et al, 1970). This inhibition has been attributed to non-specific detergent action (Taketa and Pogell, 1966; Shafrir and Ruderman, 1974) but the effect at low palmityl CoA concentrations is both specific and reversible (Goodridge, 1972; Ogiwara, 1978). Citrate activation has been questioned because the apparent Ka is 2-6mM (Numa et al, 1965) while estimated cytosolic citrate ranges from 0.1 to 0.2mM (Greenbaum et al, 1970). Nevertheless, the rate of fatty acid biosynthesis correlated with total citrate concentration in perfused liver (Brunengraber et al, 1973). Incubation with lactate increased the cytoplasmic citrate concentration in hepatocytes isolated from rats that were fasted (Siess et al, 1976) or fed (McGarry et al, 1978). Moreover, incubation of hepatocytes with oleate reduced cytoplasmic citrate to an unmeasurable concentration possibly by inhibition of mitochondrial citrate transport (Siess et al, 1976; Section 1.3.3). Much of the work on citrate activation of hepatic acetyl CoA carboxylase has been performed on the avian enzyme. There are, however, significant species differences between regulation of the avian and rodent enzyme (for discussion see Clarke et al, 1980). Chick liver contains type K-pyruvate kinase not type L (Ochs and Harris, 1978) and incubation of isolated chick hepatocytes with glucagon depleted cytoplasmic citrate by over 90% (Watkins et al, 1977). By contrast, the effect in rat hepatocytes was much less marked (Siess et al, 1977; Clarke et al, 1980). Moreover chick liver acetyl CoA carboxylase is not inhibited by multiple phosphorylation (Pekala et al, 1977) and displays an absolute requirement for citrate for activity to be measured in vitro.

The effects on rat liver acetyl CoA carboxylase of phosphorylation :

dephosphorylation and citrate : fatty acylCoA are not necessarily mutually exclusive. Both polymeric and protomeric enzyme form can be phosphorylated (Carlson and Kim, 1974b). Apparent Ka for citrate activation of protomer is increased from 0.2mM to 4.0mM by phosphorylation. Conversely, apparent Ki for fatty acyl CoA inactivation of polymer is decreased by phosphorylation. If these changes reflect in vivo conditions then the phosphorylation state of rat liver acetyl CoA carboxylase should profoundly alter the activation or inhibition by relatively constant concentrations of citrate and fatty acyl CoA.

The significance in vivo of any allosteric regulation of hepatic acetyl CoA carboxylase is still disputed despite the elaborate mechanisms proposed from in vitro data. Acetyl CoA carboxylase was inhibited by glucagon in vivo (Klain and Weiser, 1973) or by dibutyryl cyclic AMP in liver slices (Allred and Roehrig, 1972). Subsequent experiments employing elaborate precautions against modification of enzyme activity during preparation of the liver extract could not demonstrate an inhibition by glucagon in vivo (Cook et al, 1977), in perfused liver (Ma et al, 1978) or in isolated hepatocytes (Watkins et al, 1977). These precautions include freeze-clamping in liquid $N_2$ (Halestrap and Denton, 1973), fractionation of carboxylase and kinase by 70% $(NH_4)_2 SO_4$ (Carlson and Kim, 1973), inhibition of proleolysis with phenylmethyl sulphonyl fluoride, inhibition of phosphoprotein phosphatase with 50mM-NaF and inhibition of kinase by chelation of $Mg^{2+}$ with EDTA (Cook et al, 1977). Recent reports describe activation by insulin and inactivation by glucagon of acetyl CoA carboxylase in isolated rat hepatocytes (Geelan et al, 1978; Witters, 1979a,b; Beynan et al, 1980). The insulin, but not glucagon, effect is not apparent if hepatocytes are homogenised at $4^{\circ}C$ not $37^{\circ}C$. Enzyme inactivation is accompanied by phosphorylation but which kinase is responsible is not known (Witters, 1979a). Catalytic regulation of adipose enzyme is more readily shown. Injection of rats in vivo with anti-insulin serum decreased acetyl CoA activity in adipose tissue by 44% but only by 22% in liver (Stansbie et al, 1976).

## Fatty Acid Synthase

Fatty acid synthase from rat liver is a multienzyme complex of molecular weight 540,000 (Burton et al, 1968). The rates of synthesis

and degradation of hepatic fatty acid synthase vary dramatically in concert with the rate of fatty acid biosynthesis. These changes in response to variations in diet or to fasting are long term. They are due to changes in enzyme concentration and not to changes of activity of pre-existing enzyme (Volpe et al, 1973). For example, fatty acid synthase concentration increased 20-fold when fasted rats were re-fed a fat free diet. The half life of 2.8 days suggests that changes in enzyme synthesis or degradation are unlikely to regulate fatty acid biosynthesis over the short term.

A number of potential mechanisms have been proposed that might provide a more rapid control over fatty acid synthase activity. Feedback inhibition by elevated hepatic concentrations of palmityl CoA (Robinson et al, 1963) has been discounted because of the irreversible, general detergent character of the inhibition (Dorsey and Porter, 1968). Although enzyme inactivation by reversible phosphorylation has been described (Qureshi et al, 1975b), the kinase and phosphatase responsible are uncertain as is the in vivo significance. When acetyl CoA carboxylase in rat hepatocytes was phosphorylated in response to glucagon, no effect was observed on fatty acid synthase (Witters et al, 1979b). A third potential mechanism involves the prosthetic group 4' phosphopantetheine. Intermediates between malonyl CoA and palmitic acid are bound to acyl carrier protein, an integral portion of the fatty acid synthase complex in rat liver (Burton et al, 1968). The binding of one mole of 4'-phosphopantetheine by one mole of acyl carrier protein is an essential requirement for fatty acid synthase activity. An equilibrium has been proposed between an apo enzyme lacking 4'-phosphopantetheine and a holo enzyme containing 4'-phosphopantetheine (Qureshi et al, 1975a). The apo enzyme would be inactive and the holo enzyme active. Feeding rats a diet containing 5% histidine increased cyclic AMP concentration and converted hepatic fatty acid synthase from the active holo enzyme to the inactive apo enzyme (Qureshi et al, 1978).

1.3.5.  Supply of Reducing Equivalents for Fatty Acid Biosynthesis

The NADPH required for fatty acid synthase action is generated by the pentose phosphate pathway and by NADP : malate dehydrogenase. The catalytic activities of "malic" enzyme, glucose-6-phosphate dehydrogenase
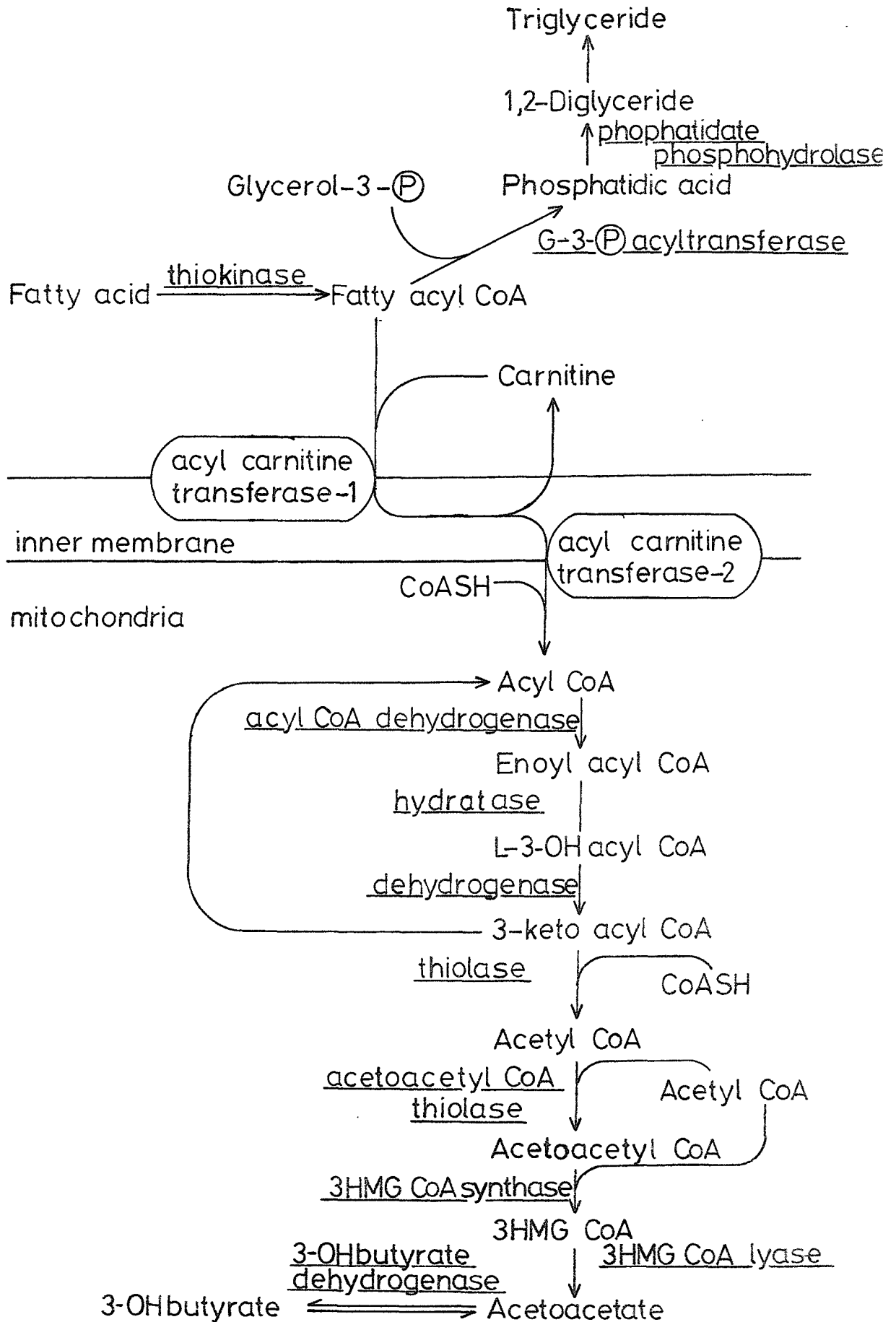
and 6 phosphogluconate dehydrogenase all vary in the liver in concert
with changes of lipogenic capacity (Hadvig and Abraham, 1980). It is
unlikely, however, that NADPH supply could limit hepatic fatty acid bio-
synthesis. Flux through the pentose phosphate pathway was estimated in
hepatocytes from fasted re-fed rats, using $[1-^{14}C]$ galactose (Rognstad
and Katz, 1976), to account for 50 to 75% of the NADPH produced. When
"malic" enzyme-derived NADPH synthesis was inhibited with 2,4-dihydroxy-
butyrate, pentose phosphate activity increased by 40 to 90% to compensate
(Rognstad and Katz, 1979). There was negligible impairment of the high
rate of fatty acid synthesis.

### 1.3.6. Fatty Acid Oxidation, Ketogenesis and Triglyceride Synthesis

Fatty acyl CoA within the liver can be either esterified to tri-
glyceride and phospholipid or oxidised to acetyl CoA. Regulation of
fluxes through these pathways are arranged in a reciprocal fashion
(McGarry and Foster, 1971). In the fasted rat fatty acyl CoA is derived
largely from adipose tissue lipolysis and is directed preferentially
towards β oxidation and ketogenesis. These rates are suppressed in liver
from fed rats and fatty acyl CoA, provided by diet or de novo hepatic
synthesis, is principaly esterified for export as very low density lipo-
protein.

After extensive investigation, the mechanism for the co-ordinated
regulation of fatty acyl CoA partition between these opposing hepatic
pathways has still not been completely clarified. Results obtained mainly
by McGarry and his colleagues suggest that oxidation is limited by mito-
chondrial fatty acyl transport. This is achieved as the carnitine ester
by the enzymes acylcarnitine transferase I and II, located on the outer
and inner surfaces respectively of the inner mitochondrial membrane
(Fig. 4; Brosnan et al, 1973). Acyl carnitine concentration is elevated
when fatty acid oxidation is stimulated (Bohmer, 1967) but no activation
of acyl carnitine transferase can be measured in broken cell preparations
(McGarry and Foster, 1974; Di Marco and Hoppel, 1975). The rate limiting
nature of acyl carnitine transferase II for fatty acid oxidation was
demonstrated by comparison of oleate and octanoate metabolism in perfused
liver (McGarry and Foster, 1971; McGarry et al, 1975). The normal physio-
logical restraints do not apply to octanoate oxidation as carnitine

Fig.4  Hepatic Metabolism of Fatty Acids



Triglyceride

1,2-Diglyceride

phophatidate
phosphohydrolase

Glycerol-3 -Ⓟ            Phosphatidic acid

G-3-Ⓟ acyltransferase

Fatty acid ——thiokinase——→ Fatty acyl CoA

Carnitine

acyl carnitine
transferase-1

inner membrane

acyl carnitine
transferase-2

CoASH

mitochondria

Acyl CoA

acyl CoA dehydrogenase

Enoyl acyl CoA

hydratase

L-3-OH acyl CoA

dehydrogenase

3-keto acyl CoA

thiolase          CoASH

Acetyl CoA

acetoacetyl CoA
thiolase          Acetyl CoA

Acetoacetyl CoA

3HMG CoA synthase

3HMG CoA

3-OHbutyrate
dehydrogenase          3HMG CoA lyase

3-OHbutyrate ⇌ Acetoacetate

activation is not required for the mitochondrial transport of octanoate. Malonyl CoA is a competitive inhibitor of acyl carnitine transferase II at physiological concentrations (McGarry et al, 1977) and the hepatic malonyl CoA concentration is increased after feeding when ketogenesis is inhibited (Guynn et al, 1972). Malonyl CoA thus provides an attractive mechanism whereby the first committed intermediate of fatty acid synthesis can regulate fatty acid oxidation and ketogenesis (Cook et al, 1978; McGarry and Foster, 1979).

Intra-mitochondrial mechanisms to regulate ketogenesis include limitation of citrate synthase activity by oxaloacetate availability or inhibition of citrate synthase by elevated ATP:ADP ratios (for review see Newsholme and Start, 1973). Direct stimulation of the 3-hydroxy-3-methyl glutaryl CoA pathway has been ascribed to activation of acetoacetyl CoA thiolase (Reed et al, 1977) and to a substrate cycle catalysed by HMG CoA synthase, HMG CoA lyase and 3 oxoacid CoA transferase (Zammit et al, 1979). The latter mechanism would explain the increased ketogenesis observed with elevated blood ratios of 3-hydroxy-butyrate to acetoacetate (Krebs, 1966). In vivo, however, these mechanisms are secondary to an increased supply of fatty acyl CoA provided by stimulated adipose tissue lipolysis.

Hepatic fatty acid esterification is depressed by fasting or experimental diabetes and stimulated by insulin. Triglyceride synthesis is, however, not absolutely dependent on insulin. When fatty acid uptake by livers from diabetic rats exceeds the capacity for β-oxidation, a high rate of triglyceride synthesis can be sustained (McGarry and Foster, 1971). The resulting hypertrigly-ceridaemia is a common observation in diabetic patients. Glycerol-3-phosphate and dihydroxyacetone phosphate concentrations in liver correlate with measured glyceride synthetic rates (Bremer et al, 1977). Although glycerol-3-phosphate acyltransferase is depressed on fasting, this enzyme does not appear to be rate limiting for esterification (Whiting et al, 1977). Phosphatidate phosphohydrolase shows the greatest increase concurrent with hepatic triglyceride synthesis after fructose or ethanol feeding and in genetic obesity (Sturton et al, 1978). This enzyme is in a position to control a branch point between triglyceride and phospholipid formation and is the only enzyme involved

shown to be elevated in the increased triglyceride synthesis seen in chronic experimental diabetes.

1.4.  Hormonal Regulation of Hepatic Lipid and Carbohydrate Metabolism

1.4.1.  Insulin

Insulin binds to specific plasma membrane receptors (Cuatrecasas, 1969; Cuatrecasas and Hollenberg, 1976). The mechanism whereby insulin binding is translated into a physiological response is unknown; no intracellular second messenger has yet been isolated. Proposed sites of insulin action are inhibition of adenylate cyclase (Jungas, 1966; Illiano and Cuatrecasas, 1972; Renner et al, 1974), activation of the low Km phosphodiesterase (Loten and Sneyd, 1970; Zinman and Hollenberg, 1974) and activation of guanylate cyclase (Illiano et al, 1973).

Hepatic fatty acid biosynthesis and glycogen synthesis are depressed by the insulin deficiency of experimental diabetes (Hers, 1976; Numa and Yamashita, 1974). Enzymes with activities decreased in diabetes include glucokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, NADP:malate dehydrogenase, ATP:citrate lyase, acetyl CoA carboxylase and fatty acid synthase. These low enzyme activities are due to decreased enzyme concentration. Restoration of activity by insulin is preceeded by the time lag required for initiation of synthesis of messenger RNA and protein.

In contrast to the long term regulation of protein synthesis, it is difficult to demonstrate many acute hepatic responses to insulin. Many of the responses of adipose tissue and muscle to insulin are secondary to stimulation by insulin of glucose transport (Levine and Goldstein, 1955; Fritz, 1972; Fain, 1974) but such a mechanism cannot apply in liver. Glucose transport across the liver cell plasma membrane is an equilibrium process and is not stimulated by insulin (Section 1.3.2). Fatty acid biosynthesis in perfused liver from fed rats could only be stimulated by insulin after the rate was depressed by addition of oleate (Topping and Mayes, 1976). Perfused livers from rats injected with anti-insulin serum have elevated cyclic AMP concentrations and stimulated rates of gluconeogenesis from lactate (Jefferson, et al, 1968).

Insulin opposes both these effects but not below a basal value. Insulin antagonizes the glycogenolytic response of added cyclic AMP but not of dibutyryl cyclic AMP (Glinsmann and Mortimore, 1968), suggesting phosphodiesterase activation (Exton and Park, 1972; Loten et al, 1978). Insulin also stimulates hepatic glycogen synthase in diabetic rats before cyclic AMP concentrations decrease (Nichols and Goldberg, 1972). Many hepatic responses to insulin are largely indirect. Feeding is usually accompanied by hyperinsulinaemia, hyperglycaemia and decrease NEFA concentrations in blood. It is the increased glucose and decreased fatty acid concentrations that are largely responsible respectively for the stimulated hepatic glycogen synthesis (Hers, 1976) and the decreased hepatic ketogenesis (Van Harken, 1969; Heimberg, 1974).

## 1.4.2. Glucagon

The catabolic hormone glucagon has, unlike insulin, a number of well defined actions on hepatic metabolism. Hepatic enzymes that are substrates for glucagon stimulated, cyclic AMP-dependent phosphorylation include phosphorylase kinase, glycogen synthase, phosphatase inhibitor I, fructose-1,6-bisphosphatase, L-pyruvate kinase and acetyl CoA carboxylase and enzymes that will be phosphorylated by cyclic AMP-independent kinases under conditions of glucagon action include phosphofructokinase, pyruvate dehydrogenase and acetyl CoA carboxylase (Section 1.2.2). The mode of glucagon activation of adenylate cyclase and elevation of hepatic cyclic AMP concentration has been extensively reviewed (Robison et al, 1972; Levebre and Unger, 1972).

The hepatic response to glucagon has been largely studied using perfused liver and isolated hepatocyte preparations. By metabolite crossover, stimulation of gluconeogenesis has been located at the conversion of pyruvate to phosphoenol-pyruvate (Exton et al, 1972; Exton et al, 1976) possibly by inactivation of L-pyruvate kinase (Engström, 1978). Inactivation of glycogen synthase and activation of phosphorylase by glucagon rapidly initiates glycogenolysis (Section 1.3.1.). The inhibition of hepatic fatty and biosynthesis by glucagon has been attributed to diminished substrate supply (McGarry and Foster, 1979; Section 1.3.3).and to inactivation of acetyl CoA carboxylase (Section 1.3.4).

Decreased lipogenesis is associated with lower hepatic concentrations
of malonyl CoA (Guynn et al, 1972) which decrease the inhibition of acyl
carnitine transferase II(McGarry et al, 1977). Ketogenesis is stimu-
lated both by increased mitochondrial transport of fatty acyl CoA and
by stimulated adipose tissue lipolysis (Section 1.3.6).

The physiological response to glucagon is modified by the insulin
concentration. The molar concentration ratio of insulin : glucagon is
considered important in determining the metabolic 'set' of the liver
(Unger, 1972). Many glucagon actions are relatively transient in vivo
as increased blood glucose and glucagon concentrations stimulate insulin
secretion (Samols et al, 1965;  Malaisse et al, 1967). Thus circulating
concentrations of ketone bodies are only slightly elevated when glucagon
is administered in vivo to fed intact rats. Adipose tissue lipolysis is
rapidly inactivated by the increased insulin concentration and hepatic
fatty acid oxidation is thus deprived of the essential substrate to main-
tain ketogenesis (Fritz and Lee, 1972). The response to glucagon is also
in many cases dependent on the presence of other hormones such as gluco-
corticoids (Section 1.4.4).

## 1.4.3. Glucocorticoid Hormones

Glucocorticoid hormones are secreted from the adrenal cortex and
affect metabolism in the long term by regulating the synthesis of specific
proteins (for reviews see Litwack and Singer, 1972;  Wick, 1974). Unlike
insulin and glucagon, corticosteroid hormones bind to intracellular
and not to extracellular receptors. Four specific cytosolic receptors
have been found in liver (Litwack and Singer, 1972). Steroid binding
induces a conformational change in the receptor and the resulting com-
plex is transported into the nucleus (Spelsberg et al, 1974). The steroid :
receptor complex binds to DNA within the nucleus and by an uncertain
mechanism either increases the synthesis (Lee et al, 1970) or decreases
the breakdown (Tompkins et al, 1967) of messenger RNA. In the rat the
major circulating glucocorticoid hormone is corticosterone. Cortico-
sterone binding to rat liver induces the synthesis of selected enzymes
primarily concerned with amino acid catabolism, gluconeogenesis and
glycogen synthesis. These hepatic enzymes include tyrosine amino-
transferase (E.C.2.6.1.5, Granner et al, 1970;  Levitan Webb, 1970),

gluta myl : alanine transaminase (E.C.2.6.1.2, Segal and Kim, 1963),
tryptophan pyrrolase (E.C.1.13.1.12, Schimke, 1970), serine dehydratase
(E.C.4.2.1.13, Inoue et al, 1971), phosphoenolpyruvate carboxykinase
(E.C.4.1.1.32, Shrago et al, 1963), glucokinase (E.C.2.7.1.2, Weinhouse,
1976) and glycogen synthase phosphatase (De Wulf and Hers, 1968).

The actions of corticosterone in regulating the synthesis of
phosphoenolpyruvate carboxykinase and glucokinase are synergistic with
the responses to insulin and glucagon. Phosphoenolpyruvate carboxykinase
in liver is induced both by glucocoriticoid and cyclic AMP (Iyendjian
et al, 1979) and direct stimulation of enzyme synthesis by cortisol has
been shown in H-35 hepatoma cells (Wicks et al, 1973). This response is
masked when steroid is injected in vivo due to the attendant hyper-
insulinaemia (Gunn et al, 1975). Hepatic glucokinase is not induced
by chronic hydrocortisone injection in vivo (Walker and Rao, 1964;
Pilkis, 1970) but is abnormally low in fasted adrenalectomized rats
(Sharma et al, 1964). The induction of glucokinase by glucose re-
feeding is impaired and delayed in these animals and is corrected by
treatment with corticosteroid (Niemeyer et al, 1965; Weinhouse, 1976).

The steroid dependence of glycogen synthase phosphatase is con-
sidered to be the mechanism behind the well-established role of gluco-
corticoid hormones in maintenance of liver glycogen reserves (Long,
1940). In adrenalectomized rats glycogen synthase is predominantly in
the inactive b form (Hornbrook et al, 1966). The initiation of cortico-
sterone production in foetal rat appears to be responsible for the
deposition of foetal hepatic glycogen after day 18 of gestation.
Glycogen synthase b can be assayed in foetal liver before this date,
but steroid induction of glycogen synthase phosphatase is required for
activation of glycogen synthase (Dopére et al, 1980).

Corticosterone has a number of other direct actions on the liver.
The fatty liver characteristic of hypercortisolaemia could be due to
induction of the triglyceride-synthesizing enzyme phosphadityl phospho-
hydrolase by steroid (Glenny and Brindley, 1978). Additionally, cortisol
or dexamethasone stimulate ketogenesis in insulin deficient man (Schade
et al, 1977), a response that in the rat is abolished by adrenalectomy

(L'Age et al, 1974). The role of corticosteroid in the regulation of protein metabolism has been extensively discussed (Steele, 1975).

Many glucocorticoid responses of hepatic metabolism are indirect due to changes in circulating concentrations of metabolites and hormones. Conditions of chronic corticosteroid excess are characterized by peripheral insulin resistance, decreased glucose uptake by adipose tissue, but also by hyperinsulinaemia (Steele, 1975). Glucocorticoid treatment stimulates insulin secretion (Malaisse and Malaisse-Lagae, 1967; Van Lan et al, 1974) while fasted plasma insulin decreases after adrenalectomy (Kawai and Kuzuya, 1977). The induction of hepatic lipogenic enzymes and stimulation of hepatic lipgenesis by corticosteroid is secondary to the insulin response (Diamant and Shafrir, 1975). No stimulated synthesis is observed when alloxan-diabetic rats are treated with steroid. Adrenalectomy decreases the rate of hepatic fatty acid biosynthesis _in vivo_ (Bouillon and Berdanier, 1980) and in perfused liver (Kirk et al, 1976). This rate can be restored by pre-treatment _in vivo_ with either cortisol or insulin. Insulin has a similar role in the stimulation of hepatic glycogen synthesis by corticosteroid. The impaired rate of glycogen synthesis in perfused livers from adrenalectomized rats can also be restored by pre-treatment _in vivo_ with either cortisol or insulin (Whitton and Hems, 1976).

## 1.4.4. Permissive Actions of Glucocorticoid Hormones

The term "permissive" was first coined for the role of corticosteroids in the increased nitrogen loss of experimental injury (Ingle, 1951). This stress response was abolished by adrenalectomy but not potentiated by excess exogenous steroid. Subsequently glucocorticoid hormones have become recognized as obligatory for many metabolic responses to a variety of hormones. This hormonal synergism is most apparent for the interaction between glucocorticoids and cyclic AMP-mediated hormones.

Hepatic glucagon actions that are depressed or abolished by adrenalectomy include stimulation of gluconeogenesis (Friedmann and Wertheimer, 1966; Friedmann et al, 1967), glycogenolysis (Exton et al, 1972) and ketogenesis (Chernick et al, 1972). Stimulation of adipose tissue lipolysis by adrenaline, glucagon or corticotropin is also steroid

dependent (Shafrir and Kerpel, 1964; Fain and Czech, 1975; Fain, 1975).
Other hormonal responses increased by glucocorticoid include stimulation
of coenzyme A synthesis by cyclic AMP in hepatocytes (Smith and Savage,
1980), thyroxine stimulated growth hormone synthesis by cultured
pituitary cells (Martial et al, 1977) and albumin synthesis in hepatocytes
in response to cyclic AMP (Brown and Papaconstantinou, 1979).

The mechanisms underlying this permissive nature of glucocorticoid
action are uncertain (for discussion see Wicks, 1974a). The direct
corticosteroid response does not appear to be mediated at all by cyclic
AMP. For instance, cortisol and cyclic AMP have opposite effects on
hepatic glycogen synthase (De Wulf and Hers, 1968a,b; Mersmann and Segal,
1969; Van den Berghe et al, 1970) and tyrosine aminotransferase is
induced by cyclic AMP in adrenalectomized as well as in control rats
(Wicks et al, 1969). There is no impaired formation of cyclic AMP when
perfused liver from adrenalectomized rats is exposed to glucagon (Exton
et al, 1972).

Some responses to α-adrenergic agonists are also blunted by
adrenalectomy. Glycogenolysis in response to adrenalin is impaired
in perfused hearts from adrenalectomized rats, a defect that is corrected
by addition of calcium (Miller et al, 1971). Adrenalectomy also impairs
the calcium mobilization and phosphorylase activation normally observed
in isolated hepatocytes in response to phenylephrine (Chan et al, 1979).
The α-adrenergic activation of kidney tubule gluconeogenesis by adrenaline
is another calcium mediated response dependent on glucocorticoid
(Macdonald and Saggerson, 1978).

1.5  Aims and Experimental Design

The rationale underlying the investigations presented in this thesis
is to quantify the absolute flux through a metabolic pathway in vivo, and
then to compare changes in this flux with changes in the activities of
selected potential regulatory enzymes. If an enzyme is rate-limiting for
that pathway, then the enzyme activity should be regulated in concert with
the overall pathway flux. This approach requires extensive prior know-
ledge of the in vitro enzyme properties to enable in vivo changes in
activity to be preserved in the tissue extract. It also requires a

method to measure the absolute rate of pathway flux. Finally, the
pathway rate must vary over a sufficiently wide range in response to
physiological manipulations for significant comparisons to be drawn
with enzyme activity changes.

These criteria are largely fulfilled for hepatic fatty acid bio-
synthesis. Incorporation of tritium into fatty acid from tritiated water
is a good measure of the absolute synthetic rate (Section 3.1.3.2) and
the lipogenic enzymes have been extensively studied (Section 1.3.4).
The rate of hepatic fatty acid biosynthesis is acutely sensitive to
environmental conditions and can be altered rapidly by feeding and by
experimental variation of hormone concentrations. In this context, the
inhibition of hepatic fatty acid biosynthesis by glucagon has been attri-
buted to depletion of hepatic glycogen (Ma et al, 1978). Any such inte-
grated regulation of these two hepatic pathways could be analysed further
if the absolute rate of hepatic glycogen synthesis could be measured.
Thus the possibility of quantifying glycogen synthesis by the rate of
tritium incorporation from tritiated water into hepatic glycogen was
explored.

The circadian variations of many metabolic parameters are exaggerated
and co-ordinated by meal-feeding compared with feeding ad libitum (Walker
and Potter, 1974). Hepatic fatty acid biosynthesis and hepatic glycogen
concentration are both at least doubled by meal-feeding (Hopkins et al,
1973; Fears and Morgan, 1976; Hopkirk and Bloxham, 1977). The extents
of these responses to meal-feeding are depressed by streptozotocin
diabetes and restored by insulin and are also affected by the circulating
concentration of corticosterone (Section 1.4). These meal-feeding
responses correlate well with the increased variation of the insulin :
glucagon ratio (Ip et al, 1977). The circadian variation of cortico-
sterone, however, is largely regulated by the light-dark cycle and not
by feeding (Acheson and Taylor, 1975). Corticosterone peaks towards the
end of the light phase in ad libitum fed rats. Rats are nocturnal
animals and feeding in the light phase can grossly disturb the normal
circadian corticosterone rhythm (Phillipens et al, 1977). In the studies
presented below it was considered advisable to feed the rats in the dark
phase to preserve the normal daily hormonal fluctuations. Consequently,

animals were housed under reversed lighting conditions with the light on
for the 12h period 16.00h - 04.00h.

In summary, the feeding and lighting conditions under which the rats
were housed stimulate hepatic fatty acid biosynthesis and hepatic
glycogen synthesis in a reproducible fashion. The extent of this stimulus
can be altered by hormonal manipulation. Comparison of pathway flux with
enzyme activity can suggest conclusions about the regulatory role of that
enzyme. Finally, these experimental conditions enable aspects of the
metabolic interactions of various hormones to be investigated.

CHAPTER 2

MATERIALS AND METHODS

## 2.1. Experimental

### 2.1.1. Animal Maintenance (Fig.5)

Male albino rats, Wistar strain (Scientific Products Farm Division, Charles Rivers U.K. Ltd., Margate, Kent), were used in all experiments except for the female rats from the same supplier used in the foetal hepatic glycogen synthesis studies. Initially weighing 100 to 125g, they were housed under a reversed lighting schedule, with the light on from 16.00 to 04.00h. The room temperature was maintained at $22^{\circ}C$. Animals were trained to eat for 2h daily, from 10.00 to 12.00h in the mid-dark period. For at least 14 days they were fed standard low fat laboratory diet (Labshure diet CRM-X, Christopher Hill Ltd., Poole, Dorset), 60g per cage of 4 rats, and the food consumption and weight gain were monitored. Experimental treatments were not started until the end of this adaptation period.

### 2.1.2. Diet

The composition of the standard diet is shown in Table 2. The major energy content was polysaccharide. On the alternative high carbohydrate diets this polysaccharide was largely replaced by sucrose, glucose or fructose. Rats were fed these diets for 7 days after the initial adaptation to meal-feeding.

Fig. 5     Feeding and Lighting Regime
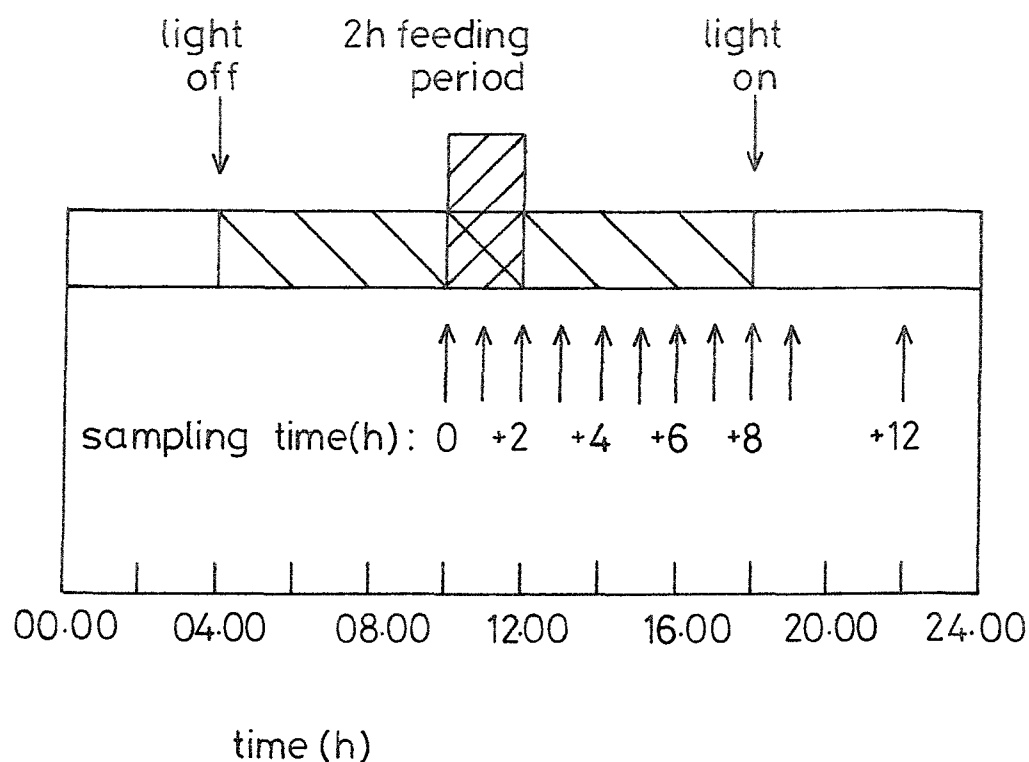


Rats were injected (i.p.) with $^3H_2O$ (2m Ci) 1h before each sampling time indicated.

Table 2     Composition of Diet

Standard diet: - CRM-X (expanded)

|                      | % diet by wt. |
| -------------------- | ------------- |
| Fat                  | 2.54          |
| Protein              | 17.54         |
| Fibre                | 3.94          |
| Ca                   | 0.80          |
| P                    | 0.71          |
| Na Cl                | 0.75          |
| Metabolizable energy | 2870 Kcal/kg  |

High carbohydrate diets contained 67% by wt. of sucrose, glucose or fructose.

2.1.3. Streptozotocin Diabetes
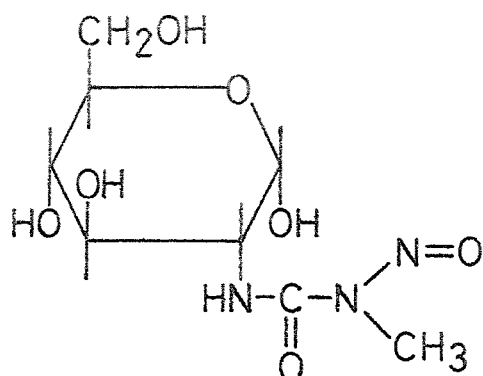


Streptozotocin

Experimental diabetes was induced in one group of rats by the antibiotic streptozotocin (Rakieten et al, 1963). Streptozotocin (55mg/kg body wt; UpJohn Co., Kalamazoo, U.S.A) was dissolved at 45mg/ml in 0.01 M-sodium citrate buffer, pH 4.5, and injected into a tail vein of a rat under light ether anaesthesia. Control animals received a similar volume of buffer with no streptozotocin.

The development of diabetes was confirmed after 2 days by measuring the blood glucose concentration (Section 2.4.2) after the 22h fast. Rats with a fasting hyperglycaemia less than 10mmol/l were excluded from subsequent experiment.

Diabetic rats were divided into two groups 3 days after streptozotocin injection. Animals in one group received a single subcutaneous injection of 3U protamine zinc insulin (40 U/ml, Wellcome Research Laboratories, Beckenham, Kent) at 08.00h, 2h before feeding. Rats in the other un-treated group were injected with an equivalent volume of 0.15M-saline at the same time.

2.1.4. Corticotropin Treatment

One group of intact rats was made hypercorticoid by daily sub-cutaneous injection of a long-acting synthetic corticotropin preparation, Tetracosactrin (Synacthen) Depot (1mg/1ml, CIBA Laboratories, Horsham, West Sussex). Corticotropin was injected at a dose of 0.5mg/kg body weight at 16.00h for 3 consecutive days.

2.1.5. Adrenalectomy

Bilateral adrenalectomy was performed by the dorsal route. Rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (40 mg/kg body weight). A single mid-line incision was made in the skin over the spine in the region of the kidneys. Through this incision two cuts were made in the body wall laterally over each kidney. The adrenal glands over the kidneys were removed using two pairs of forceps. Rats were given 0.15M saline to drink to counteract the mineralocorticoid deficiency resulting from adrenal removal. Adrenalectomized rats were left for 7 days to recover from the operation.

A number of rats were sham-operated as adrenalectomy controls. The operating proceedure was identical except that the adrenal glands were maintained intact. A second control was provided by pair-feeding intact rats with adrenalectomized animals.

2.1.6. Foetal Rats

Vaginal smears were taken from female rats, 200-250g, and the stages of the 5 day oestrus cycle plotted. When a female was in oestrus a male was introduced and mating took place. Pregnancy was confirmed by palpation 14 days later. The gestational age was numbered by taking the date of insemination as day 0.

The rate of tritiated water incorporation into foetal hepatic glycogen was measured by injecting pregnant rats with 2mCi of tritiated water. After 55 min they were anaesthetized with sodium pentobarbitone (40mg/kg body weight) and the foetuses delivered by Caesarian section. Livers were rapidly removed, frozen in liquid nitrogen and pooled for analysis.

2.1.7. Glucagon Treatment

In one series of experiments glucagon was administered to rats in vivo at a pharmacological dose (1mg/kg body weight) to elicit a maximum response. Glucagon (1mg) was dissolved in carrier 0.3M lactose (1ml) and injected subcutaneously within 10 min. An equivalent volume of 0.9% (w/v) saline was injected into control animals.

## 2.1.8. Femoral Artery Cannulation

Polyethylene cannulae (No. 1619R, Bardic I-Catheter, C.R. Bard International Ltd., Clacton-on-Sea, Essex) were inserted into the left femoral arteries of rats under ether anaesthesia. The cannulae were filled with 0.15 M-saline and tied in position. After stitching the skin incision, rats were placed in restraining cages overnight. They were supplied with both food and water. Blood samples were taken after flushing the cannula with 0.15 M-saline (200 µl) and 100 units of heparin.

## 2.2. Measurement of Rates of Biosynthetic Pathways by Tritiated Water Incorporation

In many biosynthetic pathways protons are incorporated by neutralisation of carbanions. Where the proton is derived directly from the medium water, substitution of deuterated or tritiated water will result in isotope incorporation. Methods for analysing glycogen metabolism (Boxer and Stetten, 1944; Ussing, 1937) and fatty acid metabolism (Schoenheimer and Rittenberg, 1936) were developed very soon after deuterium oxide enriched water became available. Since then measurement of fatty acid synthesis by tritium incorporation from tritiated water (Foster and Bloom, 1963) has become a widely used technique. By contrast, tritiated water incorporation has not been employed previously to measure rates of glycogen synthesis.

### Tritiated Water Incubation in vivo

At various stages of the circadian cycle rats were injected intraperitoneally with tritiated water (1 m$^{Ci}$/ 100g body weight in 0.15 M-sodium chloride), (Fig. 5 ). After an interval, typically either 30 min or 1 h, they were killed by cervical dislocation. Liver samples (2 to 4g) were freeze-clamped within 10 sec of death between aluminium tongs which had been pre-cooled in liquid nitrogen. The frozen liver was ground to a powder and stored under liquid nitrogen until analysis.

### Water Content of Liver

Duplicate samples of fresh liver (200 to 500 mg) were weighed while wet and then dried in an oven at 80°C for 24h. The water content was

calculated from the loss in weight on drying. For 40 livers the water content (mean $\pm$ S.E.M) was 71.3 $\pm$ 0.3%.

## The Specific Radioactivity of Tritium in Liver Water

Frozen liver powder (1g) was homogenized with 6% w/v perchloric acid (3g). After centrifugation at 2,000 x g for 15 min duplicate super-natant samples (100 µl) were taken for determination of total tritium by liquid scintillation counting. The specific radioactivity of tritium in liver water was calculated from the formula:

$$\frac{\text{Mean DPM/100 µl sample} \times 10 \times 100}{0.25 \times \% \text{ water content} \times 111 \times 1000} \qquad \text{DPM/ µgatom Hydrogen}$$

This calculation assumes the molecular concentration of water to be 55.5 moles /l, giving 111 x $10^3$ µgatoms of hydrogen per ml of water. The factor 0.25 was the dilution of liver in perchloric acid.

### 2.2.1. Hepatic Lipid Synthesis
#### Extraction of Saponifiable and Non-saponifiable Lipids from Liver

Frozen liver powder (1g) was homogenized with methanol (7ml) and chloroform (14ml). The homogenate was centrifuged at 2,000 x g for 20 min and the supernatant decanted. The residue was re-extracted with chloroform : methanol (2:1, v/v, 5ml). The extracts were pooled and the solvents removed by rotary evaporation.

### Saponification

The lipid residue was dissolved in 10% (w/v) methanolic KOH (5ml). The solution was saponified by heating at 70°C for 30 min. When cool, three extractions with portions (5ml) of petroleum spirit (40° - 60°C b.pt) were performed. When pooled these extracts contained the non-saponifiable, largely sterol, fraction of the liver lipid. The methanolic subnatant was acidified with concentrated hydrochloric acid and the petroleum spirit extractions were then repeated. These acidified extracts contained the saponifiable, fatty acyl, fraction of liver lipid.

The respective pooled fractions were back washed three times with 0.15M-sodium chloride (50ml total), dried with 50% (v/v) ethanol (20ml) and evaporated overnight in scintillation vials. When dry the tritium content of the extracts was measured by liquid scintillation.

## Calculation of Rates of Saponifiable and Non-saponifiable Lipid Synthesis

Biosynthetic rates were calculated as µgatoms H incorporated/h/g liver wet weight from the formula:

$$\frac{\text{d.p.m. in lipid fraction} \times 60}{\text{d.p.m. / µgatom 'H'} \times \text{wt tissue} \times \text{time of } ^3H_2O \text{ extracted (g) incubation (min)}}$$

d.p.m. / µgatom 'H' was the specific radioactivity of tissue water. The extraction from alkaline solution gave the non-saponifiable (sterol) rate and that from acid solution the saponifiable (fatty acid) rate. Tritium isotope effects were ignored in these calculations.

The fatty acid biosynthesis rate can be converted to acetyl units incorporated after division by 1.78 (Jungas, 1968) or µmoles of palmityl (C-16) fatty acyl equivalents after division by 13.3 (Windmueller and Spaeth, 1966).

The sterol biosynthesis rate can be converted to µmoles cholesterol after division by 14.5 (Lakshmanan & Veech 1977)

### 2.2.2. Hepatic Glycogen Synthesis
#### Isolation of Glycogen

Frozen liver powder (1g) was homogenized with 6% (w/v) perchloric acid (3g) and centrifuged at 2,000 x 8 for 15 min. Glycogen was precipitated from a supernatant sample (2ml) with ethanol (4ml) at 4°C and collected by centrifugation at 2,000 x g for 10 min. After being twice redissolved in distilled water (1 ml) and reprecipitated with ethanol (2 ml) the glycogen pellet was dried overnight in a vacuum dessicator. The dry pellet was dissolved in 0.04M-sodium acetate buffer, pH 4.8 (2ml) containing 50 µl α-amyloglucosidase (10.5 µkatal). The glycogen was hydrolysed to glucose by incubation of this solution at 37°C for 2h.

After freeze drying the glucose solution was reconstituted with distilled water (2 ml) and the tritium content of sample (0.2 to 1.0 ml) measured.

## Calculation of Rate of Glycogen Tritiated Water Incorporation

This was calculated from the formula:-

$$\frac{\text{DPM in glycogen glucose sample} \quad x \quad 2}{\underset{\text{liver water}}{\text{Specific activity}} \; x \; \underset{\text{vol(ml)}}{\text{sample}} \; x \; \underset{\text{dilution}}{\text{Acid}} \; x \; \underset{\text{incubation (h)}}{\text{time of }^{3}H_2O}} \qquad \begin{array}{l} \mu\text{gatoms 'H'/g} \\ \text{wet wt liver/h} \end{array}$$

Tritium isotope effects were ignored in this calculation.

## Measurement of Glucose and Total Glycogen

Free glycose and hydrolyzed glycogen were measured by the hexokinase method (Keppler and Decker, 1974).

## Free Glucose

About 1 ml of perchloric acid supernatant was neutralized with 10% (w/v) potassium hydroxide, using universal indicator. The potassium perchlorate precipitate was discarded after centrifugation at 2,000 x g for 10 min and the neutral supernatant decolourized with about 200 mg of florosil.

Samples of neutral supernatant (100 to 200 μl) were added to glass cuvettes, 1 cm path length, and made up to a final volume of 2 ml with 100 mM-Tris-H Cl, pH8.0, 10mM-Mg Cl2, 1.0mM-ATP, 1.5mM-NADP$^{+}$ and G6PdH(0·1μk); the absorbance at 340nm on a Zeiss PMQ spectrophotometer was recorded. The absorbance after the addition of 5 μl hexokinase (0.02 μkatal) was then recorded and the increase calculated. The reaction was generally complete within 30 min. The absorbance due to enzyme addition was measured in a control assay lacking glucose sample. This value was subtracted from the sample absorbance change to give the extinction (E). The glucose concentration was calculated assuming the molar extinction coefficient of NADPH at 340nm to be 6.22 x 10$^{3}$ by the formula:

$$\frac{E \quad x \quad 2}{6.22 \; x \; \text{sample vol (ml)} \; x \; \underset{\text{dilution}}{\text{Neutral}}} \qquad \mu\text{moles/g wet wt liver}$$

## Total Glycogen

The glycogen in a sample of neutralized supernatant (500 μl) was hydrolysed by 20 μl α-amyloglucosidase (0.2 μkatal) in 200 μl of 20 mM sodium acetate, pH4.8, followed by incubation at $37^{\circ}C$ for 2h. The liberated glucose was measured as before typically using samples from 5 to 20 μl. Subtraction of the free glucose concentration from this value gave the total hepatic glycogen concentration.

## Precipitated Glycogen

The glucose concentration of the precipitated, hydrolysed, reconstituted glycogen solution was determined as above, using samples of 2 to 20 μl. The recovery of precipitated glycogen was calculated by comparison with the total glycogen concentration.

## Positional Analysis of Glycogen Glucose Tritium

After enzymatic hydrolysis the precipitated glycogen glucose samples were sequentially degraded to determine the positional distribution of tritium between the glucose carbon atoms. The tritium at C-1 and C-6 of glucose was measured by chemical derivative formation; that at C-2, C-3, C-4 and C-5 by the tritium loss after specific enzyme reactions.

### C-1 Tritium

Tritium at C-1 glucose was eliminated during the oxidation of glucose to potassium gluconate (Andrews et al, 1965):



Glucose                                          Potassium gluconate
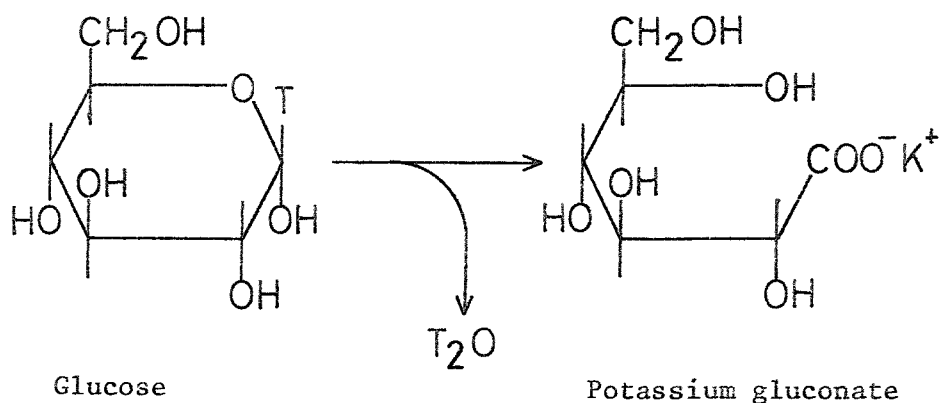
Non-radioactive carrier glucose (100 mg in 200 µl distilled water) was added to a sample of tritiated glucose and then washed with methanol (2 ml) into 6 ml of a solution of iodine in methanol (4.7 g/100 ml) at 40°C for 10 min. To this mixture were then added 6 ml of a molecular sieve-dried solution of potassium hydroxide in methanol (5g/100 ml), slowly with stirring. The white crystals of potassium gluconate formed on cooling to room temperature. The crystals were successively washed with methanol and diethyl ether and dried in a vacuum dessicator over silica gel. The recovery ranged from 73 to 89%.

The tritium at C-1 of glucose was calculated from the formula:-

$$\frac{\text{Total d.p.m. in glucose sample} - \left( \text{d.p.m. in gluconate sample} \times \frac{100}{\% \text{ recovery}} \right)}{\text{total d.p.m.}} \times 100\%$$

## C-6 Tritium

Glucose was oxidised by periodate to give formaldehyde from C-6. The tritium in a dimedone-formaldehyde complex was thus a measure of the tritium at C-6 of glycogen glucose (Bloom, 1962)



Dimedone - formaldehyde complex
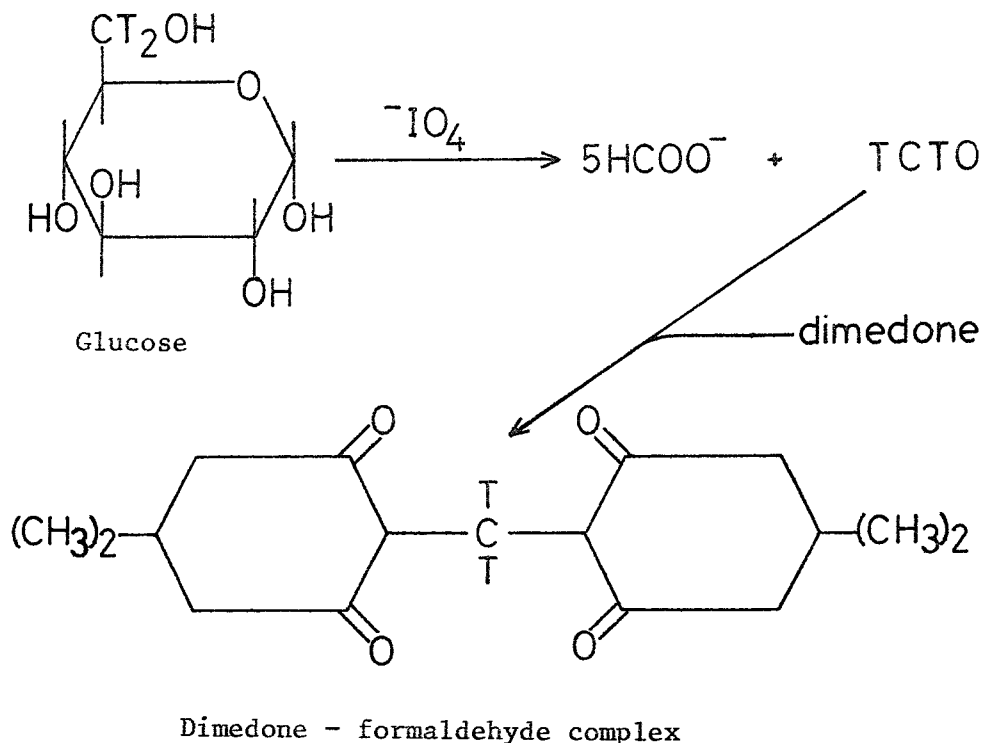
Carrier glucose (36 mg) was added to a sample of tritiated glucose
in 2 ml distilled water. To this were added 4 ml of 0.5 M-sodium phos-
phate buffer, pH7.5, and 2.4ml of 0.5 M-sodium periodate in distilled
water. After incubation at room temperature for 1h, 2 ml of dimedone
(5,5-dimethyl cyclohexane-1,3-dione) in ethanol (80 mg/ml) were then
added. The dimedone was re-crystallized twice from acetone before use.
The mixture was left to stand overnight. The resulting crystals were
filtered and re-dissolved in 3.5 ml of hot ethanol. They were re-
crystallized when cooled upon addition of 1.3 ml of distilled water.
The recovery varied between 57 and 79%.

The tritium at C-6 of glucose was calculated from the formula:-

$$\frac{\text{d.p.m.} \quad \text{in} \quad \text{dimedone complex} \quad \text{x} \quad 100}{\text{d.p.m.} \quad \text{in} \quad \text{glucose sample x \% recovery}} \quad \text{x } 100\%$$

## C-2, C-3, C-4 and C-5 Tritium

Tritiated glucose samples, containing 10 to 20 µmoles of glucose,
were sequentially degraded in a series of enzymatic reactions. During
some of these reactions substrate tritium exchanged with either water
or ethanol and was removed by freeze drying. The tritium content at the
relevant glucose position was calculated from this tritium loss expressed
as a percentage of the original total glucose tritium. The degradation
scheme is summarized in Fig. 6. The efficiencies of the reaction con-
versions were assessed by using control glucose degradations.

## C-2 Tritium

The removal of tritium from glucose C-2 was common to all procedures.
Tritiated glucose samples (200 µl) were incubated in scintillation vials
at $37^{\circ}C$ for 2h in 2 ml 0.1M-Tris H Cl buffer pH8.0 containing Mg $Cl_2$
(30 µmoles), ATP (30 µmoles), yeast hexokinase (0.25 µkatal) and
phosphoglucose isomerase (0.1 µkatal). The vials were freeze dried
and the contents reconstituted with 2 ml distilled water. The loss of
tritium represented that present at C-2 of glucose (Rose and O'Connell,
1960).

Fig. 6    Summary of Positional Analysis of Tritium at C-2, C-3, C-4 and
          C-5 of Glycogen Glucose Tritium

        Tritium at various glucose carbon atoms was exchanged with medium
water or transferred to ethanol at the reactions indicated. The tritium
content was calculated from the loss after freeze-drying. All samples were
equilibriated with hexokinase (HK) and phosphoglucose isomerase (PGI) to
remove C-2 tritium. After freeze-drying and re-constitution, the following
reactions were performed.

Fig. 7    Glucose C-5 Tritium Analysis

        The sequential actions of phosphofructokinase (PFK), aldolase(ALD)
and α-glycerophosphate dehydrogenase (GPDH) trapped tritium from glucose
C-3 as [1S-$^3$H] α-glycerophosphate. C-5 tritium was then removed by triose
phosphate isomerase (TPI) equilibrium.

Fig. 8    Glucose C-3 Tritium Analysis

        The sequential actions of phosphofructokinase (PFK), aldolase
(ALD) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) trapped tritium
from glucose C-5 as R-[2$^3$H] 3phosphoglycerate    . C-3 tritium was
then removed by triose phosphate isomerase (TPI) equilibrium.

Fig. 9    Glucose C-4 Tritium Analysis

        Tritium from glucose C-4 was transferred from [1-$^3$H] glyceralde-
hyde-3-phosphate to [4S-$^3$H] NADH by gluceraldehyde-3-phosphate dehydro-
genase (G3PDH). The HR-hydride of NADH was removed by alcohol dehydro-
genase (ADH). Because of the catalytic concentration of NAD+ used, re-
cycling of this system caused equilibriation of tritium at HS and HR of
NADH. Virtually complete transfer of tritium was obtained from substrate
to ethanol, which was removed by freeze drying.

Fig. 6   Summary of Positional Degradation Analysis of Tritium at
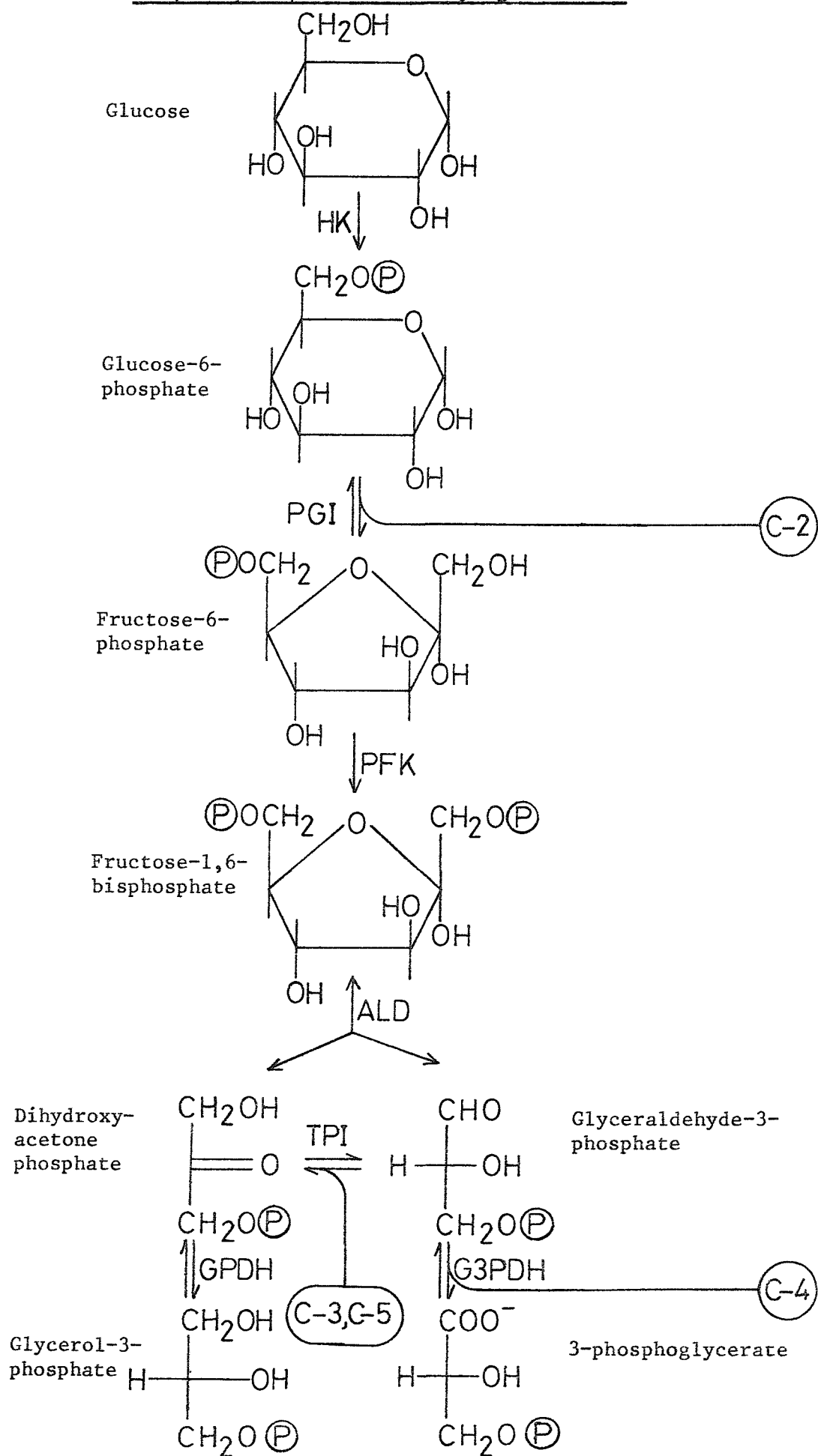C-2, C-3, C-4 and C-5 of Glycogen Glucose



Glucose

Glucose-6-
phosphate
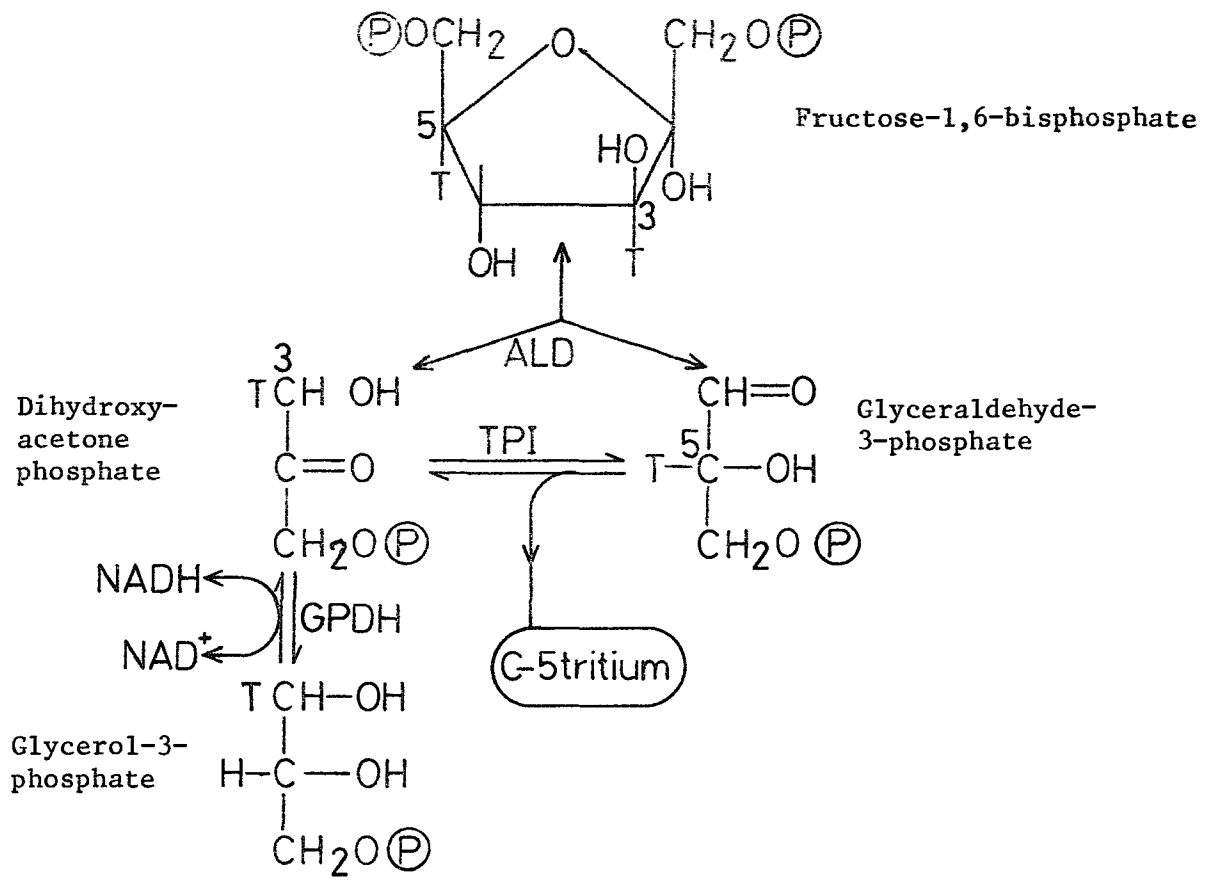
Fructose-6-
phosphate

Fructose-1,6-
bisphosphate

Dihydroxy-
acetone
phosphate

Glyceraldehyde-3-
phosphate

Glycerol-3-
phosphate

3-phosphoglycerate

Fig. 7     Glucose C-5 Tritium Analysis



Fructose-1,6-bisphosphate

Dihydroxy-acetone phosphate

Glyceraldehyde-3-phosphate

Glycerol-3-phosphate

C-5 tritium

Fig. 8     Glucose C-3 Tritium Analysis



Fructose-1,6-bisphosphate

Dihydroxy-acetone phosphate

Glyceraldehyde-3-phosphate
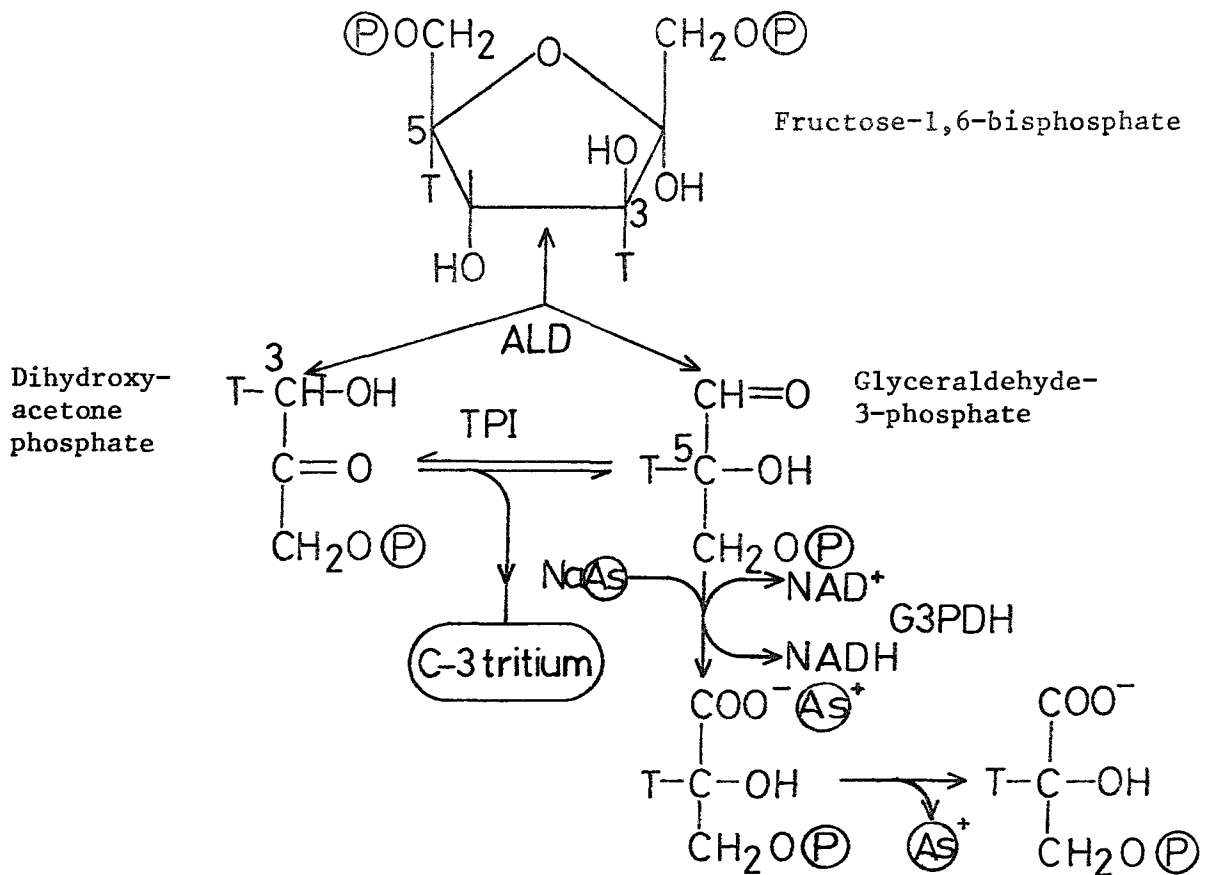
C-3 tritium

Fig.9. Scheme for Analysis of Tritium at Glucose C-4



Glucose

Glyceraldehyde
-3-phosphate

NAD+

G3PDH

S- [4-³H] NADH

ADH

CH₃CHO

CH₃CH₂OH

3-phosphoglycerate

CH₃CHTOH

[1³H]ethanol

ADH

CH₃CHO

G3P

3PG

G3PDH

R,S-[4³H]NADH
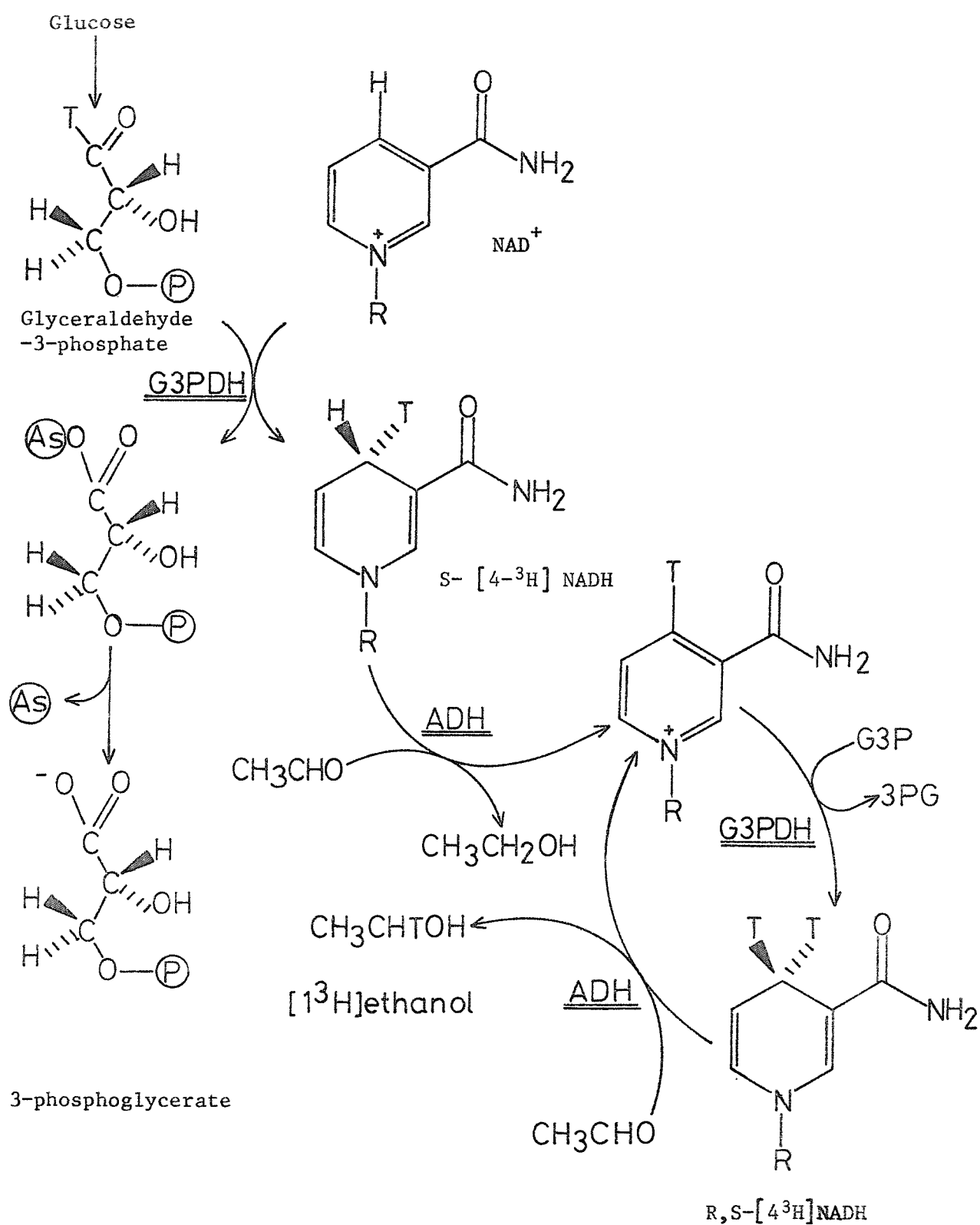
The rate of glucose phosphorylation was followed indirectly by measuring the disappearance of glucose using microbial glucose dehydrogenase (Merck). Samples from the control degradation were added to cuvettes containing 2 ml of 0.4 M-potassium phosphate buffer, pH 7.6, 0.15 M-Na Cl and $NAD^+$ (1 μmole). The reaction was initiated with 10 μlglucose dehydrogenase (0.02 μkatal). The glucose concentration was calculated from the increase in absorbance at 340 nm. Free glucose was not detectable after the first hour of incubation.

## C-5 Tritium (Fig.7)

After freeze drying and removal of tritiated water derived from [2 $^3$H] glucose, the contents of the vials for C-5 tritium analysis were re-constituted with 1 ml of water. The vials were then incubated at 37°C for 2h after the further addition of Mg $Cl_2$ (30 μmoles), ATP (30μmoles), phosphofructokinase (0.1 μkatal), fructose-1,6-bisphosphate aldolase (0.05 μkatal), α-glycerophosphate dehydrogenase (0.025 μkatal) and NADH (40 μmoles). The rate of conversion of hexose monophosphate was monitored using glucose-6-phosphate dehydrogenase. Samples from the control degradation were added to cuvettes containing 2 ml 0.1M Tris-HCl, pH80 and about 0.3 μmoles of NADH. The reaction was started by adding glucose-6-phosphate dehydrogenase (0.02 μkatal) and the decrease in absorbance at 340 nm recorded. No hexose monophosphate was detectable after the first 40 to 60 min. The vials were then heated at 70°C for 5 min to inactivate the enzymes and prevent uncontrolled removal of C-4 tritium by reversal of aldolase in the subsequent presence of triose-phosphate isomerase (Rose and Warms, 1969). Tritium at C-5 was then transferred to medium water by incubation at 37°C for 1h with triose phosphate isomerase (1.7 μkatal) and α-glycerophosphate dehydrogenase (0.025 katal) (Fig. 8; Rieder and Rose, 1959). Tritium derived from C-3 of glucose was trapped as [1-$^3$H] glycerol-3-phosphate and was retained after freeze drying. The percentage tritium at C-5 of glucose was calculated from the tritium loss after freeze drying. The efficiency of the reaction sequence was assessed by monitoring the NADH utilization in a control degradation. The total NADH oxidised was equivalent to 91% of the original glucose concentration.

C-3 Tritium (Fig.8)

To a re-constituted vial after C-2 tritium determination were added MgCl$_2$ (30 µmoles), ATP (30 µmoles), NAD$^+$ (50 µmoles), sodium arsenate (50 µmoles), phosphofructokinase (0.1 µkatal), fructose-1,6-bisphosphate aldolase (0.05 µkatal) and glyceraldehyde-3-phosphate dehydrogenase (0.15 µkatal). Tritium from C-5 of glucose was trapped at [2-$^3$H] 3-phosphoglycerate after incubation at 37°C for 2h. The vials were then heat inactivated at 70°C for 5 min. Tritium from C-3 was then transferred to water by incubation at 37°C for 1h with triosephosphate isomerase (1.7 µkatal) and removed by freeze drying (Fig. 9). The reaction progress was measured by the NADH production in a control degradation. The overall conversion of glucose to 3-phosphoglycerate was estimated at 87%.

C-4 Tritium

Tritium from C-4 of glucose was removed from C-1 of glyceraldehyde-3-phosphate and transferred via [4-$^3$H]NADH to [1-$^3$H] ethanol by the combined actions of glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase. These two enzymes have opposite stereospecificities for NADH (Section 3.1.3.5) and so using stoichiometric amounts of NAD$^+$ would result in retention of tritium in NADH. To ensure transfer of tritium to ethanol, which could be removed by freeze drying, a cyclical enzyme system was devised employing catalytic quantities of NAD$^+$ (Fig. 9 ). During the initial cycle, NAD$^+$ was reduced to [4S - $^3$H] and reoxidised to [4 - $^3$H]NAD$^+$, the 4Rhydride being donated to ethanol. In subsequent cycles, formation of [4R,S-$^3$H] NADH enabled tritium to be transferred from substrate to ethanol. Theoretically, if the NAD$^+$ concentration was 1% of the substrate concentration, then 99% of the tritium should have been transferred to ethanol not NADH.

To a reconstituted vial after C-2 determination were added Mg Cl$_2$ (30 µmoles), ATP (30 µmoles), NAD$^+$ (0.2 µmoles), sodium arsenate (50 µmoles), acetaldehyde (50 µmoles), phosphofructokinase (0.1 µkatal), fructose-1,6-bisphosphate aldolase (0.05 µkatal), glyceraldehyde-3-phosphate dehydrogenase (0.15 µkatal) and alcohol dehydrogenase (1.5 µkatal). The vial was incubated at 37°C and the reaction progress

monitored by measuring the utilization of acetaldehyde. Samples of a
control reaction (10 to 20 μl) were added to cuvettes containing 2 ml
of 0.1M-Tris H Cl, pH8.0 and NADH (about 0.3 μmoles). The reaction
was started with alcohol dehydrogenase (0.01 μkatal) and the acetalde-
hyde concentration calculated from the change in absorbance of NADH at
340 nm. Acetaldehyde conversion to ethanol was complete after 90 to
110 min; this represented an overall glucose conversion of 94%. Tritium
from C-4 of glucose was calculated from the tritium loss after freeze
drying.

## 2.3. Measurement of Enzyme Activity

### Liver Homogenization

Extracts of liver were made using a motor driven teflon-glass homo-
geniser. The buffer contained 100 mM-potassium phosphate, pH7.0,
2mM-disodium EDTA, 5mM-reduced glutathione and 50mM sodium fluoride.
Frozen liver powder (500 mg) was homogenised in buffer (5 ml final volume)
at $4^{\circ}C$ and centrifuged at 100,000 x g av. in an 18 x 6 ml angle rotor
at 10 - $15^{\circ}C$ for 10 min. After discarding the fat cake, the clear
supernatant was used for enzyme assay.

### 2.3.1. Acetyl Coenzyme A Carboxylase (Acetyl CoA : Carbon Dioxide Ligase, EC.6.4.1.2)

The activity of acetyl CoA carboxylase was measured by the rate of
fixation of[$^{14}C$]bicarbonate into malonyl CoA, after Halestrap and Denton
(1973). The assay without prior citrate activation was performed within
25 min of first thawing the liver powder to give a possible indication
of the activity inside the cell in vivo. The maximum catalytic activity
of the enzyme was exposed by preincubation of homogenate supernatant
with citrate at $37^{\circ}C$ for 30 min.

### Synthesis of Acetyl CoA Substrate

Acetyl CoA was synthesized from acetic anhydride and coenzyme A
according to Simon and Shemin (1953). Coenzyme A, trilithium salt
(10 μmoles) was dissolved in distilled water and added to a solution of

acetic anhydride (10 µmoles) in distilled water (500 µl). The reactants were kept at $4^{\circ}C$ in an ice bath and at pH 6-7 with a drop of 1M-sodium hydrogen carbonate. The loss of free thiol groups was monitored by the nitroprusside spot test. The reaction was complete by 30 min when the mixture was acidified to pH2-3 with 0.1M H Cl and extracted twice with 2 ml of diethyl ether to remove unreacted acetic acid. Residue diethyl ether was evaporated under a stream of nitrogen and the pH raised to pH 5.0 with 5-M sodium hydrogen carbonate. The acetyl CoA solution was made up to 2 ml final volume and stored in 500 µ 1 portions at $-20^{\circ}C$. The acetyl CoA concentration was measured using citrate synthase and malate dehydrogenase (Decker, 1974).

## Nitroprusside Spot Tests for Thiols

The nitroprusside reagent of Tonnies and Kolb (1951) was used. Sodium nitroprusside (1.5 g) was dissolved in 1M-sulphuric acid (5 ml) and absolute methanol (95 ml) added. Ammonia solution (0.88 S.G., 10 ml) was added and the resulting white precipitate filtered off. The clear orange filtrate was stored at $0^{\circ}C$. Aliquots of sample and then reagent were placed on a filter paper. Free thiol groups were indicated by a red colour. Thiol esters were indicated by the red colour appearing only after further treatment with 10% (w/v) methanolic KOH.

## Acetyl CoA Carboxylase Assay

Samples (50 µl) of the 100,000 x g supernatant were added to 400 µl of buffer/substrate cocktail in 1.4 ml plastic centrifuge tubes. This mixture contained final concentrations of 100mM-Tris H Cl pH7.4, 10mM-Mg $Cl_2$, 0.5mM-disodium EDTA, 1mM-glutathione, 2.5mM-ATP, 0.6mM-acetyl CoA, 1% (w/v) delipidated albumin and 16.5mM-NaH $^{14}CO_3$ (1m Ci/m mol). The incubation at $37^{\circ}C$ was terminated after 3 min by adding 50µl of 10% (w/v) perchloric acid. The tubes were centrifuged in an Eppendorf 3200 centrifuge at 12,000 x g for 30 sec, and 500 µl of the resulting supernatants transferred to scintillation vials. Substrate[$^{14}$C]bicarbonate was removed under a stream of air and the acid stable radioactivity determined by liquid scintillation counting. This value was corrected by the $^{14}$C incorporation in a blank reaction containing no acetyl CoA.

Maximum catalytic activity of acetyl CoA carboxylase was measured
after pre-incubation with 20 mM-trisodium citrate and 1% (w/v) de-
lipidated albumin at $37^{\circ}$C for 30 min. The activity without pre-incubation
of supernatant was measured in the presence of 2 mM-tri-sodium citrate
to enable direct comparisons to be made.

### 2.3.2. Pyruvate Kinase (ATP : Pyruvate Phosphotransferase, EC.2.7.1.40)

Pyruvate kinase activity in the 100,000 x g liver supernatant was
measured by following the utilization of NADH in the linked lactate
dehydrogenase reaction (Bucher and Pfleiderer, 1955). Supernatant was
diluted in homogenizing buffer to 1% (w/v). Samples of the dilute
solution (20 µl) were added to cuvettes containing 25 mM-Tris H Cl,pH7.5,
100 mM-K Cl, 10mM-Mg $Cl_2$, 0.2mM-NADH, 2mM-dithiothreitol, lactate
dehydrogenase (0.02 µkatal) and up to 10mM-phosphoenol pyruvate in a
final volume of 1.02 ml. The reaction was initiated by the addition
of 5 mM-ADP (100 µl) and the decrease in NADH absorbance at 340 nm
measured for 1 min. This assay was automated on an LKB reaction rate
analyzer, model 3,000. The instrument settings were full scale deflection
0.05 absorbance units, back-off 0.2 absorbance units, initial delay
15 sec, temperature $37^{\circ}$C and decrease absorbance mode.

The activity of pyruvate kinase was measured at phosphoenol pyruvate
concentrations from 0.125 to 10m mols/l and corrected for the activity
in the absence of added substrate. Pyruvate kinase activity was not
changed by freezing liver samples before homogenization (Fig. 10).

Hepatic pyruvate kinase was inactivated in vitro by incubation of
100,000 x g supernatant with cyclic AMP. Supernatant samples (100 µl)
were incubated at $37^{\circ}$C for 30 min in 1 ml final volume of buffer con-
taining 25 mM-Tris H Cl, pH7.5, 100 mM-K Cl, 10mM-Mg $Cl_2$, 10 mM-ATP and
10 µm cyclic AMP. Samples (20 µl) of this diluted homogenate solution
were then assayed for pyruvate kinase.
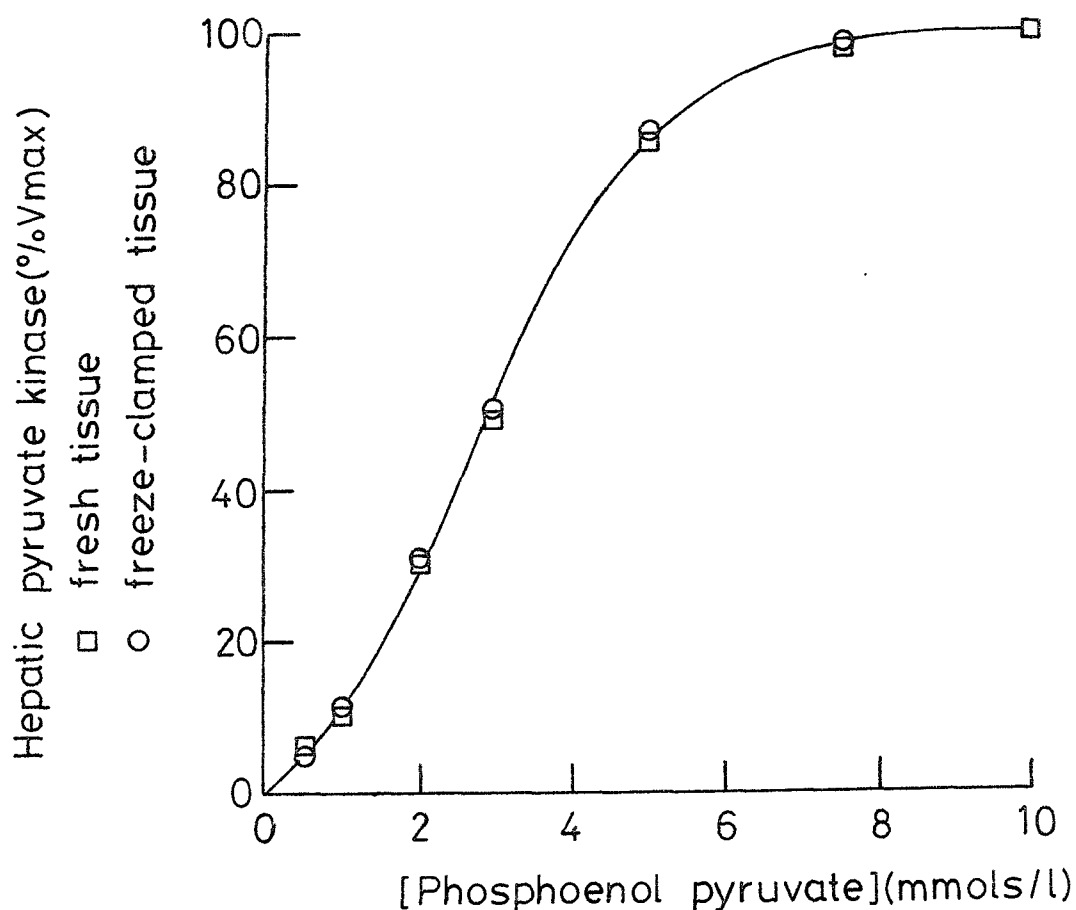
Fig. 10    Effect of Freeze-clamping in Liquid $N_2$ on Hepatic Pyruvate
           Kinase Activity

2.3.3.   ATP:Citrate Lyase (EC.4.1.3.8), Glucokinase (EC.2.7.1.2)
         and Glucose 6-Phosphate Dehydrogenase (EC.1.1.1.49)

ATP citrate lyase activity was measured by the rate of NADH utili-
zation in the linked malate dehydrogenase reaction (Foster and Srere, 1968).
Glucokinase activity was measured by the rate of formation of NADPH in
the linked glucose-6-phosphate dehydrogenase reaction (Pilkis, 1975).
The enzyme activity at a substrate glucose concentration of 100mM was

56

Fig. 11    Automated Lowry Protein Analysis



Colorimeter,700nm
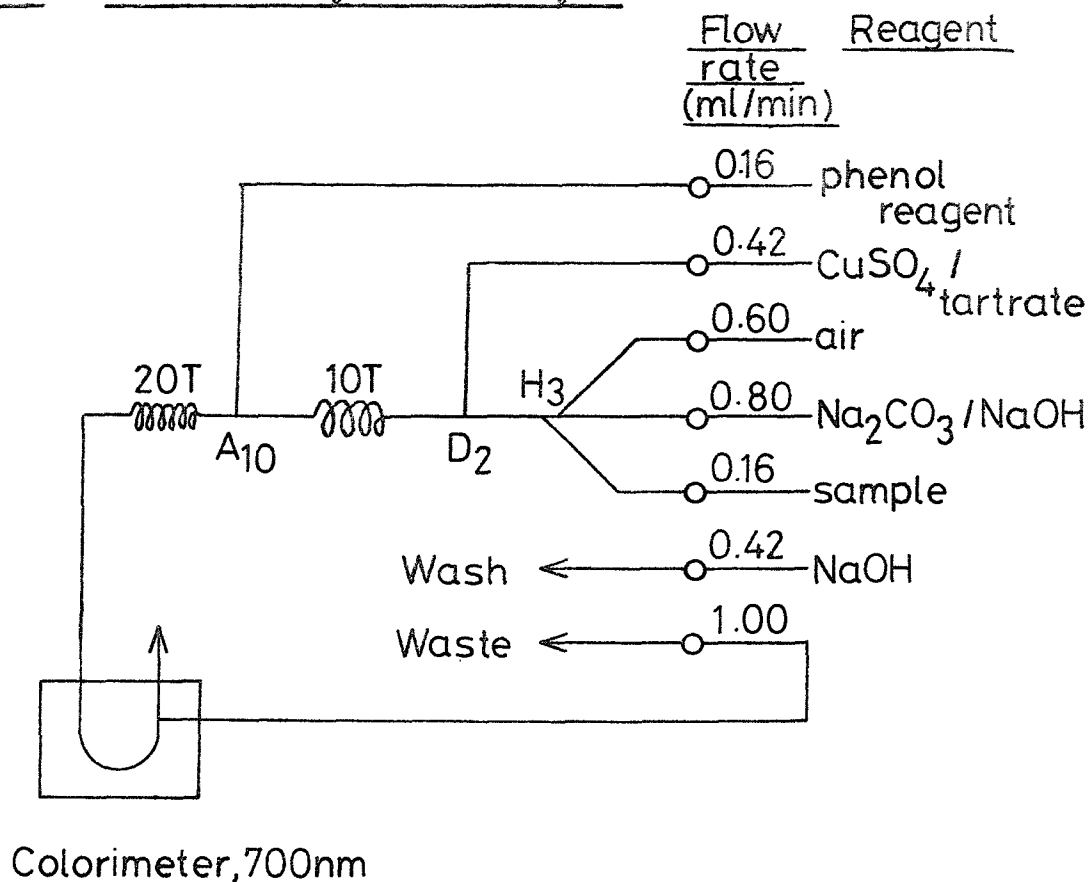
Reagent Composition

1.  50% (v/v) Folin and Ciocalteu's phenol reagent.

2.  2% (w/v) $Na_2CO_3$;  0.1M-NaOH;  0.01% (v/v) Tween 80.

3.  0.04% (w/v) Cu $SO_4$;  0.08% (w/v) NaK tartrate.

4.  0.1M NaOH.

corrected for hexokinase activity measured at 0.5mM-glucose. Glucose
6-phosphate dehydrogenase was measured by the rate of formation of
NADPH (Borner and Mattenheimer, 1959). All these assays were performed
on an LKB reaction rate analyzer.

## 2.4. Concentration Measurements

### 2.4.1. Protein

The protein concentration of the 100,000 x g supernatant was
measured by a semi-automated adaptation of the method of Lowry et al
(1951). The autoanalyzermanifold diagram and reagent composition are
shown in Fig. 11. The sampling rate was 80/h with a sample ratio of 2:1.
Carryover between samples was less than 5%. Supernatant samples (20 μl)
and bovine serum albumin standards, containing from 0.2 to 1.0 mg of
protein, were precipitated with 10% (w/v) trichloracetic acid. The
precipitates obtained after centrifugation at 12,000 x g for 30 sec
were dissolved in 1 ml of 0.1 M-NaOH and aspirated directly into the
autoanalyzer.

### 2.4.2. Blood Glucose, Lactate, Pyruvate, Alanine and 3-Hydroxybutyrate Analysis

Blood (100 μl) sampled from heart puncture or tail vein was quenched
in ice-cold 3% (w/v) perchloric acid (2 ml) and the precipitated protein
removed by centrifugation. The metabolite concentrations were measured
directly on the acid supernatant by enzymatic methods automated for
fluorometric analysis by autoanalyzer (Lloyd et al, 1978).

### 2.4.3. Triglyceride Analysis

Serum

Serum triglyceride was measured by a fully enzymatic automated
method (Postle and Goodland, 1978). The autoanalyzer manifold, reagent
composition and the reaction    sequence employed in this analysis are
shown in Fig. 12

Fig. 12    Serum Triglyceride     Flow rate(ml/min)  Reagent
           Analysis

20T        5T
           A10      H3
                         0.16   sample
                         0.80   buffer
                         0.23   air
                         0.16   lipase/esterase

           5T     A10
                  H3
                         0.16   glycerokinase
                         0.80   buffer
                         0.23   air
                         0.23   NADH /ATP/PEP

dialyser,
12″path
                         0.20   NaCl wash

waste
20T
                         1.00

waste

fluorimeter, 470nm

## Reaction sequence

Triglyceride $\xrightarrow{\text{lipase}}$ glycerol $\xrightarrow{\text{GLK}}$ glycerol-3-phosphate

ATP → ADP

lactate $\xleftarrow{\text{LDH}}$ pyruvate $\xleftarrow{\text{PK}}$ phosphoenol pyruvate

$NAD^+$  NADH

## Reagent composition

1.  50mM-triethanolamine buffer, pH 7.3;  4mM-$MgCl_2$; 0.05% (v/v) Tween 20.

2.  Lipase  90 U /ml;  esterase  150mU/ml.

3.  Glycerokinase  60 mU/ml;  LDH  3U/ml;  PK  0.5 U/ml.

4.  0.4mM-NADH;  1.8mM-ATP;  1.2mM PEP.

Fig. 13    Liver Triglyceride Analysis
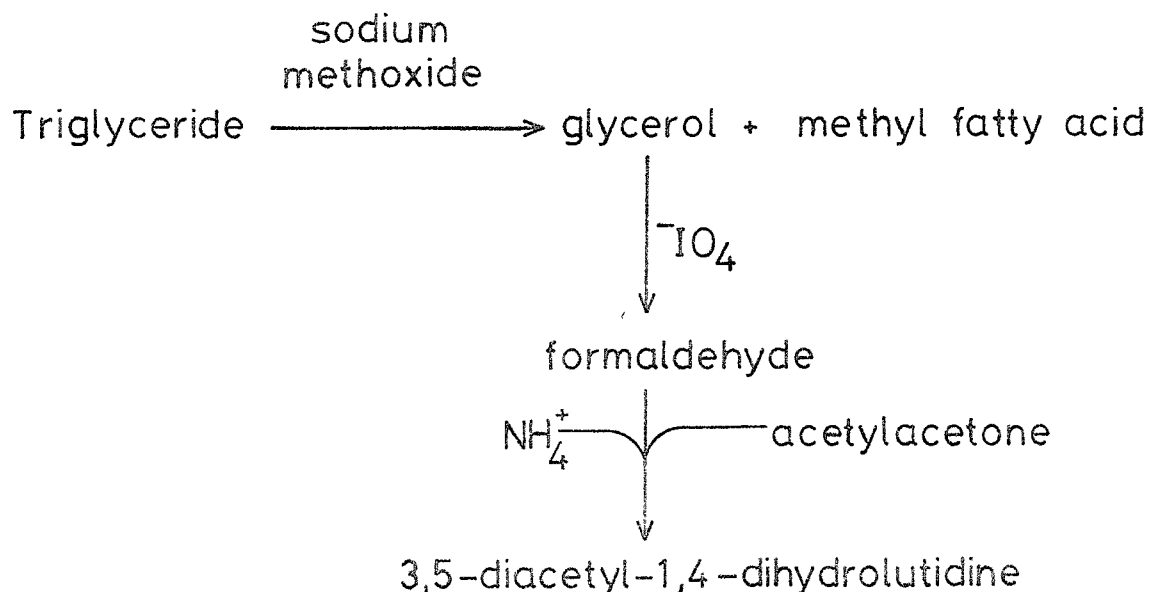


colorimeter, 410nm
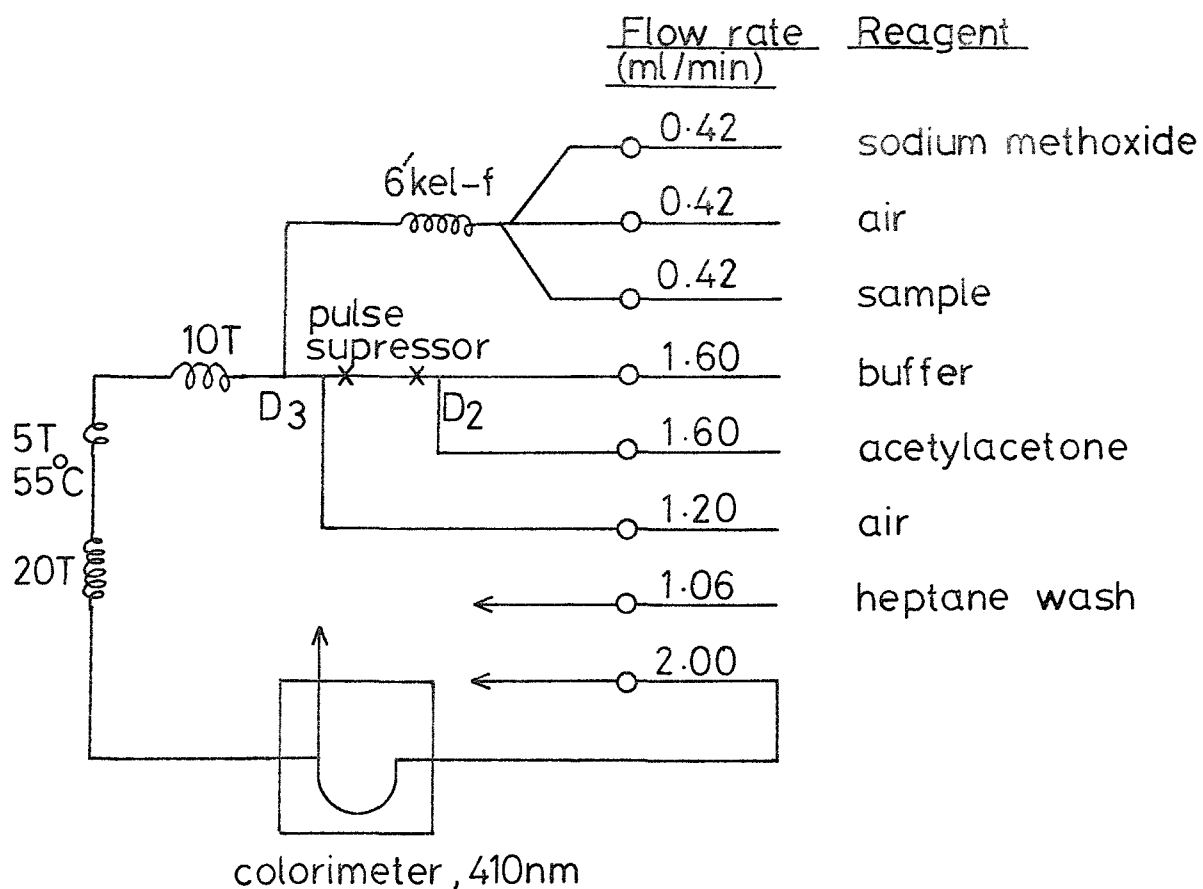
Reagent Composition

1.  0.9% (w/v) sodium methoxide in propan-2-ol:heptane (1:1,v/v).

2.  3.0 M-ammonium acetate buffer (pH 5.9), 42mM sodium periodate.

3.  2.0% (w/v) acetylacetone.

## Liver

Liver triglyceride was measured by an adaptation of a semi-automated chemical method (Postle and Goodland, 1978). Frozen liver powder was homogenized (5% w/w) in $0.04$ M-$H_2SO_4$. An homogenate sample (1.0 ml) was extracted with 3 ml of a mixture of nonane : isopropanol (2:1 v/v). The upper organic phase was aspirated directly into the autoanalyzer. The manifold diagram is shown in Fig. 13. The reaction principles are described below. The absorption of the product of the Hantzsch condensation reaction, 3,5-diacetyl-1,4-dihydrolutidine, was measured at 410 nm.

$$\text{Triglyceride} \xrightarrow{\text{sodium methoxide}} \text{glycerol} + \text{methyl fatty acid}$$

$$\downarrow ^-IO_4$$

$$\text{formaldehyde}$$

$$NH_4^+ \diagdown \diagup \text{acetylacetone}$$

$$\downarrow$$

$$\text{3,5-diacetyl-1,4-dihydrolutidine}$$

Reaction Sequence of Liver Triglyceride Analysis

### 2.4.4. Insulin Analysis

Serum insulin was measured by a micromodification of the radio-immuno assay method of Soeldner and Stone (1965).

### 2.4.5. Corticosterone Analysis

Corticosterone concentration in serum was measured by a radio-immunoassay adapted from a method for cortisone (Seth and Brown, 1978).

The method employed an [$^{125}$I] corticosterone derivative and a solid-phase antibody linked to micro-crystalline cellulose.

## Preparation of Corticosterone Derivative
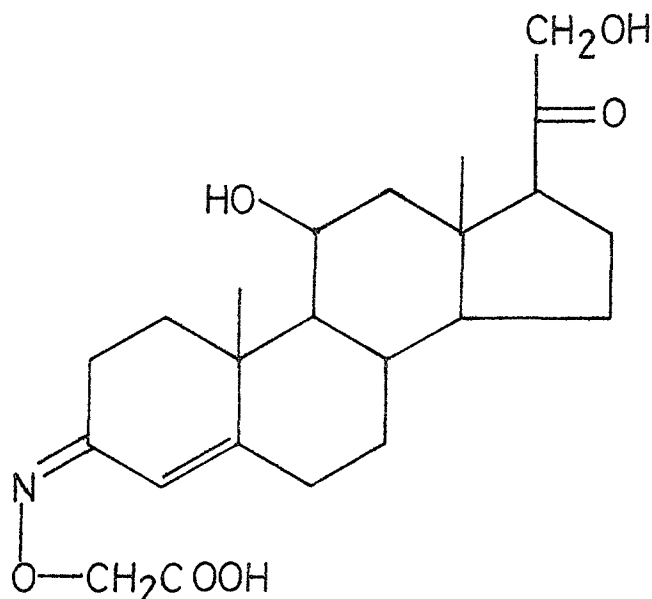


Corticosterone-3N-Carboxymethyloxime

Corticosterone-3N-carboxymethyloxime was synthesized via a
3-enamine intermediate (Janoski et al, 1974;   Fahmy et al, 1975).
Corticosterone (1.5 m mole) was dissolved in ethanol (8 ml) and reacted
with pyrrolidine (6.0 m moles), followed by 0-(carboxymethyl) hydroxy-
lamine hemihydrochloride (1.5 m moles). The mixture was heated at 50°C
for 5 min and then dried by rotary evaporation. The product was purified
by 4 alternate partitions into ethyl acetate at pH2.0 and into 1.0 M-
NaOH and then re-crystallized twice from 50% (V:V) ethanol. The yield of
colourless crystals was 23%.

The steroid oxime derivative was iodinated to give corticosterone-3-(O-carboxymethyl)oximo-[$^{125}$I]-iodohistamine (Nars and Hunter, 1973). Dioxan (250 μl) , tri-N-butylamine (10 μl) and isobutylchloroformate (10 μl) were incubated at 10°C for 30 min with corticosterone 3-N-carboxymethyl-oxime (3.35 μmoles). The mixture was diluted with dioxan (1.5 ml) and 0.1M-NaOH (10 μl). The [$^{125}$I]-iodohistamine was prepared by adding histamine (2.22 μg) and chloramine T (50 μg) dissolved in 0.25M-potassium phosphate buffer, pH8.0 (20 μl) to 1 mCi Na[$^{125}$I] (71.8 Ci/mole). After 1 min at room temperature , sodium metabisulphite (300 μg) and oxime mixture (50 μl) were added. After reacting at 0°C for 2h the [$^{125}$I] derivative was purified by thin layer chromatography on silica gel G using the solvent system benzene : ethanol : acetic acid (75:4:1). The purified label (Rf 0.3) was eluted and stored in ethanol. The recovery of radioactivity was 40% and the derivative was stable at 4°C for at least 3 months. Corticosterone and histamine were supplied by Sigma and all other chemicals by the Aldrich Chemical Co..

## Preparation of Corticosterone Antibody

Anti-corticosterone goat serum was kindly donated by Dr. Peter Wood, Department of Chemical Pathology, Southampton General Hospital. Cyanogen bromide activated microcrystalline cellulose (1.0g, Wide, 1969) was added to 500 μl serum in 2 ml 0.1M-sodium borate buffer, pH8.6 The mixture was rotary mixed at 4°C for three days and the solid-coupled antibody washed successively three times with 0.1M-sodium borate, pH8.6 (20 ml), twice with 0.1M-sodium acetate, pH4.0 (20ml) and then three times with assay buffer (20 ml). The antibody suspension was stored in assay buffer at 4°C at a 1:50 dilution.

### Assay Procedure
Reagents
1) Assay buffer, pH4.0, containing 0.1M-citric acid, 0.2M-disodium hydrogen phosphate, 0.2 g/l thiomersal, 1.0g/l gelatine.
2) Corticosterone standards from 62.5 nM to 8 μM dissolved in dexamethasone-suppressed sheep serum.
3) $^{125}$I -corticosterone derivative, approximately 200,000 c.p.m./ml in assay buffer.

4) Antiserum, 1:5,000 final dilution in assay buffer.

5) Diluent, 0.1M-NaCl, 2g/l Brij30, 2g/l cellulose.

## Method

Duplicate samples (10 μl) of test, standard and control pooled sera were dispensed into 2 ml plastic tubes, followed by label (100 μl) and antiserum (200 μl). The tubes were mixed every 15 min during incubation at $37^{o}C$ for 1h. Diluent (1.5 ml) was then added and the tubes centrifuged at 2,000xg for 15 min. The supernatant was aspirated and the pellet radioactivity measured. Non-specific binding measured in the absence of antibody was typically 2 to 5% of the total bound counts. The radioactivity in the pellets of the standard tubes was plotted against $\log_{10}$ corticosterone concentration. The sample corticosterone concentration was determined from this graph.

## 2.5. General Techniques

### Measurement of Radioactivitity

The β-radioation of samples was measured in a Packard Model 3090 liquid scintillation spectrometer(Packard Instruments Ltd., Caversham, Berks). Results were corrected for quenching by the external standards ratio method. The scintillation fluid employed throughout consisted of 2,5-diphyloxazole (5.0 g/l) and 1,4-bis- 2-(4-methyl-5 phenyloxazolyl) – benzene (0.3 g/l) dissolved in a mixture of toluene : triton x -100 (2:1,v:v). Gamma radiation was measured on an LKB-Wallac gamma counter (LKB Instruments Ltd., Croyden, Surrey).

### Optical Measurements

All spectrophotometric measurements of optical density were made on a digital single beam Zeiss PMQ-3 instrument (Carl Zeiss Oberkochen Ltd., London). A molar extinction coefficient for NADH and NADPH at 340. nm of $6.22 \times 10^{3} cm^{-1}$ was assumed in the calculation of results. The auto-analyzer equipment was supplied by Technicon Instruments Co. Ltd., Basingstoke, Hants, while the reaction-rate analyzer was manufactured by LKB Instruments Ltd.

### Centrifuges

The centrifuges employed were an MSE 65 superspeed and MSE 'Mistral'

(MSE Scientific Instruments, Crawley, Sussex) and an Eppendorf 3200 'microfuge' (Anderman & Co. Ltd., East Molesey, Surrey).

## Materials

All radioactive compounds were purchased from the Radiochemical Centre, Amersham. Enzymes and co-factors were purchased from either Sigma or Boehringer. General chemicals were purchased from BDH.

CHAPTER 3

HEPATIC GLYCOGEN SYNTHESIS

3.1. Introduction

Hepatic glycogen serves as a reserve to maintain blood glucose homeostasis and makes little contribution to the energy requirements of the liver (Krebs, 1972). This function of hepatic glycogen differs from that of muscle glycogen, which fuels the major metabolic process of muscle contraction (Rosell and Saltin, 1973). The hepatocyte is capable of storing glycogen at a concentration in excess of 10% of the total wet weight (Hers, 1976). Liver glycogen is rapidly deposited after feeding and slowly degraded during subsequent fasting to maintain normal glycaemia (Stalmans, 1976). After fasting for 48h hepatic glycogen concentration is negligible and thus makes no contribution to metabolism during more prolonged starvation (Steiner and King, 1964).

The enzymology of hepatic glycogen metabolism was discussed in Section 1.3.1. Briefly, the rate limiting enzymes of glycogen synthesis and degradation are glycogen synthase and phosphorylase (Hers et al, 1970). The system of kinases and phosphatases regulating these enzyme activities (Cohen, 1978) is arranged so that in the fed rat hepatic glycogen synthase and phosphorylase are not both active at the same time (Stalmans, 1974). For a large series of rats over a wide range of nutritional states glycogen synthase was only activated after phosphorylase was inactivated by at least 90%.

The rate of hepatic glycogen synthesis can be estimated by measuring either changes in glycogen concentration or the incorporation of radio-active precursors. The principles and advantages of such methods are discussed below.

3.1.1. Glycogen Concentration Changes

Glycogen concentration at any moment is the net sum of glycogen synthesis and degradation. Glycogen concentration changes are therefore only a measure of glycogen synthesis when the rate of degradation can be

shown to be negligible. Theoretically, if both pathways were equally highly active, a high rate of glycogen synthesis would be possible with no corresponding change in glycogen concentration. In isolated hepatocyte preparations, synthesis of glycogen is often preceded by an initial phase of glycogen degradation. In such circumstances, measurement of increased concentration would underestimate the true rate of glycogen synthesis.

### 3.1.2. Incorporation of $[^{14}C]$-Precursors

The incorporation of a $[^{14}C]$-precursor into liver glycogen is affected by a number of factors other than the absolute rate of glycogen synthesis. These include the significance of that substrate as a glycogen precursor, dilution with endogenous unlabelled substrate and the existence of exchange reactions and substrate cycles.

The classical theory of the role of liver glycogen in the regulation of blood glucose homeostasis was developed from the experiments of Soskin (Soskin et al, 1938; Stalmans, 1976; Fig. 14). It holds that when blood glucose after feeding exceeds a threshold concentration glucose is converted directly into liver glycogen. The physiological relevance of such a conversion has been questioned (Katz et al, 1979) because the glucose loads administered were massive and free glucose is a minor component of most diets. Moreover, in vivo hepatic uptake of glucose was difficult to demonstrate after rats were fed a diet containing 40% starch (Rémésey et al, 1978). The results obtained after feeding a diet comprising 80% starch confirmed earlier studies that substantial net hepatic glucose uptake only occurred after feeding a high carbohydrate diet (Landau et al, 1961).

It is evident that incorporation of $[^{14}C]$-glucose cannot measure hepatic glycogen synthesis when there is no net conversion of glucose to liver glycogen. Further evidence supporting the limited significance of glucose as an hepatic glycogen precursor comes from in vitro liver preparations.

In perfused liver from starved rats 30mM-glucose maintained a glycogen synthetic rate of 0.17 µmoles/g/min, which was at most 20% of

Fig. 14    Auto-regulation of Hepatic Glycogen Metabolism by
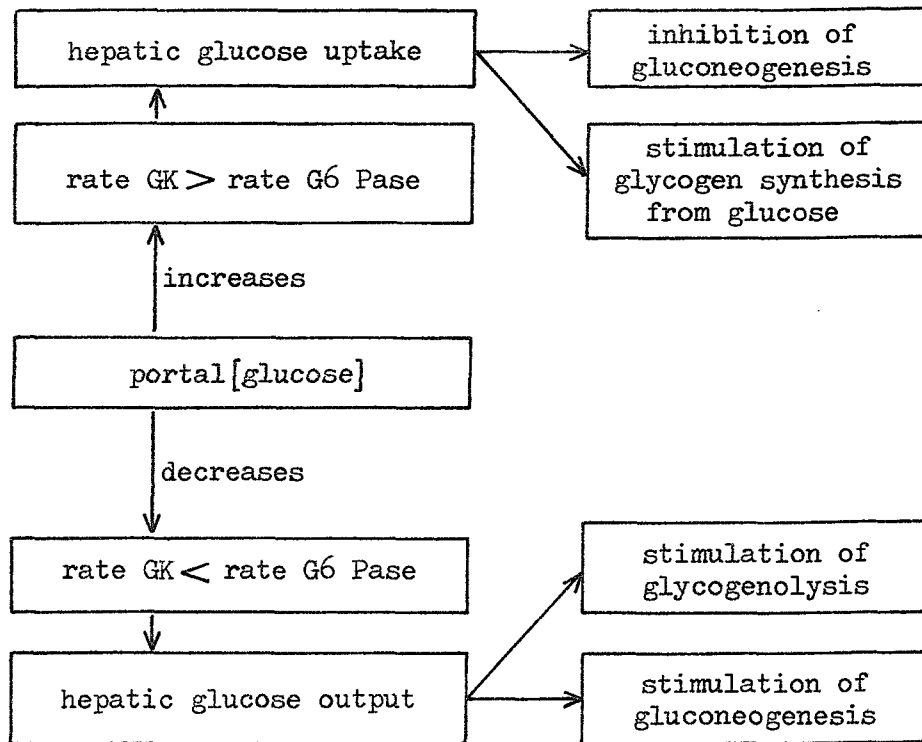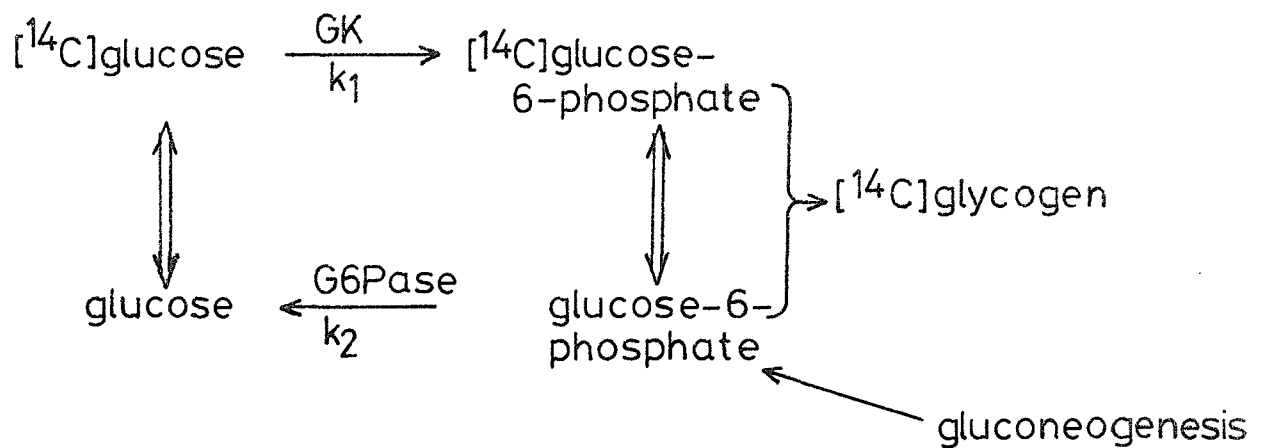           Portal [Glucose]



Fig. 15    Incorporation of $[^{14}C]$ Glucose into Hepatic Glycogen
           During Substrate Cycling



When glucokinase, kl < glucose-6-phosphatase, k2, and $[^{14}C]$ glucose
exchanges with glucose-6-phosphate, then $[^{14}C]$ glycogen can be formed
from gluconeogenesis with no net uptake of glucose by the liver.

the rate observed in vivo at a glucose concentration of about 10mM (Hems et al, 1972). Addition of a mixture of gluconeogenic precursors (pyruvate, serine and glycol, each initially 5mM)not only activated glycogen synthesis by a factor of four but also resulted in glucose production even above the initial glucose concentration of 30mM. Under these conditions glucose was unable to compete with gluconeogenic substrates as a glycogen precursor.

Similar difficulties have been observed using isolated hepatocyte preparations. Cells from both starved and fed rats make little glycogen from glucose at concentrations below 30mM. This rate could be stimulated by increasing the medium concentration of potassium ions (Hue et al, 1975) or lithium ions (Nyfeler and Walter, 1979) or by supplying additional gluconeogenic precursors (Katz et al, 1979). Of the latter, the greatest stimulation was caused by amino acids such as glutamine (Katz et al, 1976). As the addition of gluconeogenic substrates increased both glycogen and glucose production there could have been no net conversion of glucose to glycogen.

Despite the absence of any net conversion, incorporation of $[^{14}C]$-glucose into glycogen can be demonstrated (Hue et al, 1976; Katz et al, 1979). This is possible because of the existence of substrate cycles which make the entire glycolytic/gluconeogenic pathway isotopically reversible regardless of the direction of net substrate flux (Section 1.3.2; Katz and Rognstad, 1976). The exchange between glucose and glucose-6-phosphate, catalyzed by glucokinase and glucose-6-phosphatase, is rapid and enables the glucose-1-phosphate pool used for glycogen synthesis to become labelled from $[^{14}C]$-glucose even when the liver is producing glucose (Fig. 15). Further, such substrate cycles between fructose-6-phosphate and fructose-1,6-biphosphate and between phosphoenolpyruvate and pyruvate, enabled label from $[^{14}C]$-glucose to appear as $[^{14}C]$ lactate even in hepatocytes from starved rats which were producing glucose from lactate.

The argument discussed for $[^{14}C]$-glucose incorporation applies to the incorporation of any other $[^{14}C]$-substrate into glycogen. Conversion of $[^{14}C]$-glycogen into an absolute rate of synthesis requires knowledge

of the specific radioactivity of the substrate α -D-glucose-1-phosphate pool in the cytoplasm. This measurement would be difficult and imposes a technical limitation on the incorporation of [$^{14}$C] substrates as a measure of rates of hepatic glycogen synthesis.

### 3.1.3. Measurement of Absolute Rates of Biosynthetic Pathways by the Incorporation of Tritiated Water

There are reactions in many metabolic pathways which result in the incorporation of protons from cellular water into their products. Substitution of deuterated or tritiated water results in the respective incorporation of deuterium or tritium, which can be used as a measure of the rate of synthesis of the pathway concerned. Tritiated water incorporation has a number of general advantages for measuring biosynthetic rates. Water equilibriates rapidly throughout the body and is not subject to intra-cellular compartmentation. The specific radioactivity of the single tritiated water substrate pool is readily calculated from the tritiated water content of tissue water. Thus, in direct contrast to [$^{14}$C] substrate incorporation, tritiated water incorporation can be directly converted to an absolute rate. Tritiated water incorporation is equally applicable to _in vivo_ or _in vitro_ measurements. One limiting factor is the extent of any kinetic isotope effect, discussed below. This is followed by theoretical considerations of tritiated water incorporation in the measurement of fatty acid biosynthesis and glycogen biosynthesis.
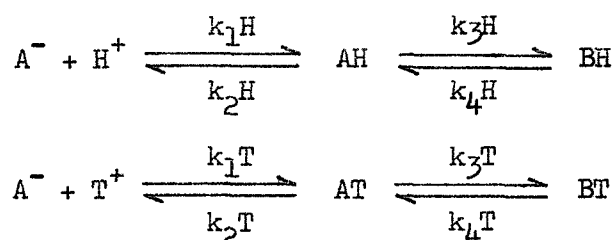
### 3.1.3.1. Kinetic Tritium Isotope Effects

The thermodynamic properties of reactions are substantially altered by substitution of isotopes in the reactants (Rose, 1970; Richards, 1970). The ground state vibrational energy of a covalent bond decreases as the mass of a bound isotpoic atom increases, but the transition state energy does not. A greater activation energy is required to form or break a bond containing a heavier isotope, which leads to a slower rate of reaction. Such primary isotope effects are most prominent for hydrogen, as the relative increase in isotope mass is greater than for other atoms. The rate of cleavage of covalent hydrogen bonds decreases in the order hydrogen > deuterium > tritium.

Carbon-hydrogen bonds are formed by protonation of a carbanion:-

$$A^- + H^+ \underset{k_2H}{\overset{k_1H}{\rightleftharpoons}} AH$$

$$A^- + T^+ \underset{k_2T}{\overset{k_1T}{\rightleftharpoons}} AT$$
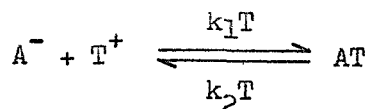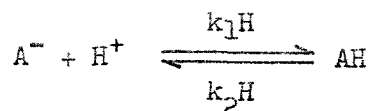
When this reaction takes place in tritiated water, $k_1H > k_1T$ and a primary tritium isotope effect is observed. Enzymatic reactions, however, normally proceed by multi-step mechanisms, for instance:-

$$A^- + H^+ \underset{k_2H}{\overset{k_1H}{\rightleftharpoons}} AH \underset{k_4H}{\overset{k_3H}{\rightleftharpoons}} BH$$

$$A^- + T^+ \underset{k_2T}{\overset{k_1T}{\rightleftharpoons}} AT \underset{k_4T}{\overset{k_3T}{\rightleftharpoons}} BT$$

Any tritium isotope effect measured in this reaction depends on the relative values of the respective rate constants. If $k_1 > k_3$ and $k_2 > k_4$, then the protonation step is not rate-limiting and a rapid proton exchange will occur. In the forward direction $k_1H > k_1T$ and so discrimination against tritium incorporation will occur. Any tritium incorporated, however, will tend to be retained in the reverse reaction, as $k_2H > k_2T$. A rapid protonation equilibrium will tend to reduce the magnitude of the observed isotope effect. A majority of enzymes are at equilibrium in vivo and the overall reaction flux is determined by a non-equilibrium rate-limiting reaction. If the activity of a reversible enzyme which incorporates tritium from tritiated water greatly exceeds the in vivo flux through that enzyme, then the observed tritium isotope effect will again be lower than the predicted value. These considerations demonstrate that the theoretical discrimination against tritium incorporation need not necessarily apply in vivo.

### 3.1.3.2. The Measurement of Fatty Acid Synthesis using Tritiated Water Incorporation

The incorporation of tritiated water is a widely used technique for the quantitation of rates of fatty acid biosynthesis. As the method for

measuring glycogen synthetic rates described in this is also based on
tritiated water incorporation, the principles of the fatty acid synthesis
method are presented below for comparison. In the classical studies of
Schoenheimer and Rittenberg (1936; 1937), rats fed a fat free diet were
injected with deuterated water daily. After 21 to 98 days the percen-
tage of fatty acid hydrogen derived from the body water approached a
plateau at 43‰. Subsequently the use of tritiated water has enabled
radioactivity incorporation to be measured conveniently over relatively
brief incubation periods (Foster and Bloom, 1963; Windmueller and
Spaeth, 1966).

## Possible Reactions of Fatty Acid Synthesis incorporating Tritiated Water

## Acetyl CoA Carboxylase

The mechanism of acetyl CoA carboxylase consists of two half reactions

## Fig.16. Acetyl CoA Carboxylase

1) Biotinyl carboxylase:-

$$\text{Biotin-E+HCO}_3^- + \text{ATP} \xrightleftharpoons{\text{Mg}^{2+}} \text{Biotin-E-CO}_2 + \text{ADP}$$

2) Carboxyl transferase:-

$$\text{Acetyl CoA} + \text{Biotin-E-CO}_2 \rightleftharpoons \text{Malonyl CoA} + \text{Biotin-E}$$

Acetyl CoA                                                        Malonyl CoA
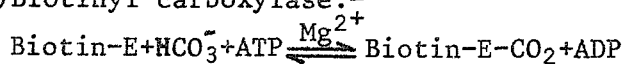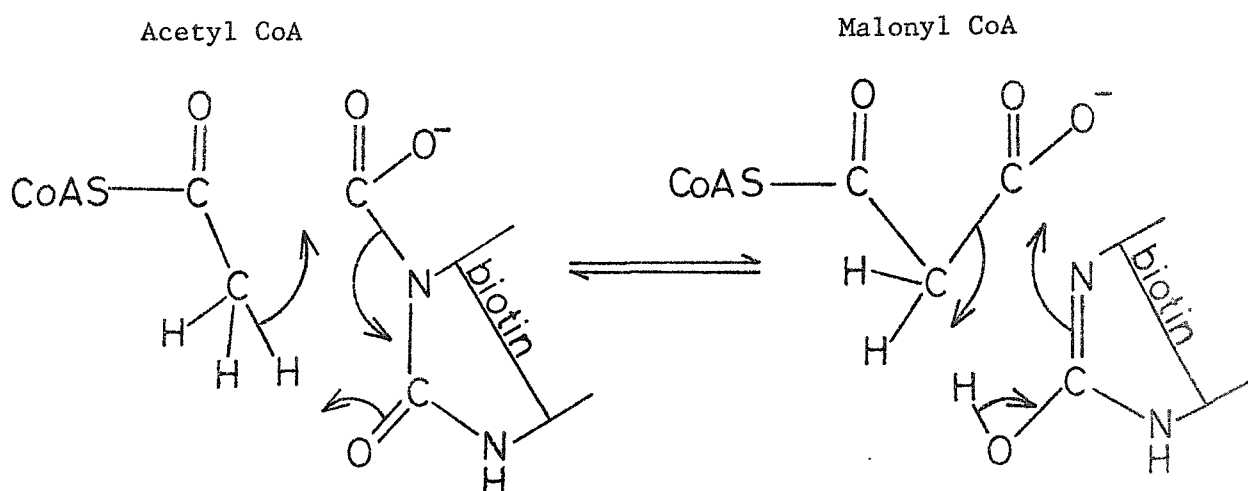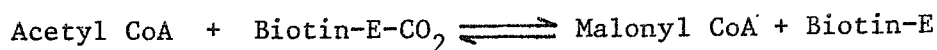
(Section 1.3.4). The second half reaction consists of a concerted electrophilic displacement (Fig. 16; Rétey and Lynen, 1965). Equilibriation during this reaction would result in proton exchange between medium water and the methyl protons of acetyl CoA. As these three protons are equivalent any incorporated tritium from tritiated water would become randomised. The kinetic isotope effect for this reaction is small (Sedgwick and Cornforth, 1977) and two thirds of any $[2-^3H]$ acetyl CoA would be preserved as $[2-^3H]$ malonyl CoA. A maximum of half of this remaining tritium could eventually contribute to fatty acid tritium.

Fig.17 Fatty acid synthase.
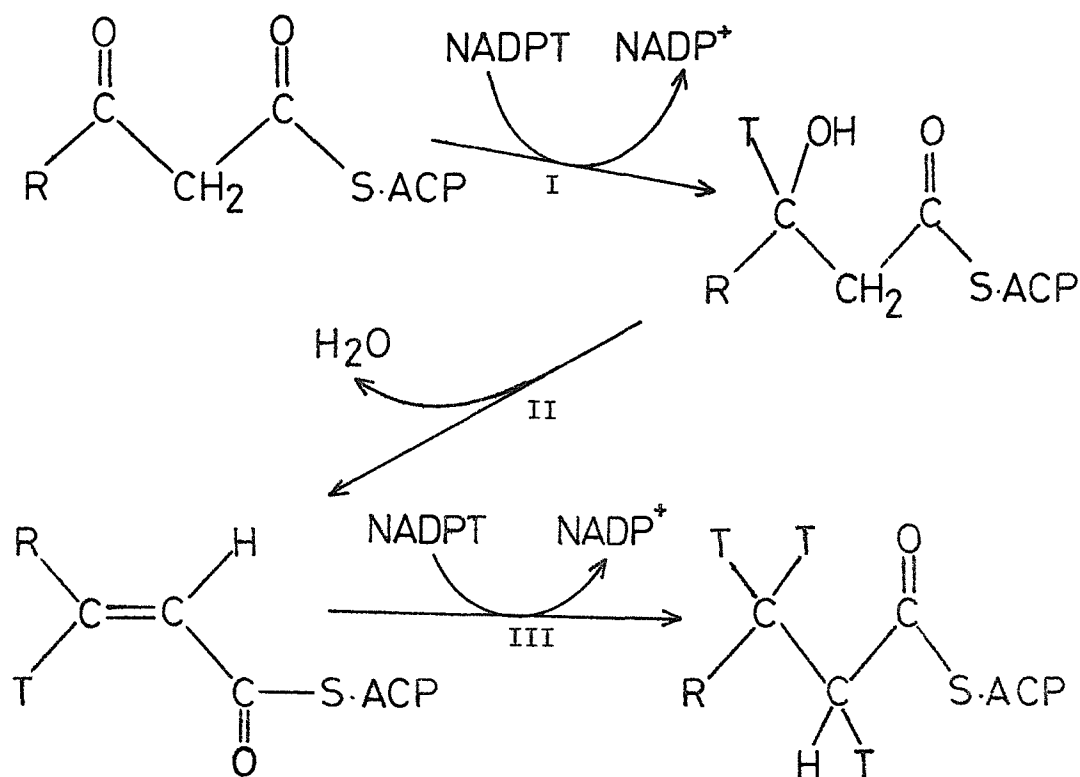


Enoyl-ACP Reductase (III)

For every malonyl CoA molecule converted to fatty acid there must be one protonation from the medium water at the enoyl reductase step of the fatty acid synthase complex (Fig. 17). In tritiated water, tritium

is incorporated specifically at the even carbon numbers of fatty acids.
A considerable kinetic isotope effect has been described for this
tritiated water incorporation by comparison of the tritiations observed
either in deuterated water or normal water (Jungas, 1968).

## NADPH-dependent Reductases (I and III)

During the reactions catalyzed by β ketoacyl-ACP and enoyl-ACP
reductases, a hydride ion is transferred specifically to the odd-
numbered carbon atoms of fatty acids (Foster and Bloom, 1963; Jungas,
1968; Windmueller and Spaeth, 1966). In the presence of tritiated water,
this pool of NADPH becomes tritiated by a series of exchange reactions
such as:-

aldolase ——> triosephosphate isomerase ——> α glycerophosphate
dehydrogenase——>malate dehydrogenase ——>NADP : malate dehydro-
genase
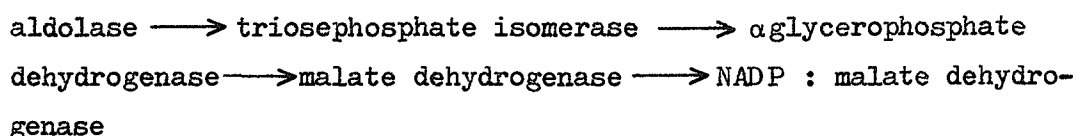
(Flatt and Ball, 1966; Section 2.1.3.6). Pentose phosphate cycling does
not exchange tritium between tritiated water and NADPH and provides about
60% of the NADPH for fatty acid synthesis (Jungas, 1968). The tritium
NADPH specific activity approaches at most about 50% of the tritiated
water specific activity. As two hydrogens from NADPH are incorporated
at each odd-number fatty acid carbon, there should be theoretically up
to 0.5 x 2 = 1 tritium atoms incorporated. The practical result was in
close agreement, giving 0.72 to 0.87 tritium atoms at 3-palmitic acid
(Jungas, 1968). The kinetic isotope effect was low for this NADPH tritium
transfer.

## Non-specific Tritium Exchange

When tritiated water was incorporated into adipose tissue fatty
acid in deuterated water solution, there were 1.84 tritiations at the
α-carbon atom. In normal water solution this value was 0.98 tritium
atoms (Jungas, 1968). As the enoyl reductase can incorporate at most
1 tritium atom on the even fatty acid carbon atoms, the remaining tritium
must have been derived from some unspecified tritium exchange reaction.
An alternative approach to this exchange has been to investigate the

loss of methylene tritiums from $[2-^3H]$ malonyl CoA during fatty acid synthesis. The average retention of methylene tritium has been reported as 18% (D'Adamo et al, 1961) and 26% (Foster and Bloom, 1962; Abraham et al, 1962). Using purified avian enzyme, Sedgwick and Cornforth (1977) calculated that 35.6% of the tritium of $[2-^3H]$ malonyl CoA remaining after the enoyl reductase step was exchanged in a subsequent unspecified reaction. A normal large kinetic isotope effect was observed for this reaction. In tritiated water, such exchange would result in tritium incorporation.

## Overall Stoichiometry of Fatty Acid Tritiated Water Incorporation

Despite the number of reactions where tritiated water incorporation into fatty acid is possible, and the variation in the isotope effects observed, the number of tritiations per mole of fatty acid was constant over a wide range of fatty acid synthetic rates. For an average fatty acid chain length of 16.0 (Martin et al, 1961) a value of 13.3 $\mu$ gatoms of hydrogen per $\mu$ mole fatty acid formed was calculated for perfused liver (Windmuellar and Spaeth, 1966). For adipose tissue synthesis, this value was measured at 14 (Jungas, 1968), giving 0.87 tritium atoms for each carbon incorporated into fatty acid.

### 3.1.3.3. The Measurement of Glycogen Synthesis using Tritiated Water Incorporation

The incorporation of deuterated water into hepatic glycogen was first demonstrated over 40 years ago (Ussing, 1937). Subsequently it was used to evaluate the relative contributions of blood glucose and gluconeogenic precursors to hepatic glycogen synthesis in vivo (Boxer and Stetten, 1944; Stetten and Boxer, 1944). Rats were fasted for 24h, injected with deuterated water and fed glucose or lactate by stomach tube. They were killed after 4h and the deuterium content of liver and carcass glycogen and of liver fatty acid determined. The results (Table 3) showed a greater deuterium incorporation into lactate-derived glycogen than into that formed from glucose. From deuterated water incorporation into liver glycogen in ad lib fed rats over 16 days, they concluded that a considerable proportion of liver glycogen was synthesized from gluconeogenic precursors. This supported previous results which

showed [$^{11}$C] bicarbonate incorporation into liver glycogen when fasted rats were re-fed glucose (Vennesland et al, 1942).

Table 3

Incorporation From Deuterated Water into Liver Glycogen

| Diet | Liver (Glycogen) | Body Deuterated Water | Liver Glycogen Deuterium | Liver Glycogen |
|------|------------------|----------------------|--------------------------|----------------|
| | % wet wt | atom % D | atom % D | % body water |
| None | 0.04 | 1.19 | 0.30 | 25.1 |
| Glucose | 0.82 | 1.29 | 0.49 | 38.1 |
| Lactate | 0.21 | 1.28 | 0.73 | 57.0 |

(Ref: Boxer & Stetten, 1944)

Technical problems in measuring low deuterium concentrations limited further application of these techniques. The incorporation of tritiated water instead of deuterated water overcomes these difficulties. The sensitive detection of tritium decay enables low rates of glycogen synthesis to be measured and also facilitates positional analysis of glycogen tritium. This is necessary for any rigorous investigation of the measurement of glycogen synthetic rates by incorporation of isotopic water.

The direct conversion of blood glucose to glycogen should not incorporate any tritium from tritiated water into glycogen. Substantial incorporation can occur at several reactions in the formation of glucose-1-phosphate prior to glycogen synthesis. These reactions are discussed below.

### 3.1.3.4. Reactions of Carbohydrate Metabolism Incorporating Tritium from Tritiated Water

Ketol Isomerase Reactions

A common mechanism for ketol isomerization was proposed for phospho-glucose isomerase (Rose and O'Connell, 1960) and triose phosphate isomerase

Fig. 18    Incorporation of $^3H_2O$ at Phosphoglucose Isomerase



fructose-6-
phosphate

$T_2O$

$[2^3H]$glucose-
6-phosphate
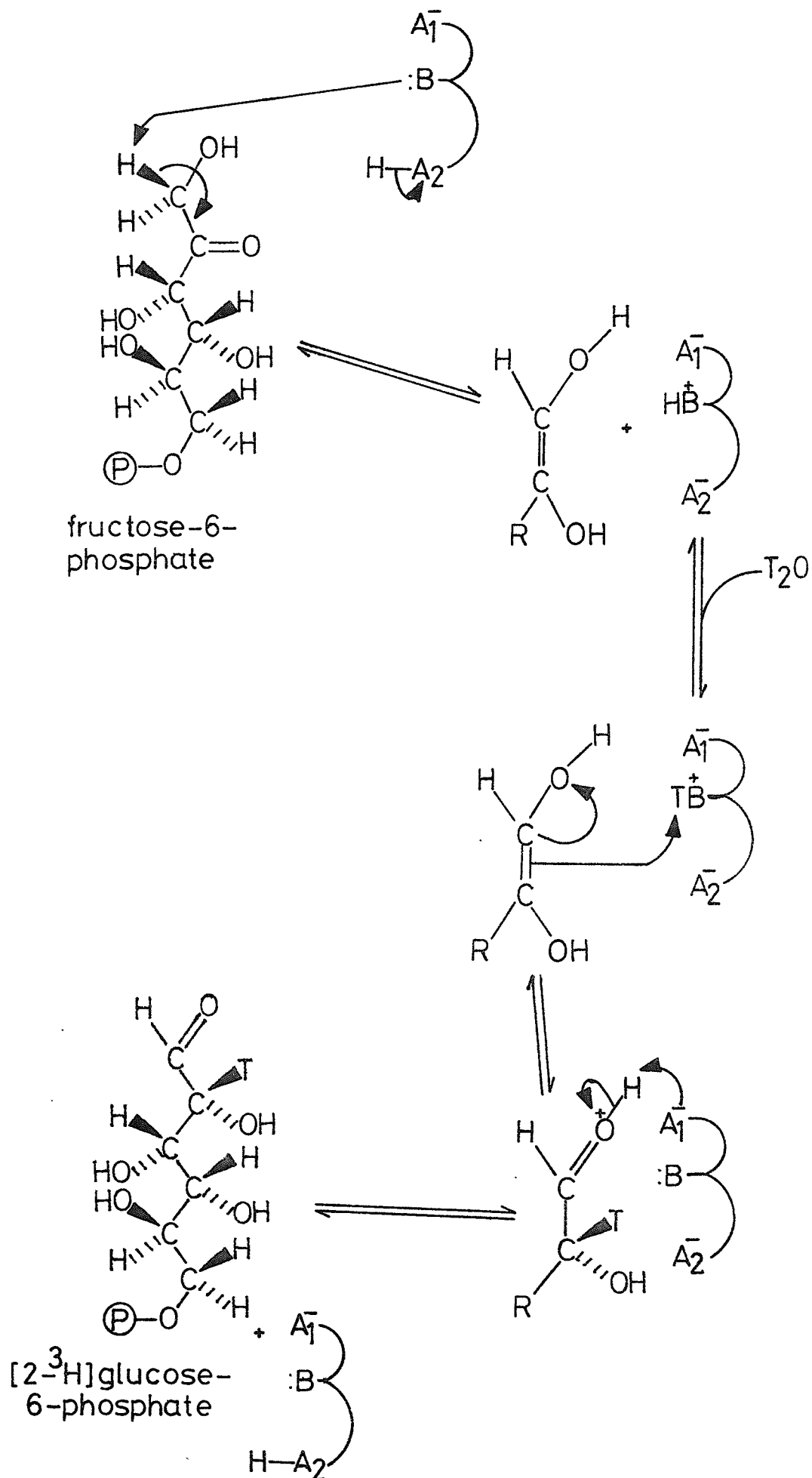
(Rieder and Rose, 1959). This envisaged the addition of water to a cis-enediol intermediate (Fig. 18). An enzyme base group catalyzes the proton abstraction and addition. Tritiated water can be incorporated into either product after exchange of tritium ions for protons on this enzyme base.

One additional limitation on tritiated water incorporation during these reactions is the occurrence of intramolecular hydrogen transfer. Where the exchange of tritiated water with the enzyme proton is slow compared with the overall reaction rate, little tritiated water will be incorporated. Such transfer has been detected by measuring tritium retention during the unidirectional conversion of tritiated substrate to product. Results using this technique indicated intramolecular hydrogen transfer to be complete for ribulose-5-phosphate 4-epimerase (McDonough and Wood, 1960) and D-xylose isomerase (Rose et al, 1969), partial for phosphoglucose isomerase (Rose and O'Connell, 1961) and very low for triose phosphate isomerase (Rieder and Rose, 1959) and ribulose-5-phosphate-3-epimerase (McDonough and Wood, 1960). Tritium incorporation by phosphoglucose isomerase from tritiated water is shown in Fig. 18. The mechanisms of triosephosphate isomerase and ribulose-5-phosphate isomerase are similar. The partial intramolecular hydrogen transfer of phosphoglucose isomerase is not observed when the enzyme is allowed to equilibriate (Rose and O'Connell, 1961). Such intramolecular hydrogen transfer should not affect tritiated water incorporation in vivo as the catalytic enzyme activity has been estimated to be two orders of magnitude greater than the overall pathway flux (Scrutton and Utter, 1968).

Tritium isotope discrimination has been extensively studied for triose-phosphate isomerase (Knowles et al, 1971). Incorporation from tritiated water was measured in both directions by trapping the products. The isotope effect for the irreversible conversion of dihydroxyacetone phosphate to [2-$^3$H] glyceraldehyde-3-phosphate was negligible (1.3) but that for the reverse reaction was considerable (8.0). In their mechanism, breakdown of the enzyme-glyceraldehyde-3-phosphate complex was rate-limiting, while that of the enzyme-dehydroxyacetone phosphate complex was 12 times more rapid. In support of this mechanism tritiated water exchange into unreacted substrate dehydroxyacetone phosphate was

78

Fig. 19    Incorporation of Tritium During Aldolase Cleavage



Fructose-1,6-bisphosphate

Glyceraldehyde-
3-phosphate

[1S-$^3$H]dihydroxy-
acetone phosphate
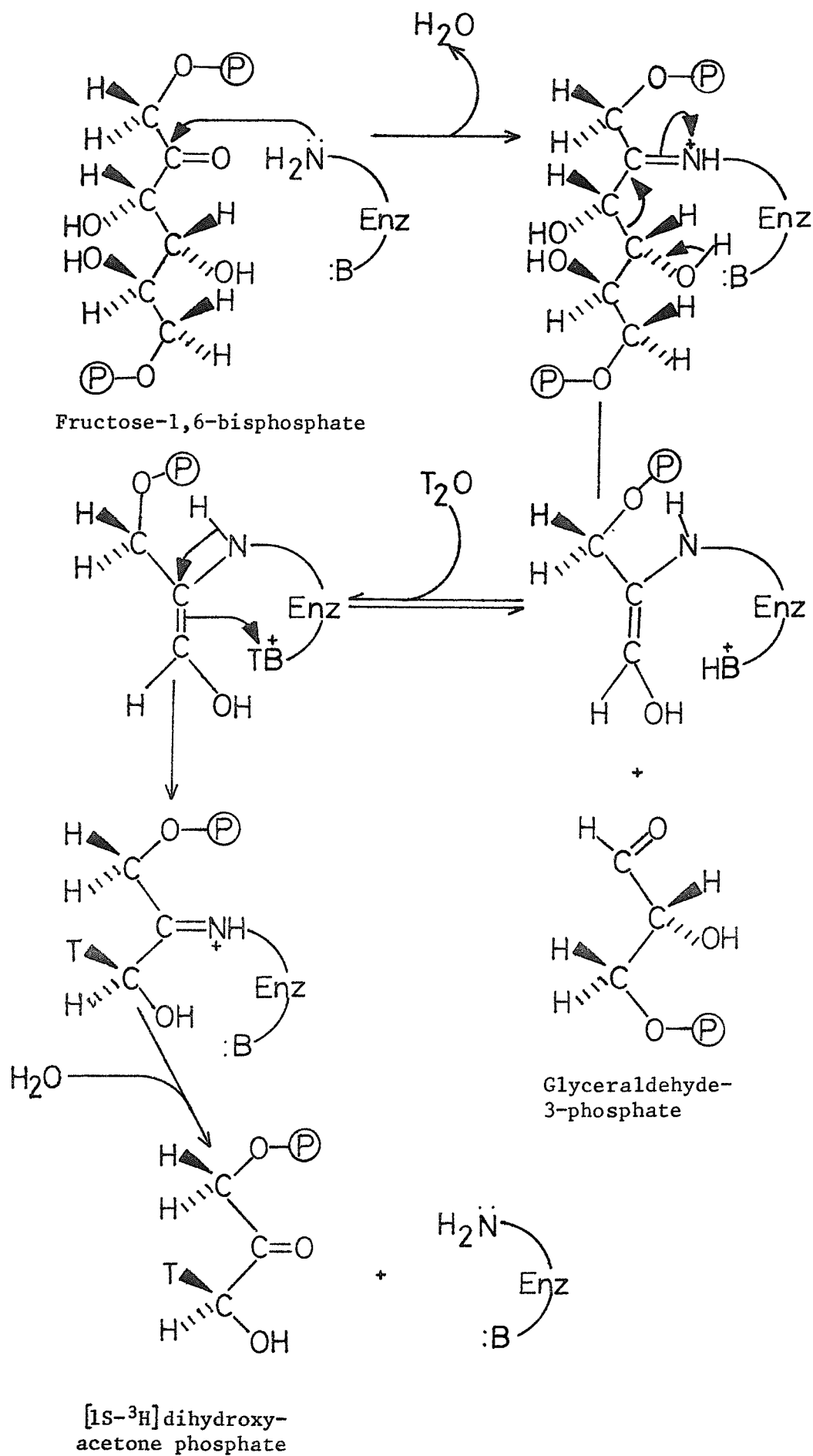
considerable but did not occur with glyceraldehyde-3-phosphate as sub-
strate. When the reaction was allowed to equilibriate no tritium
isotope effect was observed in the products.

## Fructose-1:6-Biphosphate Aldolase

Fructose-1:6-biphosphate binds to aldolase with the formation of
a Schiff's base intermediate (Horecker et al, 1962; Fig. 19). Substrate
cleavage results in formation of free glyceraldehyde-3-phosphate and
enzyme bound dihydroxy-acetone phosphate. In tritiated water this in-
corporates tritium to form[1S-$^3$H] dihydroxy-acetone phosphate. As
triose phosphate isomerase removes the pro R hydrogen of dihydroxyacetone
phosphate, this pro S tritium will be retained on subsequent conversion
to [1,2-$^3$H] glyceraldehyde-3-phosphate.

## Enolase
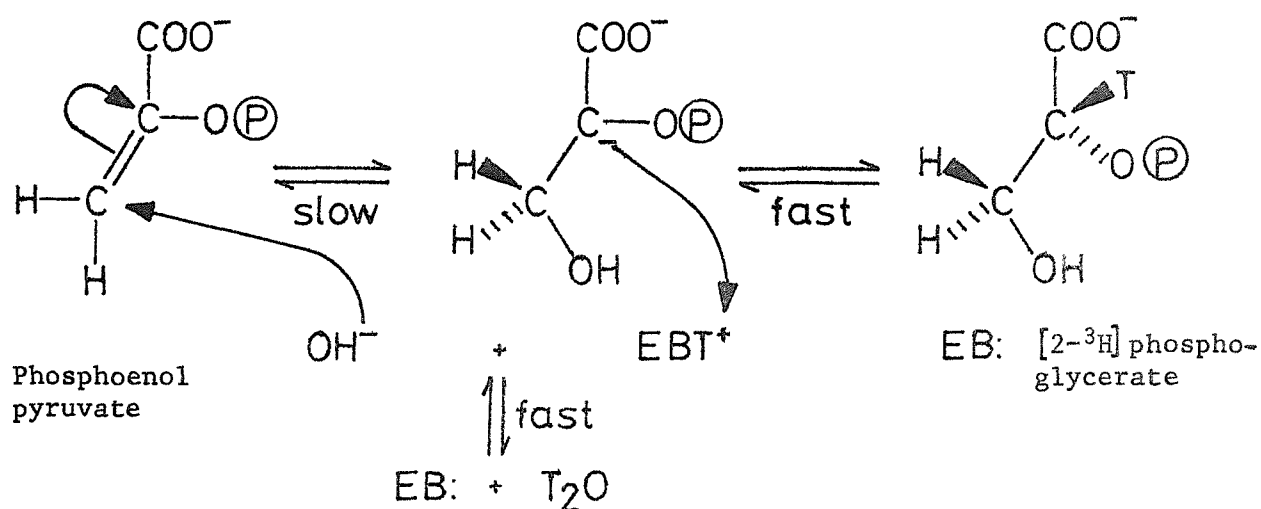


Fig.20. Enolase Mechanism

In the direction of gluconeogenesis, enolase hydrates the double
bond of phospho-enol pyruvate. A carbanion intermediate has been described
(Dinovo and Boyer, 1971; Fig. 20). In tritiated water the exchange
of tritium is rapid compared with the overall rate and so a low isotope
discrimination is observed. The product formed by the si,si addition of
tritiated water is [2R-$^3$H]-2phosphoglycerate.

Fumarase
<u>Fumarase</u>



Fumerate               [3R-$^3$H]malate
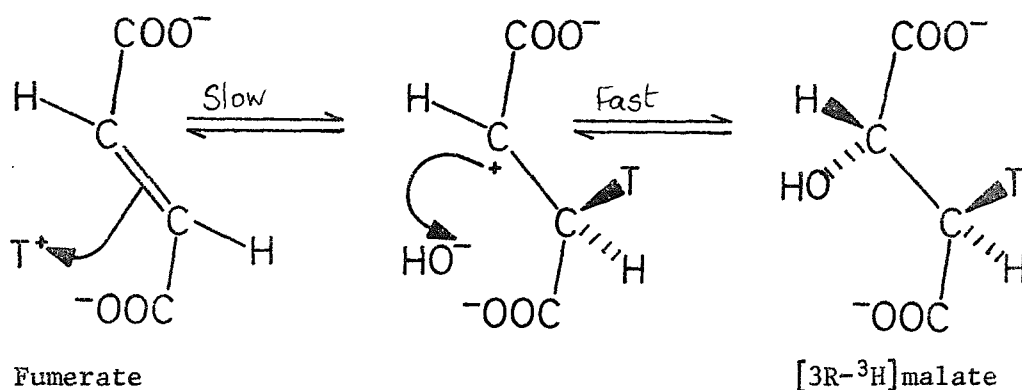
Fig.21. Fumarase Mechanism

Fumarase hydrates fumarate stereospecifically by a trans mechanism to form L-malate. In tritiated water fumarase incorporates tritium into [3R-$^3$H]-malate. It is not certain whether the reaction proceeds via a carbanion or a carbonium ion intermediate (Fig. 21). The $^{18}$O-exchange rate with water of [2-$^{18}$O] malate is more rapid than the tritium exchange rate with water of [2R-$^3$H]-malate, suggesting the carbonium intermediate (Alberty et al, 1957; Hansen et al, 1969). Such exchange rate comparisons do not preclude the possibility of an initial cleavage of the C-3 carbon-hydrogen bond of malate, thus forming a carbanion intermediate, followed by a slow proton release from the enzyme (Rose, 1970). The kinetic isotope effect for malate detritiation was negligible for fumarase (Alberty et al, 1957; Hansen et al, 1969). During gluconeogenesis from lactate in tritiated water tritium incorporated at fumarase will be preserved at C-1 and C-6 of glucose by sequences of reactions such as pyruvate ⟶ malate ⟶ fumarate ⟶ malate ⟶ oxaloacetate ⟶ phosphoenol pyruvate ⟶ glucose or glycogen (Fig. 26).

<u>NAD Dependent Dehydrogenases</u>

In pyridine nucleotide dependent dehydrogenation the hydride exchange is direct between substrate and nucleotide. There is no exchange with medium water (Kaplan, 1960).

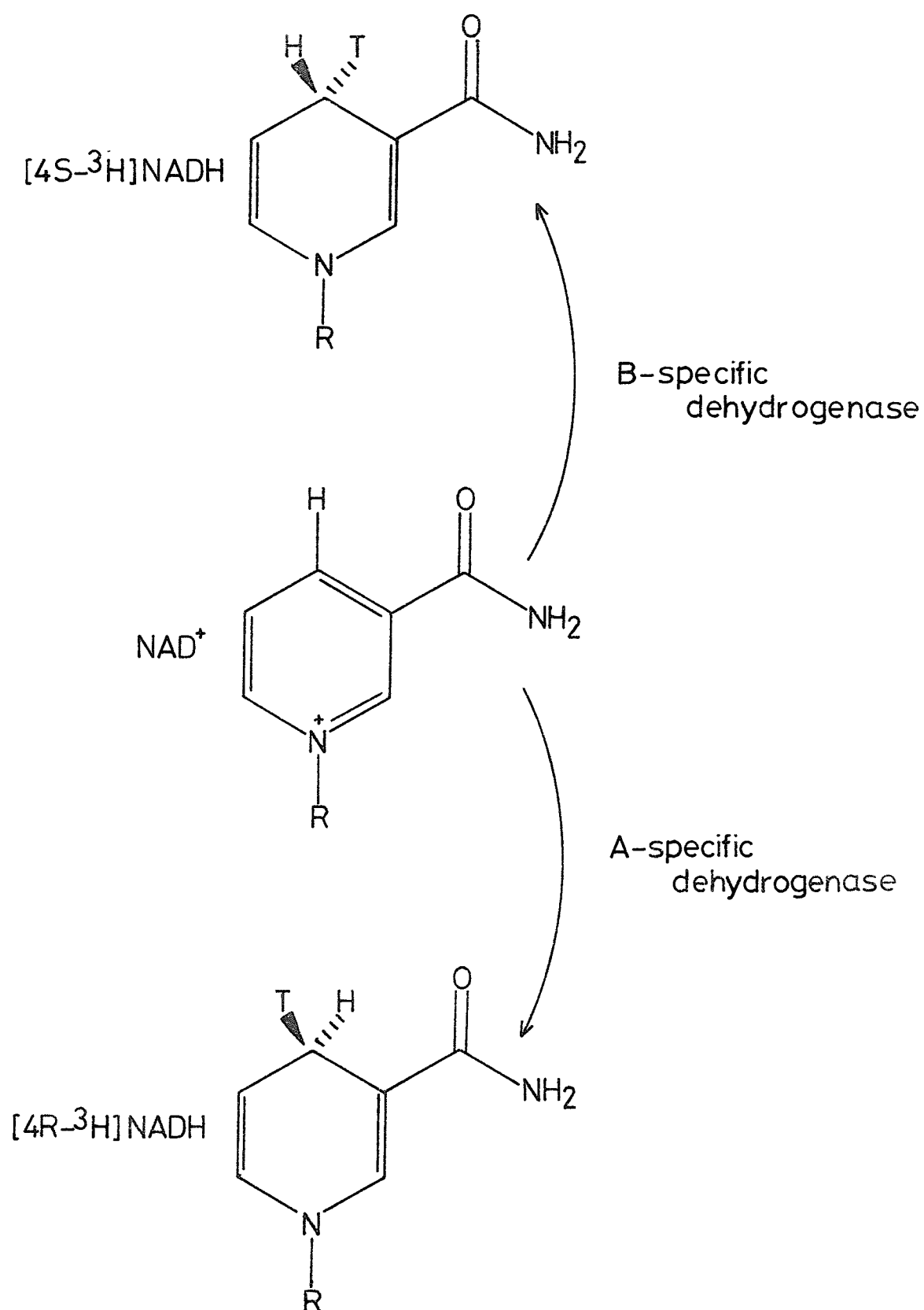Fig.22. Stereo-specificity of NADH-dependent Dehydrogenase

Table 4

Stereospecificities of Nicotinamide-dependent Dehydrogenase Reactions

| Enzyme | Tissue | Stereo-specificity |
|---|---|---|
| Alcohol dehydrogenase | liver | A |
| L-Lactate dehydrogenase | heart | A |
| L-Malate dehydrogenase | heart | A |
| 3-Glycerophosphate dehydrogenase | muscle | B |
| Glyceraldehyde-3-phosphate dehydrogenase | muscle | B |
| L-Glutamate dehydrogenase | liver | B |
| L-3-Hydroxybutyryl CoA dehydrogenase | heart | B |

Dehydrogenases are divided into two groups on the basis of their biological stereospecificity towards the paired hydrogens at C-4 of the reduced nicotinamide ring of NADH (Fig. 22). The group A dehydrogenases transfer the 4R hydride ion from substrate, while group B transfer the 4S hydride ion (Table 4). Tritiated water cannot be incorporated directly into the products of dehydrogenase reactions. Tritium incorporation is possible, however, if the NADH pool becomes tritiated. This would be possible by the sequential action of dehydrogenases of opposite specificities. For example the actions of aldolase, triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase will form $[4S-^3H]$ NADH in tritiated water (Fig. 22). Oxidation of $[4S-^3H]$ NADH by malate or lactate dehydrogenases will form $[4-^3H]$ NAD$^+$. Repeating the cycle of B and then A specific dehydrogenases will result in an equilibrium mixture of $[4R-^3H]$-NADH, $[4S-^3H]$ NADH and $[4R,S-^3H]$ NADH.

3.1.3.5. Theoretical Pattern of Glycogen Tritiation during Glycogen Synthesis in the Liver

Tritiated water incorporation during different reactions will result in tritiation of different glycogen carbon atoms. Positional glycogen tritium can therefore theoretically provide information about the ultimate precursor source for the synthesized glycogen. The tritium incorporations resulting from the activities of a variety of metabolic pathways in tritiated water are presented below.

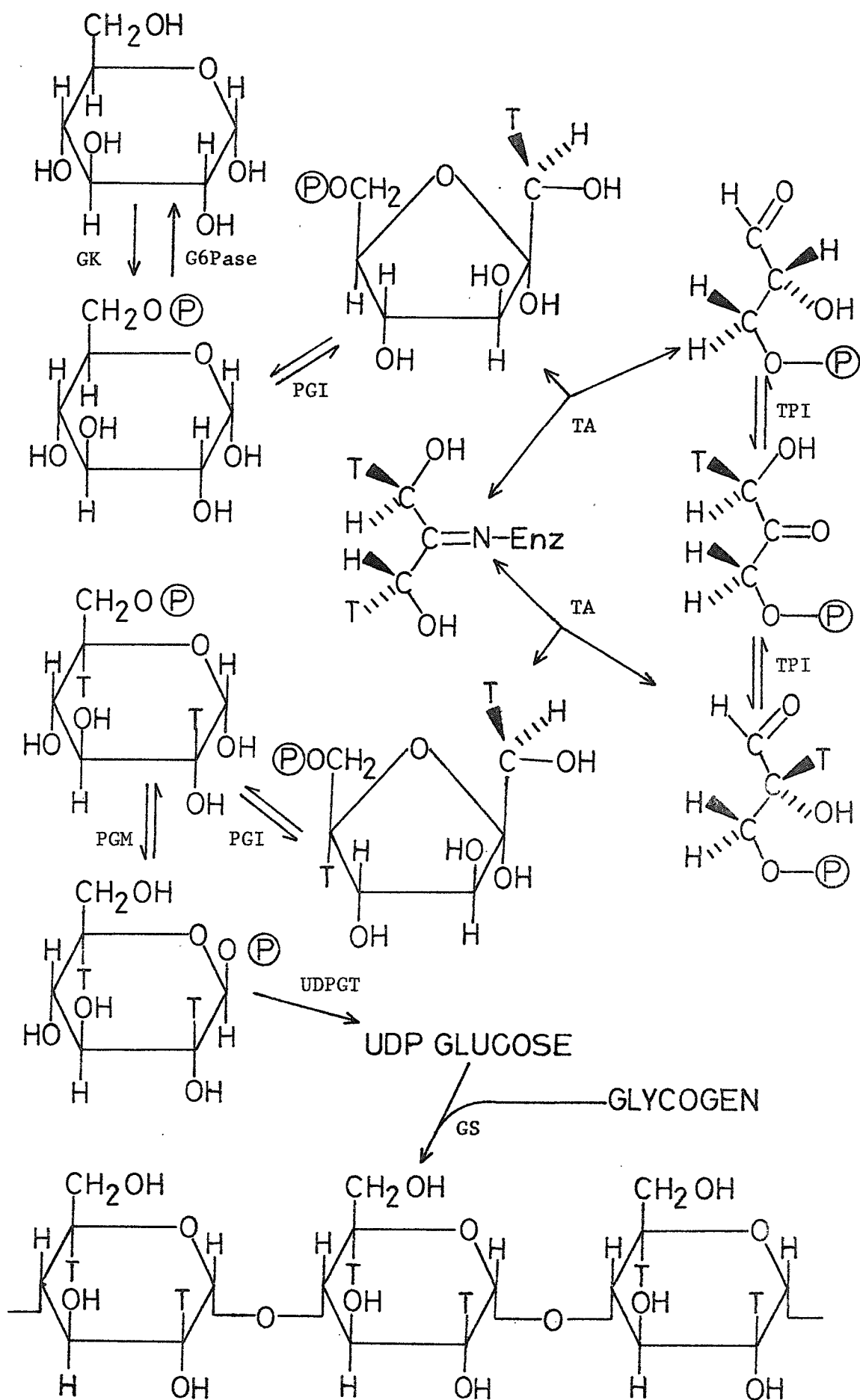## Conversion of Blood Glucose to Glycogen (Fig. 23)

The phosphoglucose isomerase catalyzed equilibrium between glucose-6-phosphate and fructose-6-phosphate is rapid in the liver. In the gluconeogenic direction [2R-$^3$H] glucose-6-phosphate will be formed in tritiated water. As a result of this equilibrium, all glycogen formed in tritiated water from glucose or any other substrate, should be tritiated at C-2.

A further equilibrium catalyzed by transaldolase in the liver inter-converts fructose-6-phosphate and enzyme-bound dihydroxyacetone and free glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate will be tritiated at C-2 by triose phosphate isomerase and this tritium will eventually be retained, after reversal of transaldolase, at C-5 of glycogen (Hue and Hers, 1974). Thus the minimum expected tritium distribution from tritiated water in newly formed liver glycogen will be at C-2 and C-5. No [5-$^3$H] glycogen would be made in muscle as transaldolase activity is absent.

## Recycling of Glucose via Fructose-1:6-Biphosphate

The operation of a substrate cycle between fructose-6-phosphate and fructose-1:6-biphosphate in the liver would result in incorporation of tritiated water at additional glycogen glucose carbon atoms (Fig. 24). By the action of phosphoglucose isomerase [1R-$^3$H]-fructose-6-phosphate would be formed. The action of phosphofructokinase followed by aldolase would result in [1S,3R-$^3$H]-dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. After triose phosphate isomerization these become [1RS,3R-$^3$H]- dihydroxyacetone phosphate and [1,2,3R-$^3$H] glyceraldehyde-3-phosphate. Reversal of aldolase followed by the actions of fructose-1:6-biphosphatase and phosphoglucose isomerase would result in the formation of [2,3,4,5,6R-$^3$H] glucose-6-phosphate. This labelling pattern should be preserved in glycogen.

A distinction between metabolism of glucose by the transaldolase or fructose phosphate pathways can possibly be drawn on the basis of the tritium at C-3 of glycogen glucose. Transaldolase equilibrium should incorporate no tritiated water at C-3, but for fructose phosphate recycling
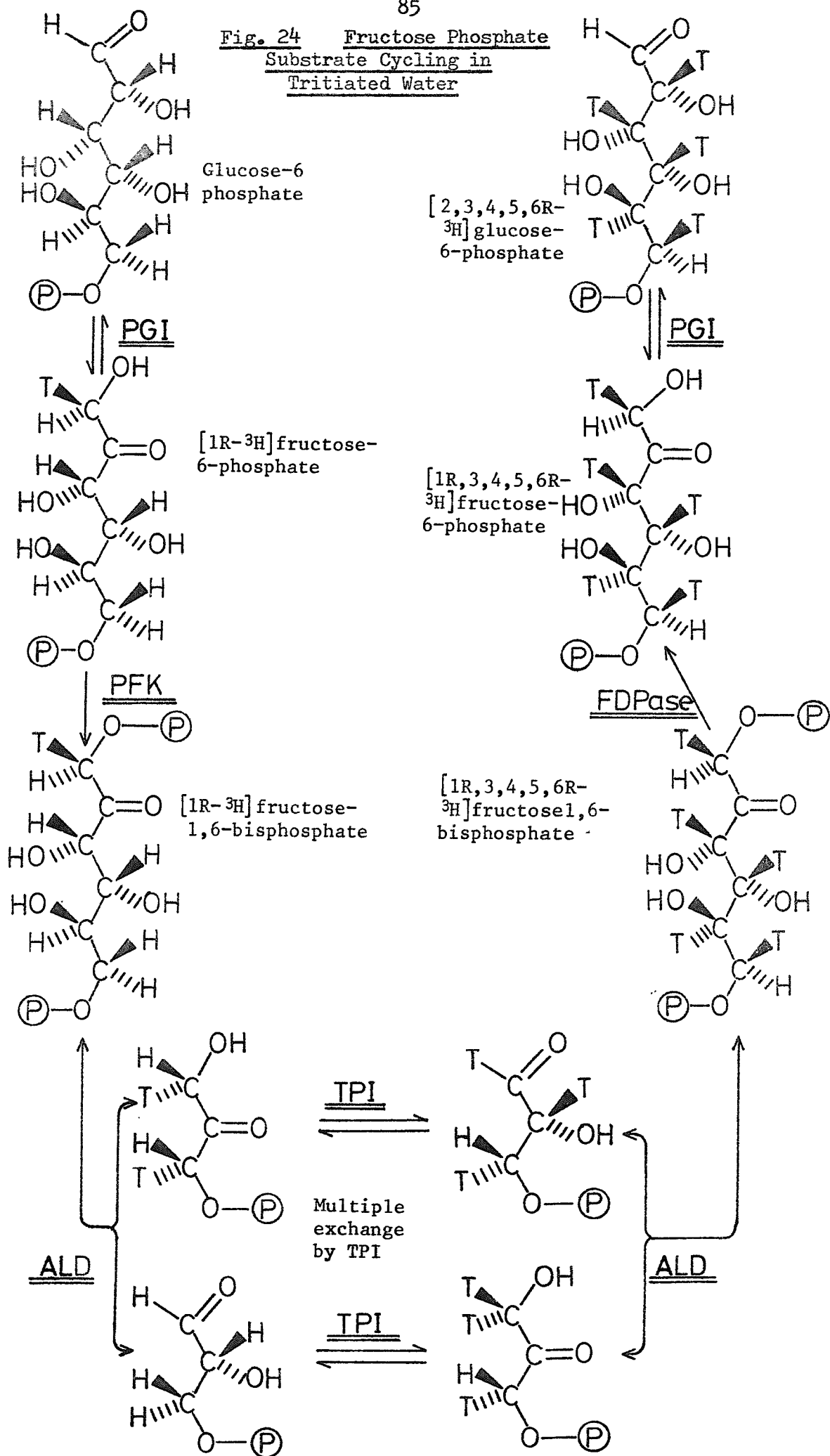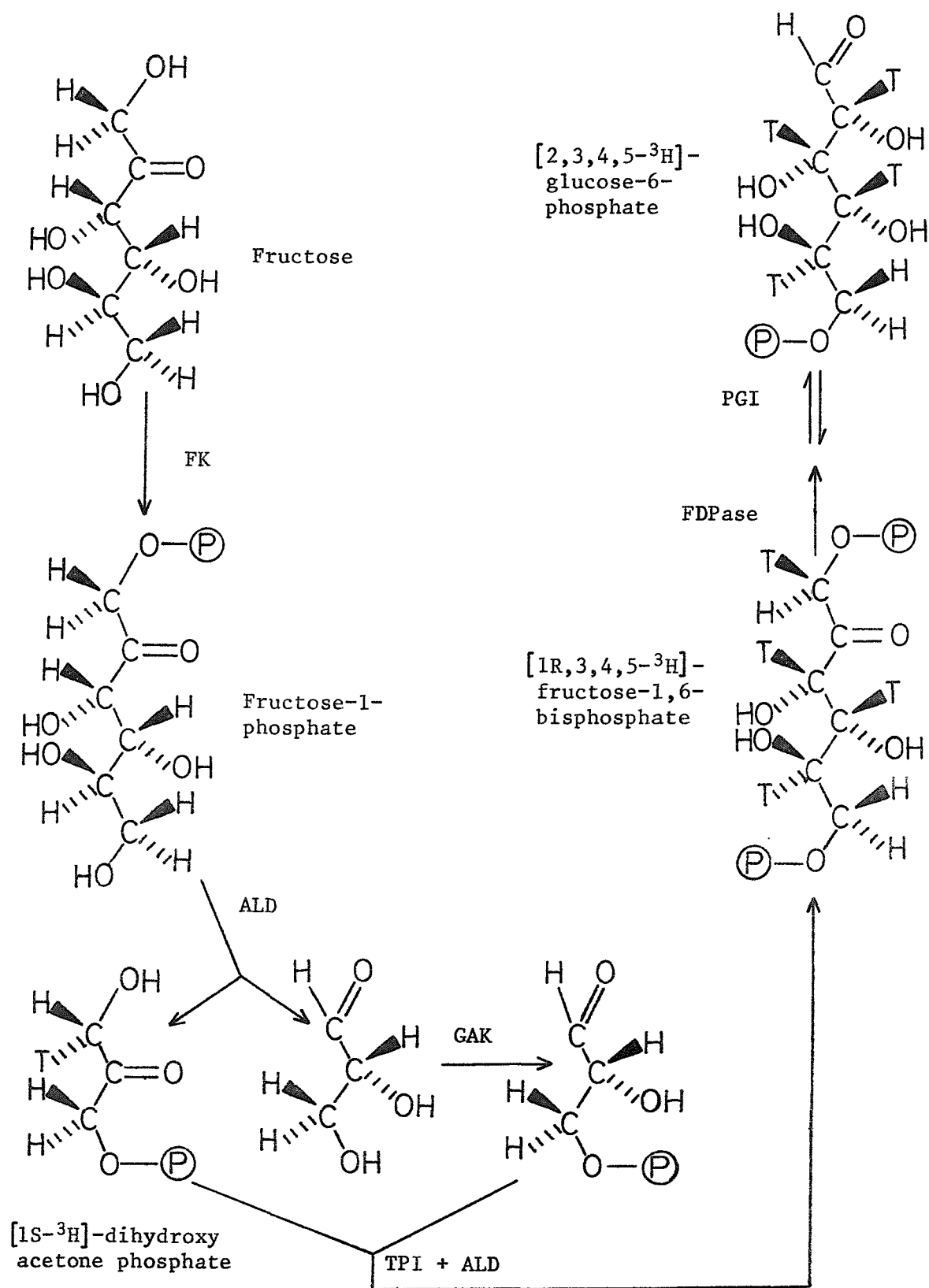
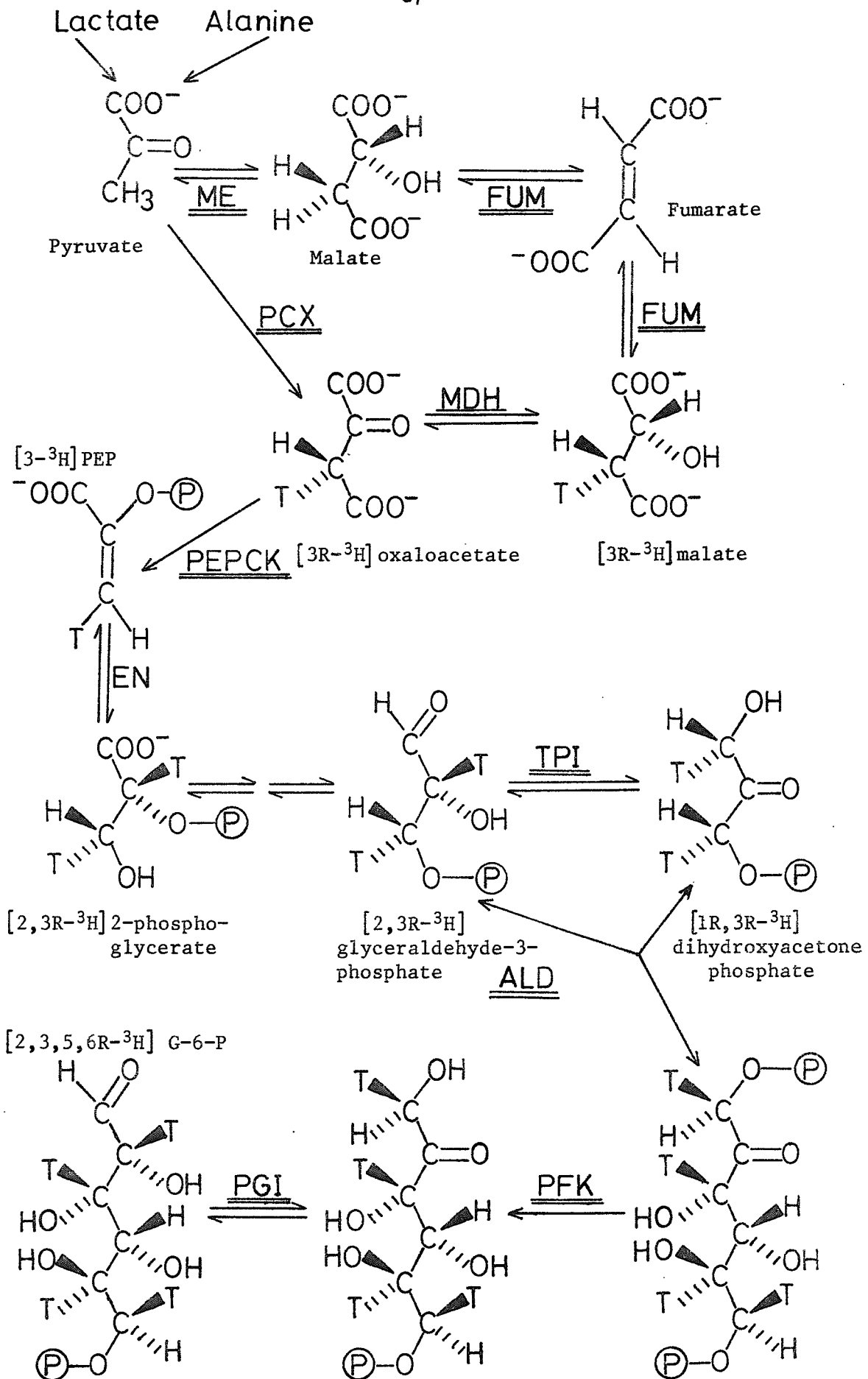Fig. 23    Conversion of Glucose to Glycogen - Expected
Tritiation Pattern from Tritiated Water

Fig. 24   Fructose Phosphate
Substrate Cycling in
Tritiated Water

Glucose-6
phosphate

[2,3,4,5,6R-
$^3$H]glucose-
6-phosphate

PGI

PGI

[1R-$^3$H]fructose-
6-phosphate

[1R,3,4,5,6R-
$^3$H]fructose-
6-phosphate

PFK

FDPase

[1R-$^3$H]fructose-
1,6-bisphosphate

[1R,3,4,5,6R-
$^3$H]fructose1,6-
bisphosphate

TPI

TPI

Multiple
exchange
by TPI

ALD

ALD

Fig.25. Conversion of Fructose to Glucose-6-phosphate in
Tritiated Water.

Fig. 26    Lactate Gluconeogenesis - Expected Tritiation Pattern

the tritium at C-3 should equal that at C-5 of glycogen glucose. In-
direct evidence favours the transaldolase mechanism. [5-$^3$H] glucose was
detritiated by liver homogenates substantially faster than [3-$^3$H] glucose
(Hue and Hers, 1974). This result is inconsistent with elevated rates
of fructose phosphate recycling in liver.

Fructose and Glycerol

Fructose is phosphorylated in the liver by fructokinase to form
fructose-1-phosphate. Aldolase cleavage in tritiated water could form
[1S-$^3$H] dihydroxyacetone phosphate and glyceraldehyde (Fig. 25). Glycer-
aldehyde is phosphorylated by glyceraldehyde kinase to glyceraldehyde-
3-phosphate. Triose phosphate isomerization would then result in [1R-$^3$H]
dihydroxyacetone phosphate and [2,3S-$^3$H] glyceraldehyde-3-phosphate.
After conversion to hexoses the final labelling pattern of glycogen
glucose formed from fructose in tritiated water should be [2,3,4,5-$^3$H]
glucose.

Glycerol is phosphorylated in the liver to $\alpha$ glycerophosphate by
glyceral kinase and then converted to dihydroxyacetone phosphate by
$\alpha$ glycerophosphate dehydrogenase. In tritiated water this would become
tritiated on conversion to glycogen in a manner analagous to fructose
(Fig. 25 ). The final glycogen glucose labelling pattern would be
[2,3,5-$^3$H] glucose.

As both fructose and glycerol must be metabolized via triose phos-
phates the tritiated water incorporation at C-3 and C-5 of glycogen
glucose formed from either precursor should be similar.

Lactate and Alanine

Both lactate and alanine are metabolized in the liver by initial
conversion to pyruvate. The conversion of pyruvate to glycogen in
tritiated water should incorporate tritium both in the direct route and
by side reactions involving tricarboxylic acid cycle enzymes (Fig. 26).
The conversion pyruvate ─→ oxaloacetate ─→ phosphoenol pyruvate ─→
2 phosphoglycerate will incorporate tritium during enolase to form
[2-$^3$H]-2-phosphoglycerate. This tritium should be preserved at C-5 of

Fig.27. Pentose Phosphate Recycling in $^3H_2O$

The enzymes involved in this scheme are:-

1. glucose-6-phosphate dehydrogenase (EC.1.1.1.49)

2. 6-phosphogluconate dehydrogenase (EC.1.1.1.44)

3. ribulose-5-phosphate 3-epimerase (EC.5.1.3.1)

4. ribose-5-phosphate isomerase (EC.5.3.1.6)

5. transketolase (EC.2.2.1.1)

6. transaldolase (EC.2.2.1.2)
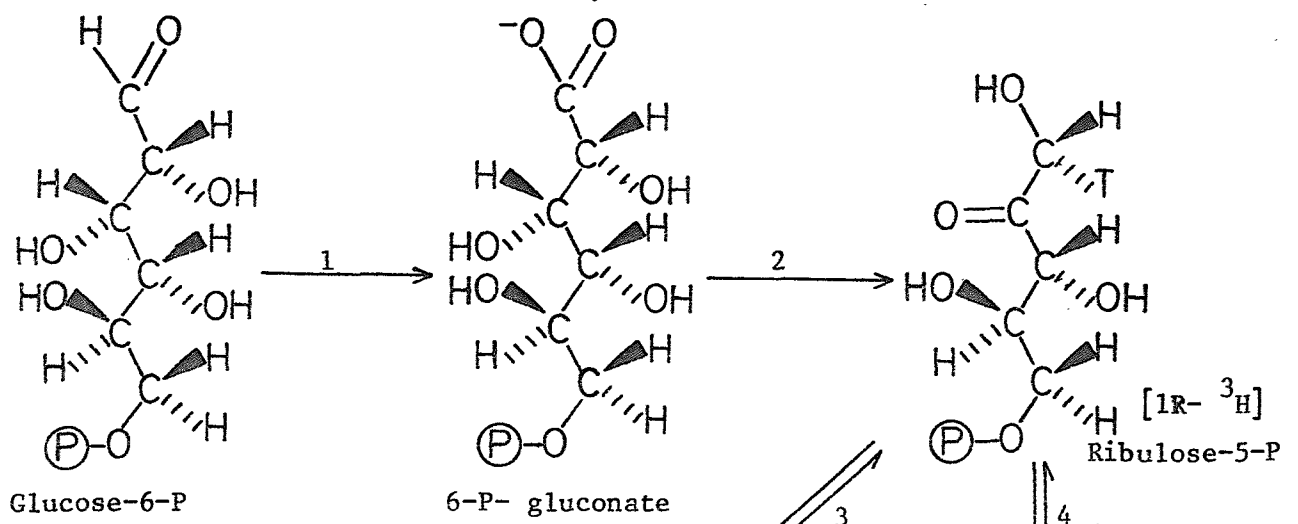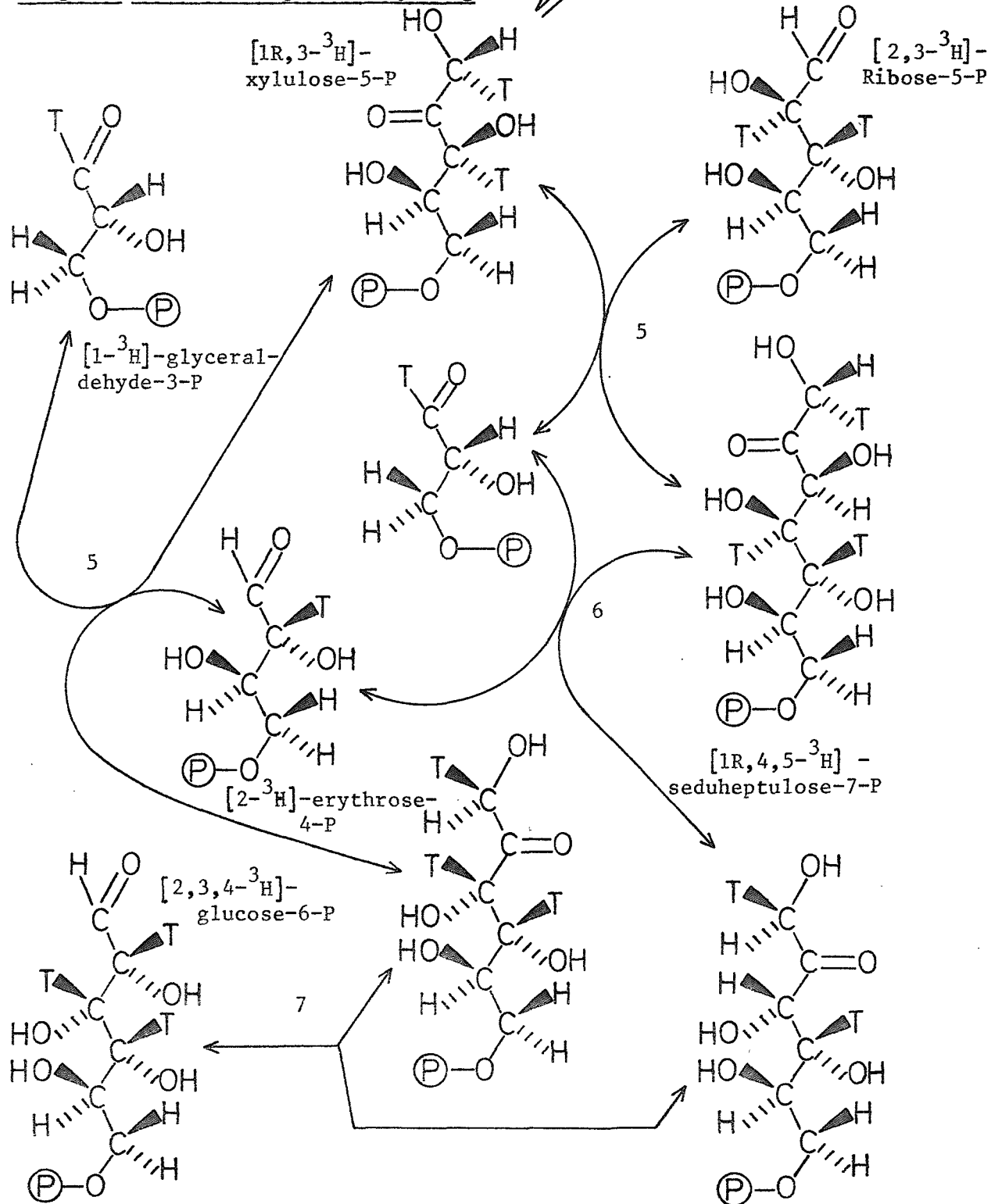
7. phosphoglucose isomerase (EC.5.3.1.9)

Glucose-6-P    6-P- gluconate    [1R- $^3$H] Ribulose-5-P

Fig.27. Pentose Phosphate Recycling

[1R,3-$^3$H]-xylulose-5-P

[2,3-$^3$H]-Ribose-5-P

[1-$^3$H]-glyceral-dehyde-3-P

[1R,4,5-$^3$H]-seduheptulose-7-P

[2-$^3$H]-erythrose-4-P

[2,3,4-$^3$H]-glucose-6-P

glycogen glucose. Further tritiation at C-3 should occur during triose phosphate isomerization and at C-2 during phosphoglucose isomerization.

Tritiated water incorporated into $[3R-^3H]$-malate during fumarase equilibriation should be preserved as $(2si, 3re)-[3-^3H]$ phosphoenolpyruvate, which gives rise to glycogen glucose labelled in the C-1 and C-6 positions. Operation of the substrate cycle pyruvate ——> oxaloacetate ——> phosphoenol pyruvate ——> pyruvate causes randomization of incorporated tritium between the equivalent methyl hydrogens of pyruvate. The scheme shown (Fig. 26) ignores this randomization to show the stereospecificities of the various reactions.

### Pentose Phosphate Pathway Activity (Fig. 27)

Tritiated water will be incorporated into the reaction products of ribose-5-phosphate isomerase and ribulose-5-phosphate 3-epimerase during the oxidation of glucose-6 phosphate by the pentose phosphate pathway. Tritium retained during the subsequent transformations catalyzed by transaldolase and transketolase will be distributed at positions 2, 3 and 4 of the re-formed glucose 6-phosphate.

### 3.2. Results

#### 3.2.1. Specific Radioactivity of Tissue Water

For tritium incorporation to be a valid estimate of the rate of glycogen synthesis, the specific radioactivity of liver water must be constant throughout the incubation with tritiated water. Results from two preliminary experiments indicated that this condition was approximately maintained.

For the first experiment 4 rats were cannulated under ether anaesthesia by the insertion of a polyethylene cannula into the right femoral artery. The rats were then placed in individual restraining cages overnight and allowed free access to food and water. The next day, each rat was injected i.p. with 2mCi of tritiated water in 0.15M-saline. Serial blood samples were taken from the cannula from 10 to 120 min after injection. For each sample 100 µl was deproteinised by quenching in 2 ml of ice cold 3% (w/v) perchloric acid. After centrifugation at

Fig.28. Specific Radioactivity of Blood after $^3H_2O$ Injection

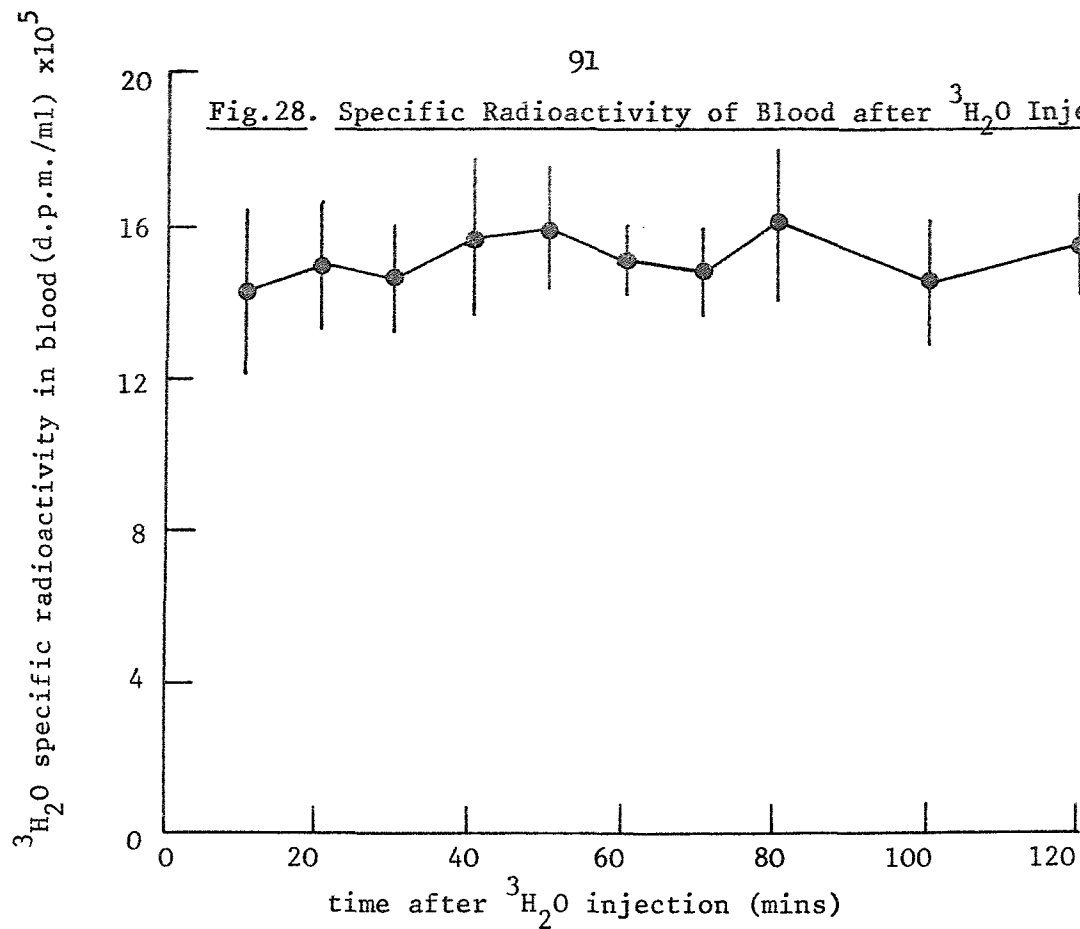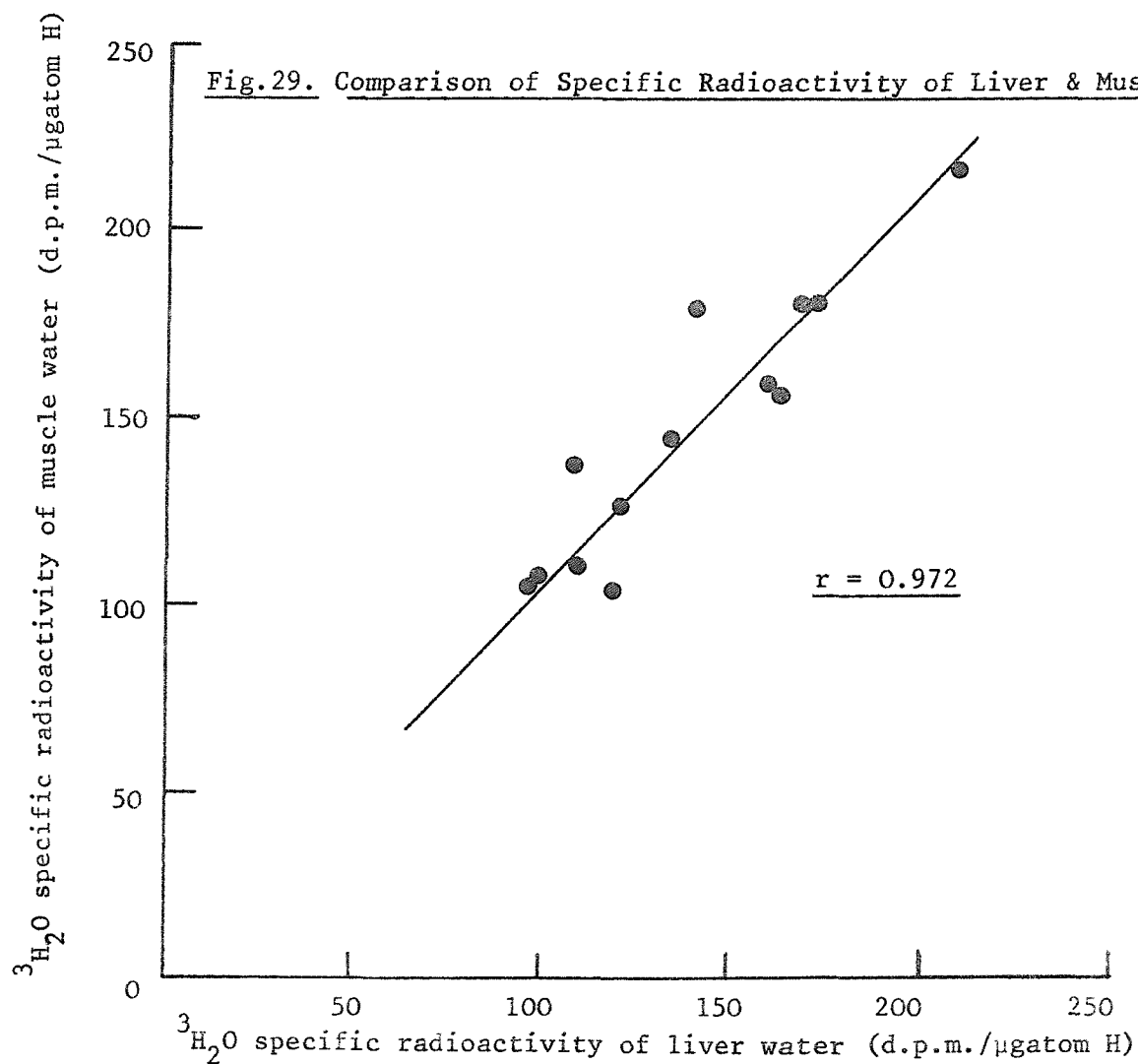$^3H_2O$ specific radioactivity in blood (d.p.m./ml) $\times 10^5$

time after $^3H_2O$ injection (mins)



Fig.29. Comparison of Specific Radioactivity of Liver & Muscle

$^3H_2O$ specific radioactivity of muscle water (d.p.m./µgatom H)

r = 0.972

$^3H_2O$ specific radioactivity of liver water (d.p.m./µgatom H)

2,000 x g for 10 min the tritium content of supernatant aliquots was determined. The specific radioactivity (DPM/ml blood) was essentially constant over the period measured (Fig. 28).

For the second experiment 13 rats were injected at various stages of the feeding cycle with 2m Ci of tritiated water and killed 1h later. Samples both of liver and skeletal leg muscle were removed and freeze-clamped immediately. Tissue was extracted with perchloric acid as described in the methods section (2.2). The specific radioactivity of tissue water was calculated from the tritium content of the perchloric acid homogenate supernatant. The specific radioactivity of muscle was 97% that of liver, correlation coefficient calculated by the least squares method, $r = 0.97$ (Fig. 29). The times measured ranged from before feeding to + 8h after feeding. The results demonstrated that, after i.p. injection, tritiated water was evenly equilibriated between liver and muscle.

### 3.2.2. Isolation of Liver Glycogen

The efficiency of glycogen precipitation by ethanol was monitored by measuring the recovery. Total glycogen concentration in the liver was determined by measuring the glucose formed after hydrolysis with $\alpha$ -amyloglucosidase of a sample of neutralised perchloric acid extract of liver. This value was corrected for free liver glucose. Glycogen from the perchloric acid supernatant was precipitated and washed with ethanol (Section 2.2.3). The glucose content of precipitated glycogen was then measured after $\alpha$ -amyloglucosidase hydrolysis. Glycogen recovery was calculated by comparison of total and precipitated concentrations. It was estimated at $96.9 \pm 1.6\%$, indicating that losses of glycogen on isolation were small.

Table 5

Hepatic Glycogen Synthesis in Fasted Rats

| Experimental Condition | (n) | (Glycogen) ($\mu$ moles/g) | Glycogen $^3H_2O$ incorporation ($\mu$ gatoms 'H'/g/h) |
|---|---|---|---|
| Control rats (standard diet) | (8) | $109 \pm 28$ | $45 \pm 6$ |
| Control rats (sucrose diet) | (3) | $133 \pm 8$ | $10 \pm 1$ |
| Control rats (glucose/fructose diet) | (4) | $74 \pm 7$ | $21 \pm 5$ |
| Diabetic rats (untreated) | (5) | $35 \pm 12$ | $25 \pm 6$ |
| Diabetic rats (insulin-treated) | (5) | $17 \pm 4$ | $17 \pm 2$ |
| Corticotropin-treated rats | (6) | $104 \pm 27$ | $38 \pm 9$ |
| Adrenalectomized rats | (4) | $12 \pm 5$ | $5 \pm 2$ |

Table 6

Maximum Rates of Hepatic Glycogen Synthesis in Fed Rats

| Experimental Condition | (n) | Time (h) | (Glycogen) ($\mu$moles/g) | Glycogen $^3H_2O$ incorporation ($\mu$gatoms 'H'/g/h) |
|---|---|---|---|---|
| Control rats (standard diet) | (4) | +3 | $383 \pm 35$ | $253 \pm 71$ |
| Control rats (sucrose diet) | (4) | +2 | $234 \pm 6$ | $233 \pm 8$ |
| Diabetic rats (untreated) | (4) | +5 | $168 \pm 46$ | $168 \pm 74$ |
| Diabetic rats (insulin-treated) | (6) | +2 | $234 \pm 47$ | $266 \pm 34$ |
| Corticotropin-treated rats | (7) | +2 | $513 \pm 54$ | $198 \pm 29$ |
| Adrenalectomized rats | (4) | +4 | $302 \pm 35$ | $170 \pm 20$ |

Fig.30    Time-course of Incorporation of $^3$H from $^3$H$_2$O into Hepatic Glycogen
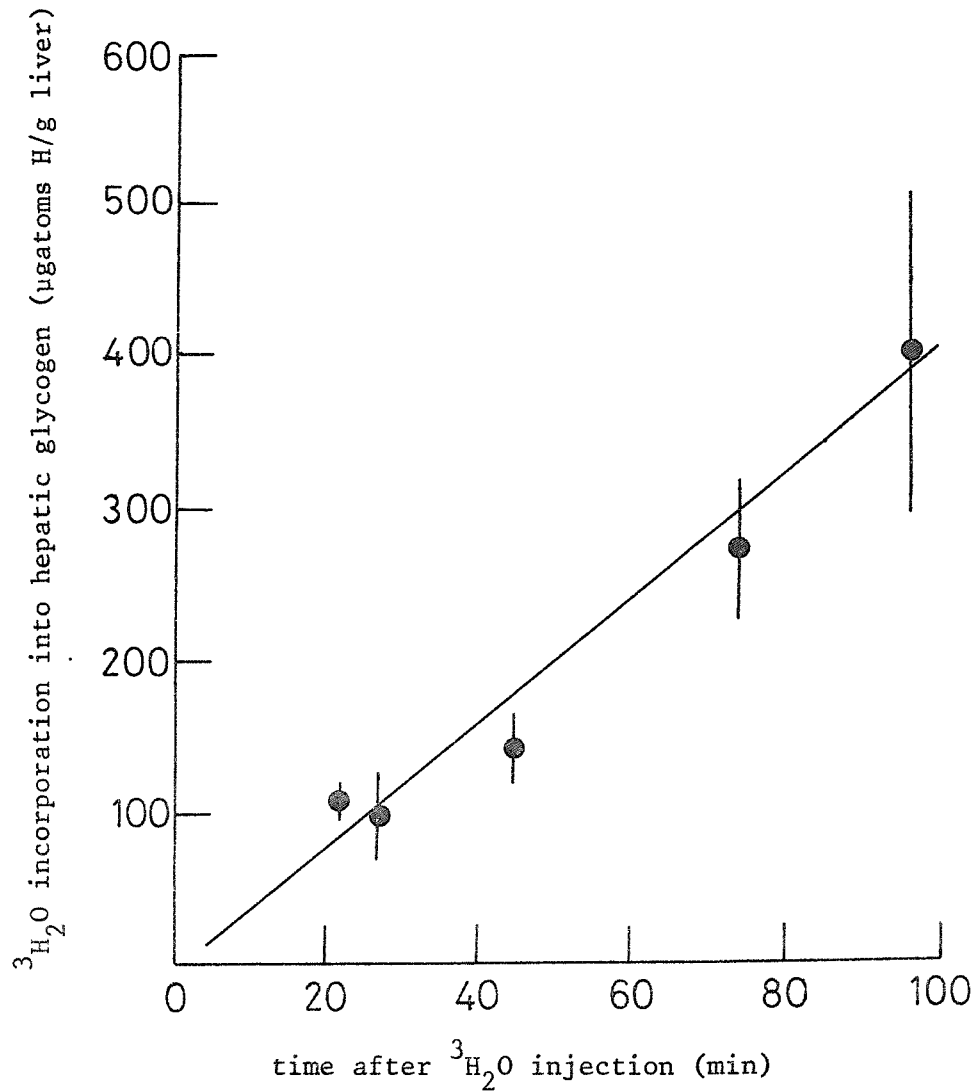


Rats were injected i.p. with $^3$H$_2$O at about +1h (11.00h). Groups of four rats were killed at the times indicated and the $^3$H in liver glycogen determined. Results are expressed as means ±S.E.M. The line was calculated by the least squares method; the correlation coefficient r = 0.75.

3.2.3. Time Course of Tritiated Water Incorporation in vivo into
Liver Glycogen

Hepatic glycogen concentration increases during the 6h after meal-feeding (Hopkins et al, 1973). An interval in this period was therefore chosen for the measurement of the time course of tritiated water incorporation into liver glycogen in vivo (Fig. 30). Twenty rats were injected i.p. with 2m Ci of tritiated water at +1h after feeding. They were killed in groups of 4 at intervals from 20 to 100 min later and their livers freeze-clamped. The tritium content of liver glycogen was measured. Tritiated water incorporation was reasonably linear with time.

3.2.4. Glycogen Synthesis in Fasted Rats

Fasting reduced the concentration of hepatic glycogen in all experimental groups of rats. For control rats fed either standard or high sucrose diet and for corticotropin treated rats fasted glycogen was about 20 to 25% of the fed concentration (Table 5). For diabetic and adrenalectomized rats this value was even lower. In no group was there any substantial incorporation of tritiated water into liver glycogen. This applied equally well to control rats with relatively high and to diabetic rats with low fasted glycogen concentrations. Interestingly, elevated corticosterone in the corticotropin-treated group, did not stimulate glycogen synthesis in the fasted state. This is in contrast to pharmacological cortisol doses which reportedly stimulate hepatic glycogen synthesis even in the fasted state (Long et al, 1940).

3.2.5. Glycogen Synthesis in Response to Meal-feeding

Meal-feeding stimulated glycogen synthesis in the livers of rats under all experimental conditions. The increase of glycogen concentration was accompanied by a stimulation of tritiated water incorporation into glycogen. The maximum values for both parameters, and the times after feeding of these maxima, are shown in Table 6. A typical analysis of the rate of glycogen synthesis in the liver of a fed control rat is presented in Table 7.

Table 7

Representative Example of the Calculation of $^3H_2O$ incorporation into Glycogen

1) Time $^3H_2O$ incubation *in vivo* = 66 min

2) Dilution liver (W/W) in 6% (W/V) $HClO_4$ = 0.250

3) Dilution liver after neutralization with 10% (W/V) KOH = 0.191

4) Dilution liver after hydrolysis with α-amyloglucosidase = 0.154

5) Water content of liver = $\dfrac{(70.3 + 70.9)}{2}$ = 70.6%

6) Tritium in 100 μl of $HClO_4$ supernatant = $\dfrac{(351,863 + 344,921)}{2}$ = 348,392 d.p.m.

∴ Specific radioactivity = $\dfrac{348,392}{70.6 \times 0.250 \times 111}$ = 178 d.p.m/ μgatom 'H'

7) [Glucose] = $\dfrac{E \times vol.\ cuvette}{6.22 \times vol.\ neutral \times neutral\ sample\ \ \ \ \ dilution}$

= $\dfrac{0.618 \times 2}{6.22 \times 0.1 \times 0.191}$ = 10.4 μmoles/g

8) [Glycogen + glucose] = $\dfrac{E \times vol.\ cuvette}{6.22 \times vol.\ glycogen \times dilution\ hydrolysate}$

= $\dfrac{0.833 \times 2}{6.22 \times 0.005 \times 0.154}$ = 348 μmoles/g

∴ Total [glycogen] = 348 - 10 = 338 μmoles/g

9) Precipitated [glycogen] = $\dfrac{E \times vol.\ cuvette}{6.22 \times vol.\ \times acid\ sample\ \ \ dilution}$

= $\dfrac{1.271 \times 2}{6.22 \times 0.005 \times 0.25}$ = 327 μmoles/g

∴ Recovery of precipitated glycogen = 96.7%

10) Tritium content of sample (10%) of precipitated glycogen = 3360 d.p.m.

∴ Total glycogen tritium = $\dfrac{3360 \times 10}{0.50 \times 0.967}$ = 69,483 d.p.m.

∴ Rate of $^3H_2O$ incorporation into glycogen = $\dfrac{69,493 \times 60}{66 \times 178}$ = 355 μgatoms 'H'/g wet wt. liver/h

Fig.31. Hepatic Glycogen Synthesis in Meal-fed Control Rats.
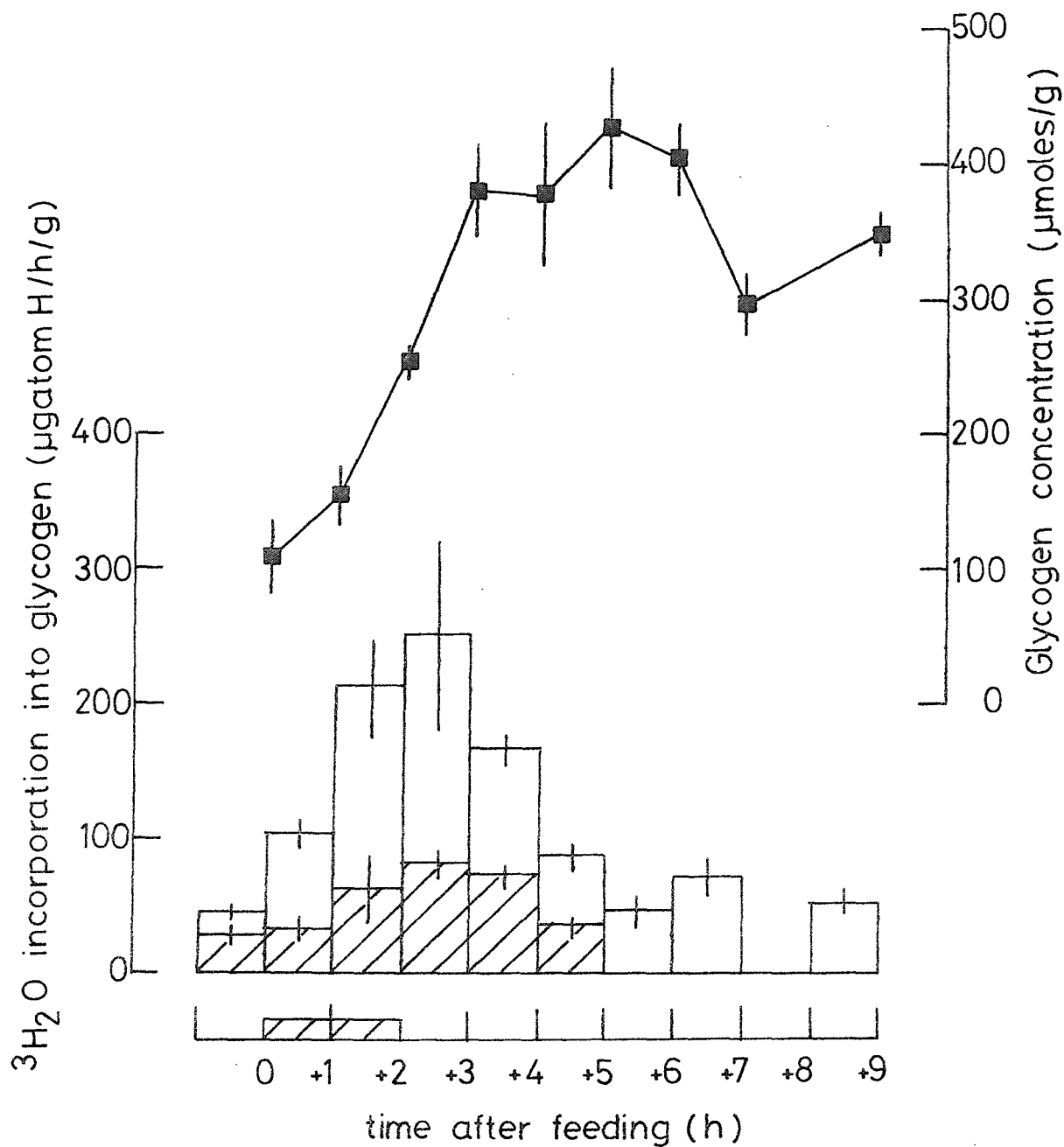


The open bars of the histogram represent the total ^{3}H incorporation into glycogen over the 1h period indicated. The hatched bars represent that proportion found at C-2 of glycosyl glycogen. The graph denotes the glycogen concentration at the end of the incubation period. Results are expressed as mean ± S.E.M. of between four and eight rats.

Fig.32. Hepatic Glycogen Synthesis in Control Rats Meal-fed a High-sucrose
Diet
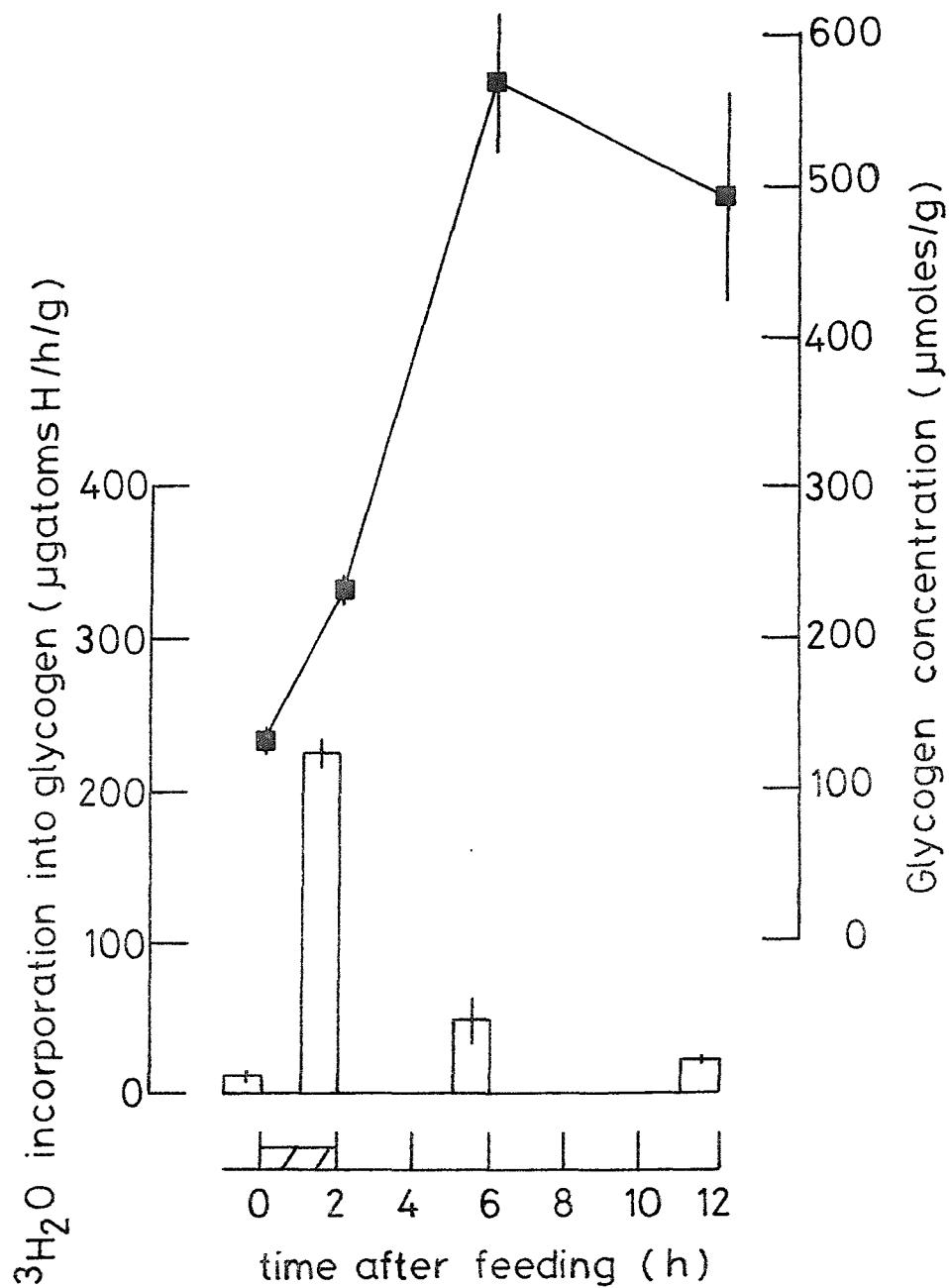


Experimental conditions and symbols are the same as in Fig. 31.
These rats were fed a diet containing 67% sucrose for seven days.

### 3.2.6. Control Rats

#### Standard Low Fat Diet

Glycogen concentration in the liver increased after feeding to a maximum by +4h (14.00h) (Fig. 31; Table 6). This plateau concentration was maintained from +4 to +9h (14.00 to 19.00h). The rate of tritiated water incorporation into liver glycogen also increased after feeding. This was measured over consecutive 1h incubation periods from pre-fed (10.00h) to +9h after feeding (19.00h). The tritiated water incorporation rate reached a maximum of 6 times the pre-fed rate by +3h after feeding (13.00h). By contrast with the continued maintenance of an elevated hepatic glycogen concentration, the glycogen tritiated water incorporation fell from the +3h maximum to the pre-fed rate by +5h (15.00h). This indicated that tritiated water was only incorporated during active glycogen synthesis and did not exchange with pre-formed glycogen. Throughout the elevated plateau of glycogen concentration, tritiated water incorporation into glycogen was low.

#### High Sucrose Diet

Groups of rats were fed the high sucrose diet for 7 days and then killed before feeding and at +2, +6 and +12h after feeding. Hepatic glycogen concentrations and incorporations of tritiated water over the preceding hour are shown in Fig. 32 and Table 6. The results indicated that glycogen synthesis increased after feeding at a similar rate for sucrose-fed rats as for standard diet fed rats. Hepatic glycogen concentration and tritiated water incorporation were comparable by +2h after feeding (compare Figs. 31 and 32). By +6h after feeding (16.00h) the liver glycogen concentration of sucrose-fed rats was 100 moles/g higher than that for control rats. This elevated level of glycogen concentration was maintained until at least +12h after feeding (22.00h). There was no significant tritium incorporation into glycogen during the 1h incubation with tritiated water before rats were killed at either +6h or +12h. This confirmed the lack of exchange of tritiated water with pre-formed glycogen. The peak phase of glycogen synthesis must have occurred between +2 and +5h. Demonstration of this would have required a complete time course of hepatic glycogen synthesis in sucrose

Fig.33. Hepatic Glycogen Synthesis in Meal-fed Diabetic Rats.
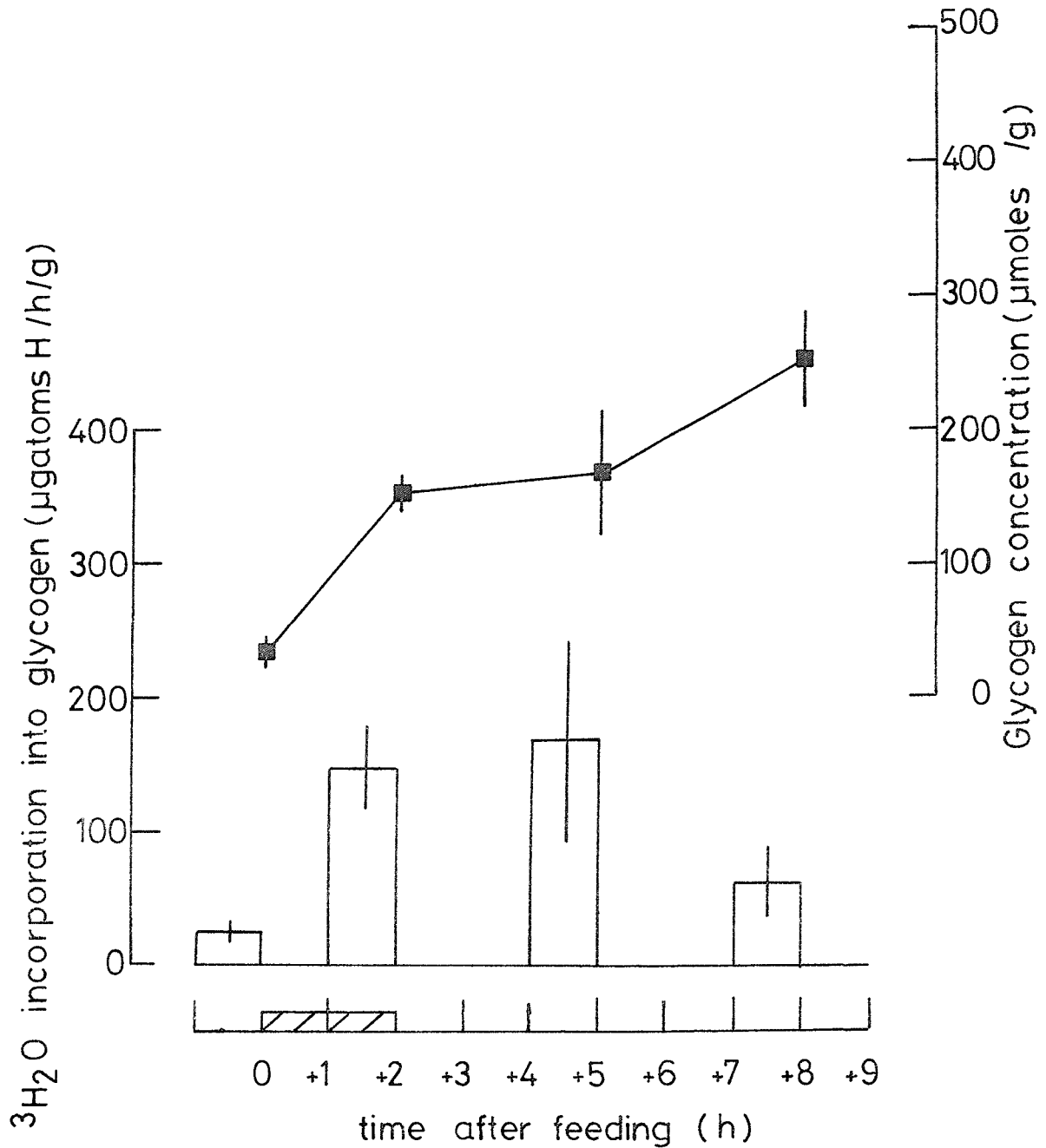


Experimental conditions and symbols were the same as in Fig. 31. Rats were made diabetic three days before the experiment by intravenous injection of streptozotocin (55 mg/kg body wt.).All rats displayed fasting hyperglycaemia in excess of 10 mmol/l.

Fig.34. Hepatic Glycogen Synthesis in Meal-fed Diabetic
Rats after Insulin Pre-treatment.
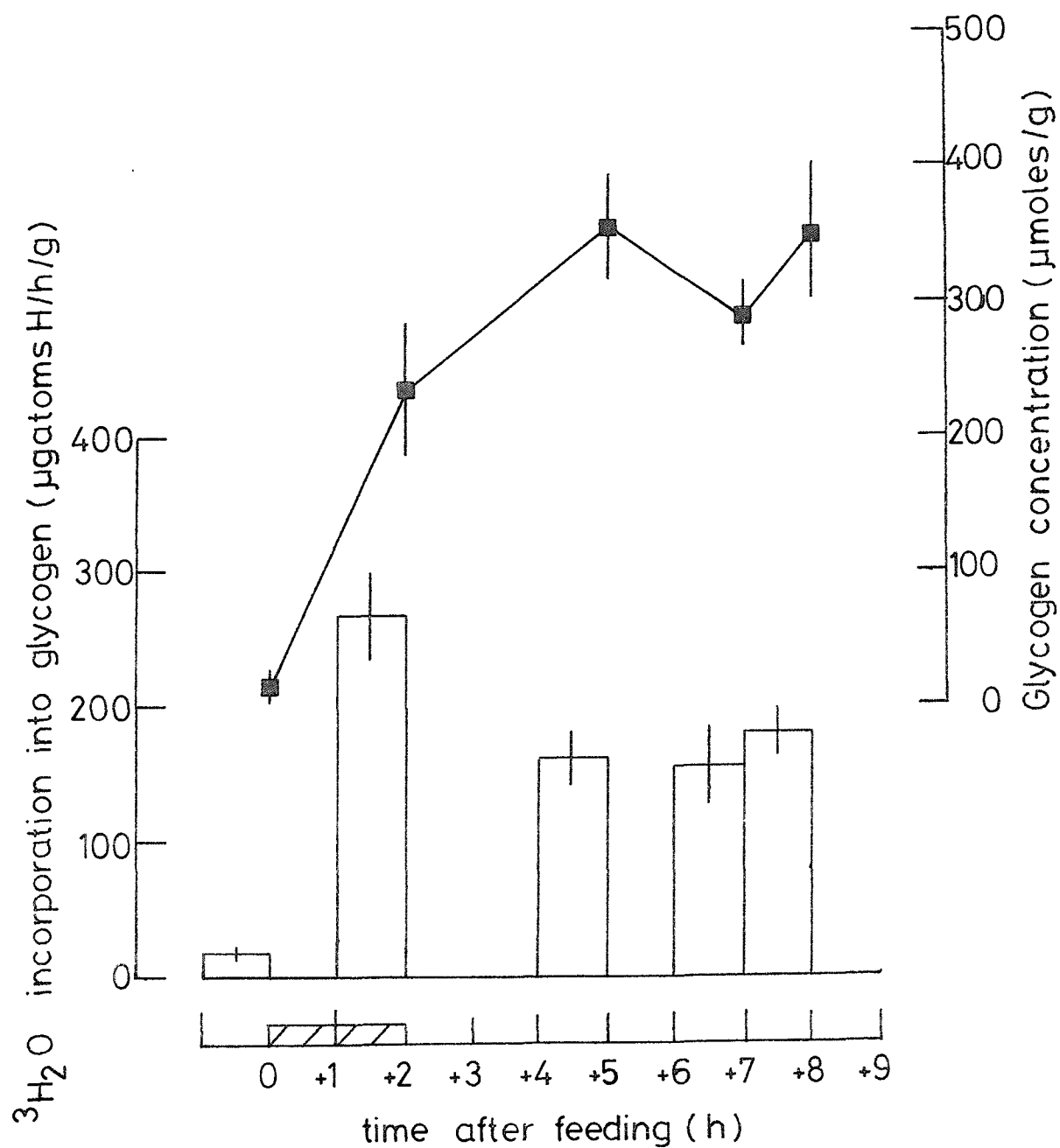


These rats were injected with insulin (P.Z.I., 3U) intraperitoneally
at -2h (08.00h).

fed rats after feeding. This was not performed.

### 3.2.7. Diabetic Rats

There is no absolute impairment of hepatic glycogen synthesis by
alloxan or streptozotocin diabetes (for review see Hers, 1976). In the
untreated diabetic animal both glycogen synthesis and storage are de-
pressed in the liver but could be restored to normal either by short term
insulin therapy or by providing a substrate such as fructose that does
not require insulin to be metabolized (Stalmans, 1976). The streptozotocin
diabetic rat was considered a good model to assess further the validity
of tritiated water incorporation as a measure of _in vivo_ glycogen syn-
thesis. It would be expected that the reduced amount of glycogen formed
in the liver would be accompanied by a proportionately lower tritium
content. All diabetic rats were fed the standard diet.

#### Untreated Diabetic Rats

The response of hepatic glycogen synthesis to meal-feeding was
both reduced and delayed by diabetes (Table 6; Fig. 33). Hepatic
glycogen concentration in diabetic rats was 66% that in control rats at
+2h after feeding (12.00h) and only reached maximum concentration by
about +8h after feeding (18.00h). Incorporation from tritiated water
into liver glycogen was also diminished compared with control rats at
+2h (12.00h). This low rate of glycogen synthesis was maintained until
+5h (15.00h) and then declined to the pre-fed rate by +8h (18.00h).
The considerable individual variation of tritiation at each time point
probably was a reflection of the variation of the degree of severity of
the diabetic lesion.

#### Insulin-treated Diabetic Rats

Injection of protamine zinc insulin (3U, i.p.) 2h before feeding
(08.00h) did not alter the fasted glycogen concentration or the glycogen
tritiated water incorporation (Table 6, Fig. 34). After feeding the
glycogen synthesis response was similar to that of controls. Glycogen
concentration was maintained within the normal range until at least +8h
after feeding (18.00h). Tritiated water incorporation reached a maximum

Fig.35. Hepatic Glycogen Synthesis in Meal-fed Corticotropin-treated Rats.
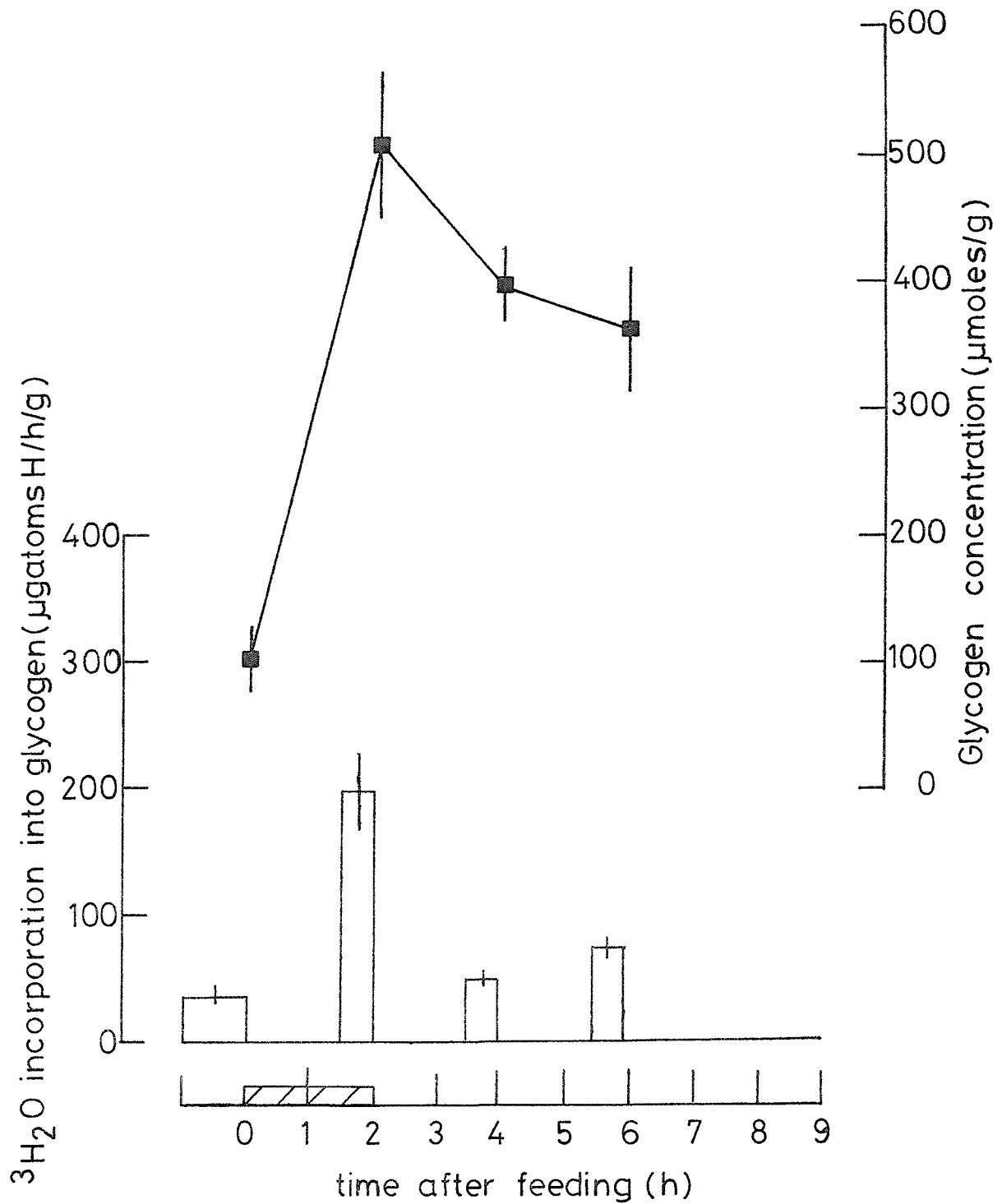


$^3$H incorporation in fed rats was measured over a 30min incubation period. Rats were injected with corticotropin ( Tetracosactrin-depot, 0.5 mg/ kg s.c.) for three days before the experiment.

at +2h after feeding (12.00h) and afterwards declined. The rate was still elevated at +8h (18.00h).

### 3.2.8. Glucocorticoid Hormones and Glycogen Synthesis

The dependence of hepatic glycogen synthesis on glucocorticoid hormones has been known for many years. An early method for bioassay of glucocorticoid activity involved injecting the steroid compound concerned into fasted, adrenalectomized rats (Bergman and Klein, 1943; Pabst et al, 1947). The increased muscle protein degradation provided sufficient amino acid precursors to support hepatic gluconeogenesis and glycogen synthesis. The amount of glycogen formed was dependent on the steroid dose administered. Adrenalectomy does not irreversibly abolish glycogen synthesis. The decreased glycogen concentration in livers from fed adrenalectomized rats was partially due to the low insulin concentration as it could be restored to control values by treatment with either cortisol or insulin (Whitton and Hems, 1976). Experimental modification of corticosterone concentration in rats, by treatment with corticotropin or by adrenalectomy, provided an additional opportunity to vary _in vivo_, the rate of glycogen synthesis. These procedures validated further the tritiated water incorporation method. The animals in these studies were all fed standard diet. They were used primarily to investigate the inhibition of lipogenesis by glucagon (Chapter 5); for this reason they were incubated with tritiated water for 30 min not 1h.

### Corticotropin Treatment

Meal-feeding induced a rapid increase of hepatic glycogen concentration in corticotropin-treated rats (Table 6; Fig. 35), which was maximal at +2h after feeding (12.00h). Tritiation of glycogen was also stimulated at +2h but did not accurately reflect the rapidity of the glycogen concentration increase. This discrepancy could be caused either by most liver glycogen being synthesized during the first 1.5h of feeding, when no incorporation from tritiated water would have occurred, or by a steroid induced increased substrate cycling between glucose and glucose-6-phosphate (Issekutz, 1977). Such substrate cycling would exchange tritiated glucose with un-labelled blood glucose and so cause tritium incorporation to be under-estimated (Section 3.3.3).

Fig.36. Hepatic Glycogen Synthesis in Meal-fed Adrenalectomised Rats.
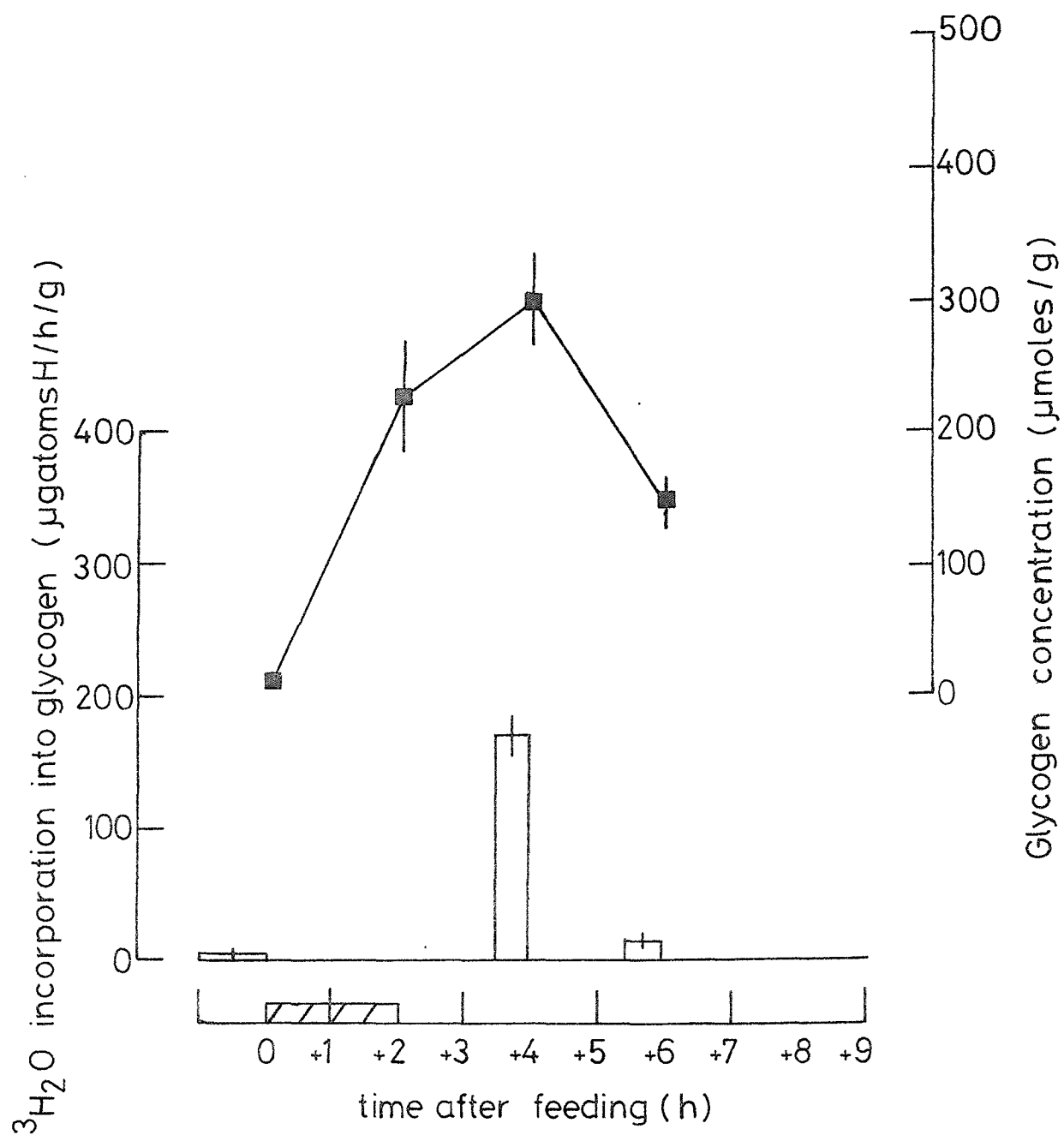


Experimental condition were the same as in Fig. 35. Rats were subject to bilateral adrenalectomy seven days before the experiment.

## Adrenalectomized Rats

Hepatic glycogen synthesis in response to meal-feeding was not significantly depressed by adrenalectomy at +2h and +4h after feeding (Table 6; Fig. 36). Adrenalectomized rats could not maintain their liver glycogen reserves, however, in the post-prandial period. By +6h after feeding tritium incorporation from tritiated water into glycogen was close to zero and hepatic glycogen concentration had fallen to 50% of the maximum value observed 2h earlier. This was reflected by the very low 22h fasted glycogen concentration before feeding (Table 5).

Table 8

### Inhibition of Hepatic Glycogen Synthesis by Glucagon

| Time (h) | Injection | Glycogen $^3H_2O$ Incorporation ( $\mu$ gatoms 'H'/g/h) | | | |
|---|---|---|---|---|---|
| | | Control Rats | Cortico-tropin-treated Rats | Adrenal-ectomized Rats | Pair-fed Rats |
| Pre-fed | – | 45±6 (8) | 38±9 (6) | 5±2 (4) | |
| +2h | saline | 189±23 (8) | 198±29 (7) | | |
| | glucagon | *39±26 (4) | *97±24 (7) | | |
| +4h | saline | 156±8 (4) | 49±11 (4) | 170±20 (4) | 132±8 (4) |
| | glucagon | †23±2 (4) | ‡12±1 (7) | †17±3 (3) | ‡12±1 (4) |
| +6h | saline | 46±12 (4) | 74±14 (6) | 18±5 (4) | |
| | glucagon | *16±1 (4) | | | |

Rats were injected with glucagon (1mg/kg) or saline 30 min before being killed. Pair-fed rats were controls for the adrenalectomized rats. Results are expressed as mean S.E.M. (number of rats). Significance ± glucagon by Students' 't' test:

*, $0.05 > p > 0.01$; †, $0.01 > p > 0.001$; ‡, $p < 0.001$.

Table 9

### Glucagon Stimulation of Hepatic Glycogenolysis

| Time (h) | Injection | Glycogen Concentration ( µmoles/g) | | | |
|---|---|---|---|---|---|
| | | Control Rats | Cortico-tropin-treated Rats | Adrenal-ectomized Rats | Pair-fed Rats |
| Pre-fed | - | 109±28 | 104±27 | 12±5 | |
| +2h | saline | 199±28 | 513±54 | 226±41 | |
| | glucagon | ‡40±8 | 379±56 | †16±6 | |
| +4h | saline | 432±13 | 400±32 | 302±35 | 336±16 |
| | glucagon | †265±28 | 319±28 | 241±17 | *248±30 |
| +6h | saline | 407±26 | | 146±19 | |
| | glucagon | ‡227±18 | | | |

| Table 10 | Calculated Glycogenolytic Rate ( µmoles/g/h) | | | |
|---|---|---|---|---|
| +2h | 268 | 234 | | |
| +4h | 334 | 150 | 70 | 136 |
| +6h | 350 | | | |

Experimental conditions, numbers of animals and probability symbols are the same as described in Table 8.

3.2.9. <u>Glucagon</u>

Glucagon action on the liver inhibits glycogen synthase and acti-
vates phosphorylase by co-ordinated cyclic AMP-dependent enzyme protein
phosphorylation (Hers, 1976). As this results in inhibition of glycogen
synthesis and stimulation of glycogenolysis there should be little
incorporation from tritiated water into liver glycogen in glucagon-
treated animals. The response of hepatic glycogen synthesis to glucagon
injection <u>in vivo</u> in fed rats (1 mg/ Kg) was measured for control,
corticotropin treated and adrenalectomized rats (Table 8). In all these
groups glucagon inhibited glycogen synthesis, measured by tritiated
water incorporation. The only group of rats for which glucagon did not
reduce the glycogen tritiated water incorporation below the pre-fed
rate was the corticotropin treated group at +2h. These animals also
displayed a large range of glycogen concentration reflecting the rapid
response of glycogen synthesis in this group to feeding (Section 3.2.9).

The difference in glycogen concentration between saline-treated
and glucagon-treated rats (Table 9) consists of two components. These
are the continued glycogen synthesis in the 35 min after saline injection
and the stimulated glycogen degradation after glucagon injection. In-
corporation from tritiated water into glycogen enables these two components
to be distinguished.

There were a mean of 2.87 tritiations for each glycosyl residue
incorporated into glycogen (Section 3.2.10). The glycogen glucose
formed in the control groups after saline injection (X) can thus be cal-
culated from the equation

$$
X = \frac{\text{glycogen tritium} \ - \ \text{glycogen tritium}}{2.87} \quad \text{x} \quad \frac{60}{\text{time of incubation}} \ \mu\text{ moles/g}
$$

(saline)     (glucagon)

The glycogenolytic response to glucagon is this product (X) subtracted
from the difference of glycogen concentration between the two groups.
This value was calculated for rats in the various experimental groups
(Table 10). The difference of hepatic glycogen concentration due to

glucagon was apparently caused by stimulated glycogenolysis and not to inhibition of glycogen synthesis. The data do not enable any inference to be drawn about the permissive role of glucocorticoid in the glucagon stimulation of glycogenolysis (Section 1.4.4; Exton et al, 1972; Chan et al, 1979). These studies with isolated perfused liver and hepatocytes found that adrenalectomy impaired the glycogenolytic response to glucagon. The effect was overcome by increased glucagon concentration and consequently would not be expected to be apparent in the studies presented above, as a maximal dose of 1mg/kg of glucagon was employed.

### 3.2.10. The Ratio of Tritiated Water Incorporated to Glycogen Deposited

The incorporation of tritiated water into hepatic glycogen was calculated as $\mu$gatoms 'H' per g of liver. Division of this value by the increase in glycogen concentration over the same period ( $\mu$moles glucose per g liver) gave the number of tritiations per glycogen glucose formed. This ratio was calculated during the phase of glycogen synthesis for control and diabetic rats and for control rats fed either high glucose diet or high fructose diet and the results are shown in Table 11. Between all groups the ratio was reasonably constant, the mean being $2.84\pm0.47$ with a range of 2.5 to 3.6. This constancy lends support to the use of glycogen tritiated water incorporation as a direct measure of the rate of hepatic glycogen synthesis. Division of the tritiated water incorporation by 2.84 would give a reasonable estimate of the $\mu$moles of glycogen glucose synthesized.

### 3.2.11. The Positional Analysis of Glycogen Tritium

The complete positional analysis of glucogen tritium was performed for control rats fed standard diet, high glucose diet or high fructose diet. These animals were injected i.p. with 2m Ci of tritiated water at 10.00h, before the feeding period. They were killed and their livers freeze-clamped at 14.00h. Enabling all the glycogen synthesized in response to feeding to become tritiated had two advantages. Firstly the specific radioactivity of the glycogen was sufficiently high for convenient analysis and secondly the number of tritiations per glycogen glucose could be readily calculated. Tritiation of glycogen during the

Table 11

Representative Example of the Positional Analysis of Glycogen Glucose
Tritium

1)  The following parameters were calculated (after Table 7):-

     [Glycogen]                                     = <u>510 $\mu$ moles/g</u>

     Glycogen $^3H_2O$ incorporation          = 1136 $\mu$ <u>gatoms</u>
                                                                 <u>'H'/g</u>

2)  <u>Percentage tritium distribution</u>

    Each [ $^3H$ ]-glucose sample (100 $\mu$l) for analysis contained
    12.0  moles, 5727 d.p.m.

<u>C-1 tritium</u>

    Gluconate:  recovery  =  79.1%             tritium  = 4195 d.p.m.

    ∴ Glycogen tritium at C-1 = $\frac{5727-(4195-0.791) \times 100}{5727}$    =   <u>7.4%</u>

<u>C-6 tritium</u>

    Dimedone complex : recovery  =  70.9%;     tritium  =  621 d.p.m.

    ∴ Glycogen tritium at C-6 = $\frac{621 \times 100}{0.709 \times 5727}$          =  <u>15.3%</u>

<u>C-2 tritium</u>

    Tritium after incubation with PGI  =  4038 d.p.m.

    ∴ Glycogen tritium at C-2 = $\frac{(5727-4038) \times 100}{5727}$    =  <u>29.5%</u>

<u>C-5 tritium</u>

    Tritium after incubation with  GDH and TPI  =  2652 d.p.m.

    ∴ Glycogen tritium at C-5 = $\frac{(4038-2652) \times 100}{5727}$    =  <u>24.2%</u>

<u>C-3 tritium</u>

    Tritium after incubation with G3-PDH and TPI  =  3311

    ∴ Glycogen tritium at C-3 = $\frac{(4038-3311)}{5727}$        =  <u>12.7%</u>

<u>C-4 tritium</u>

Tritium after incubation with G3-PDH and ADH = 3689 d.p.m.

∴ Glycogen tritium at C-4 = $\dfrac{(4038 \times 3689) \times 100}{5727}$ = <u>6.1%</u>

3) <u>Ratio tritium : glycogen glucose</u> = $\dfrac{\text{glycogen tritium}}{\text{(glycogen)}}$

$= \dfrac{1,136}{510-109}$  $= \underline{2.83 \,\mu\text{gatoms}}$ $\underline{\text{'H'} / \mu\text{mole}}$ <u>glycosyl</u>

4) <u>Number of tritium atoms / mole at each glycosyl carbon</u>

| Glucose carbon no. | 1 | 2 | 3 | 4 | 5 | 6 | total |
|---|---|---|---|---|---|---|---|
| % tritium | 7.4 | 29.5 | 12.7 | 6.1 | 24.2 | 15.3 | 95.2 |
| no. tritium atoms/ mole glycogen glucose | 0.21 | 0.83 | 0.36 | 0.17 | 0.68 | 0.43 | 2.68 |

routine 1h incubations with tritiated water meant that more glycogen glucose sample had to be used in the glucose degradation analysis. Such samples were used only to measure tritium at C-2 of glycogen glucose from control and diabetic rats during the feeding response of hepatic glycogen synthesis. An example of the complete positional analysis calculations for glycogen tritium is shown in Table 11.

The positional distribution of hepatic glycogen tritium from rats in all experimental groups is summarized in Table 12. A feature common to all groups was the substantial tritium content at C-2, which ranged from 26 to 34% and was equivalent to between 0.77 and 0.92 tritium atoms at C-2 per molecule of glycogen glucose synthesized. This value approached one full tritiation at C-2 and provided further evidence for the validity of measuring glycogen synthesis by tritiated water incorporation. In control animals throughout the period of elevated glycogen synthesis, the glycogen tritium incorporated at C-2 accurately reflected the pattern of total tritiated water incorporation with a mean of 34% of that value (Fig. 31).

For control rats fed any of the three diets there was consistently substantial further tritiation at C-5 of glycogen glucose. This incorporation represented from 0.6 to 1.0 tritium atoms at C-5 per glucose molecule. The distribution of the remaining tritium between the other glucose carbon atoms varied with the diet eaten. For rats eating the standard diet this tritium was evenly divided between C-1, C-3 and C-6; on the high glucose diet tritium was distributed between C-1 and C-6; on the high fructose diet tritium was located largely at C-3.

The generally consistent pattern of glycogen tritiation between groups enabled the rate of glycogen synthesis ( $\mu$moles/g/h) to be calculated from the rate of glycogen tritiated water incorporation ( $\mu$gatoms 'H'/g/h). Unfortunately it also meant that the positional tritium distribution in glycogen was of limited value in assigning significance to the relative contributions of different precursors to glycogen synthesis <u>in vivo</u>. The minimum expected tritium distribution would be at C-2 and C-5, incorporated by equilibriation of glucose-6-phosphate through phosphoglucose isomerase, transaldolase and triose

Table 12

Pattern of $^3$H in Glucose Derived from Glycogen

| | Intact rats | | | Diabetic rats (Standard diet) | |
|---|---|---|---|---|---|
| | Standard diet (4) | Glucose diet (3) | Fructose diet (3) | Untreated (6) | Insulin-treated (6) |
| Hepatic glycogen concentration (μmol/g) | | | | | |
|   Fasting | 109 ± 28 | 73 ± 7 | 74 ± 8 | 35 ± 12 | 17 ± 2 |
|   Fed | 446 ± 43 | 375 ± 30 | 532 ± 11 | 147 ± 29 | 234 ± 47 |
|   Increase on feeding | 337 | 302 | 458 | 112 | 217 |
| Incorporation of $^3$H from $^3$H$_2$O into glycogen (μg-atoms of H/g) | 1016 ± 54 | 783 ± 136 | 1650 ± 85 | 280 ± 49 | 542 ± 64 |
| Number of $^3$H atoms per glucose molecule in glycogen | 3.0 | 2.6 | 3.6 | 2.5 | 2.5 |
| Percentage of total $^3$H at glucose carbon atom number | | | | | |
|   1 | 10.4 ± 1.4 | 14.3 ± 1.3 | 7.8 ± 1.3 | – | – |
|   2 | 30.4 ± 2.7 | 29.7 ± 2.1 | 25.7 ± 0.8 | 34.4 ± 2.7 | 31.9 ± 1.5 |
|   3 | 12.7 ± 2.2 | 8.6 ± 2.0 | 19.2 ± 1.4 | – | – |
|   4 | 3.2 ± 1.9 | 7.8 ± 0.7 | 9.5 ± 2.7 | – | – |
|   5 | 22.7 ± 3.2 | 21.8 ± 0.5 | 29.0 ± 3.1 | – | – |
|   6 | 13.6 ± 1.6 | 24.2 ± 2.8 | 10.3 ± 0.4 | – | – |

Number of atoms of $^3$H incorporated
 at glucose carbon atom
 number

| | | | | | |
|---|---|---|---|---|---|
| 1 | 0.31 | 0.37 | 0.27 | - | - |
| 2 | 0.91 | 0.77 | 0.92 | 0.86 | 0.80 |
| 3 | 0.38 | 0.22 | 0.69 | - | - |
| 4 | 0.10 | 0.20 | 0.34 | - | - |
| 5 | 0.68 | 0.57 | 1.04 | - | - |
| 6 | 0.41 | 0.60 | 0.37 | - | - |

Rats were injected with 2mCi of $^3$H$_2$O at 10:00h; normal rats were killed at 14.00h; diabetic rats were killed at 12:00h. The number of $^3$H atoms incorporated per glucose molecule from glycogen was calculated by dividing the $^3$H incorporation by the corresponding increase in glycogen concentration. Results are expressed as means $\pm$ S.E.M. where appropriate. The values in parentheses indicate the number of rats per group. Each liver sample was analysed on at least three separate occasions.

phosphate isomerase (Section 3.1.3.5). This pattern was displayed
by all glycogen samples analyzed. The additional tritium at C-3 of
glycogen isolated from liver of fructose fed rats is interesting. As
fructose is obligatorily metabolised via triose phosphates, (Hers, 1957),
glycogen synthesized from fructose should be tritiated at C-3 and C-5
during triose phosphate isomerization (Section 3.1.3.5).

### 3.2.12. Muscle Glycogen Synthesis

Table 13

#### Incorporation of Tritiated Water into Muscle Glycogen

| Time after feeding | (Glycogen) ($\mu$ moles/g) | Glycogen $^3H_2O$ incorporation ($\mu$ gatoms 'H'/ g/h) |
|---|---|---|
| Pre-fed | 12.5±1.9 | 56.6±10.8 |
| + 2 | 21.4±3.6 | 58.5± 3.1 |
| + 4 | 26.3±3.3 | 43.3± 1.7 |
| + 6 | 28.8±1.25 | 58.9±11.8 |
| + 8 | 28.1±2.6 | 71.5± 9.3 |

In one series of experiments muscle glycogen synthesis in control
rats was measured at intervals during the feeding cycle. After a 1h
pulse with 2m Ci of tritiated water, the rats were killed and samples
of skeletal muscle from the leg were removed and freeze-clamped. The
glycogen concentration and tritiated water incorporation in these
muscle samples were then estimated and are summarized in Table 13.
Although muscle glycogen concentration increased on feeding from 12.5 to
23.4 μmoles/g, this was only between two and four percent of the increase
in liver glycogen concentration under identical conditions. The
associated rates of tritium incorporation into muscle glycogen were
correspondingly low and so no significant analysis was possible.

3.2.13. Glycogen Synthesis in Foetal Rat Liver

The rate of glycogen synthesis in foetal rat liver was estimated in vivo by the incorporation of tritiated water. Female rats, 200 to 250g, were fed ad lib and housed in the light from 08.00h to 20.00h. The five day oestrus cycle was charted by microscopical examination of vaginal smears. Rats were mated overnight when in oestrus; this time was considered day 0 of gestation. Pregnancy was confirmed by palpation 14 days later.

Groups of pregnant rats were injected with 2m Ci of tritiated water at about 11.00h on days 18.5, 19.5 or 20.5 of gestation. They were anaesthetized 55 min later with sodium pentobarbitone (20-30 mg/kg, i.p). Anaesthetized foetuses were delivered by Caesarian section and their livers rapidly removed and frozen in liquid nitrogen. Liver samples from sibling foetuses (n = 4 to 10) were pooled for analysis. The time between administration of the anaesthetic and removal of the last foetal liver never exceeded 15 min.

The glycogen concentration and tritiated water incorporation of foetal rat liver is shown in Fig. 37. Both were very low on day 18.5 and increased during the subsequent 2 days. Glycogen concentration was at meal-fed adult values by day 20.5 ($380\pm6$ $\mu$moles/g). The incorporation from tritiated water was elevated on day 19.5 ($138\pm30$ $\mu$ gatoms 'H'/g/h) and remained so even on day 20.5 ($133\pm15$ $\mu$ gatoms 'H'/g/h) when glycogen concentration was maximal. The proportion of incorporated tritium at C-2 of glycogen glucose was $27.0\pm4.4\%$, implying that the number of tritiations per glycosyl moeity incorporated into foetal glycogen could be similar to the ratio calculated for the adult rat. Using the adult value of 2.84 (Section 3.2.10), the absolute rate of glycogen synthesis on days 19.5 and 20.5 of gestation can be calculated from the tritium incorporation to be between 30 and 40 $\mu$moles of glycosyl units/g of liver/h.

The increase in glycogen concentration of foetal liver from day 19.5 to 20.5 was 247 $\mu$moles/g. If this increase was linear, the net rate of glycogen deposition over that 24h period would be about 10 $\mu$moles/g of liver/h. The absolute rate of glycogen synthesis, calculated from tritium

Fig.37. Hepatic Glycogen Synthesis in Foetal Rats.

Maternal rats were injected with 2mCi $^3H_2O$ 1h before being killed.
Foetuses were delivered by Caesarian section and foetal liver freeze-
clamped in liq $N_2$. Each point represents the mean ± S.E.M. of pooled
liver samples from 3 litters.
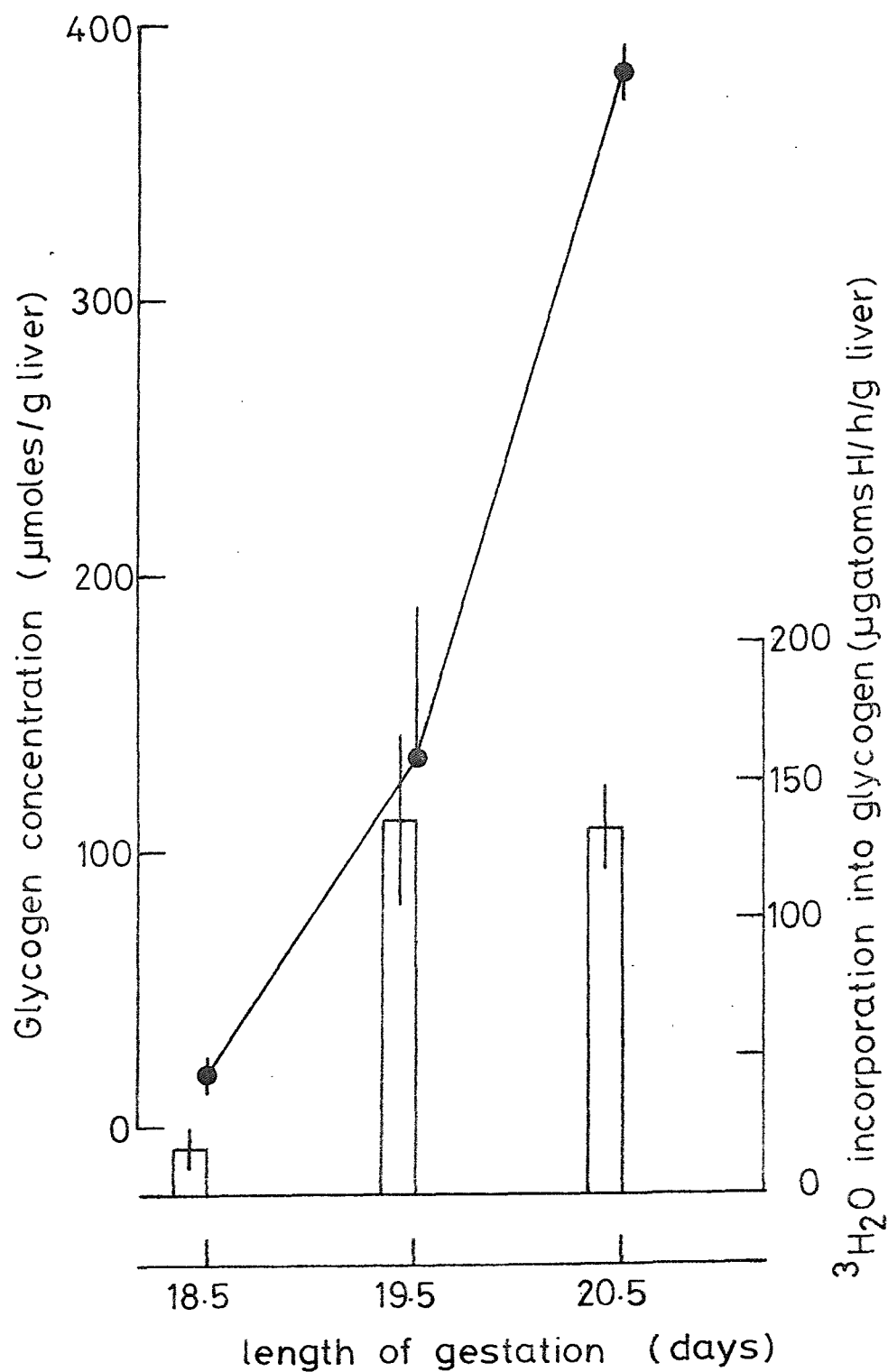
incorporation, was some three-fold higher. Thus either glycogen synthesis was disproportionately elevated during the incubation with tritiated water or that the rate of simultaneous synthesis and degradation of glycogen is considerable in foetal rat liver.

This conclusion is unlikely to be affected by any glycogen degradation in the period between anaesthetizing the mother and freezing the foetal liver. A glycogenolytic rate of $2\,\mu$moles/min/g liver was reported in anaesthetized foetal rats (Devos and Hers, 1974). The maximum time taken for isolation of foetal glycogen was 15 min, implying a maximum loss of glycogen of $2 \times 15 = 30\,\mu$moles/g liver. The same authors have elegantly shown, however, an ordered sequence of glycogen synthesis and degradation, with the latest glycogen glucose incorporated being the first glucose released (Devos and Hers, 1974; Devos et al, 1979). In the foetal livers there would be preferential degradation therefore of newly synthesized, tritiated glycogen. Such a degradation would lead to the underestimation of the tritiated water incorporation into glycogen and thus also of the rate of glycogen turnover in foetal rat liver.

## 3.3. Discussion

These results confirm that glycogen synthesis can be measured by incorporation of tritium from tritiated water. This method proved particularly applicable to the measurement of _in vivo_ rates of hepatic glycogen synthesis as rapid and large increases of hepatic glycogen concentration were observed in response to feeding. A greater body load of tritiated water would have been required to measure adequately the changes in muscle glycogen synthesis which were much smaller under similar feeding conditions. The tritiated water dosage to estimate glycogen synthesis in muscle would be 100m Ci per rat in comparison to the 2m Ci per rat required for the same measurement in liver. Such tritium concentrations would be too great for use in a routine method.

Hepatic glycogen metabolism was efficiently regulated by the meal-feeding and lighting regime under which rats were housed. The amplitude of the diurnal variation of hepatic glycogen concentration was increased

in meal-fed rats compared with rats fed ad libitum (Hopkins et al, 1972; Babcock and Cardell, 1974). The pattern of rapidly increasing glycogen concentration in response to feeding followed by a prolonged period of elevated glycogen concentration enabled a number of aspects of hepatic glycogen metabolism to be evaluated by incorporation of tritium from tritiated water. These aspects included the occurrence of any significant rate of glycogen turnover in vivo, the effects of experimentally altered hormonal states on glycogen metabolism and the effect of different diets on the glycogen tritiation pattern.

## 3.3.1. Hepatic Glycogen Turnover

A significant rate of glycogen re-cycling in the liver would imply that glycogen synthesis and degradation were both active at the same time. As glycogen concentration changes are the net result of the rates of glycogen synthesis and degradation, they cannot be used directly to estimate glycogen turnover. The turnover rate could be estimated, however, by comparison of the incorporation from tritiated water into glycogen with changes in glycogen concentration.

The acinal model of hepatic anatomy comprises three functional zones (Rappaport, 1960). The periportal hepatocytes in zone I are situated around the terminal portal veins, while the pericentral cells in zone III are arranged around the terminal hepatic arteries and veins. Intermediate cells in zone II are located between pericentral and periportal cells. Distinct enzyme profiles between these different zones have been demonstrated by microdissection of frozen liver (Guder et al, 1976). Gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase are preferentially located in the periportal cells and glycolytic enzymes such as pyruvate kinase in the pericentral cells. This cell heterogeneity is thought to be maintained dynamically by the gradients of substrates and oxygen within the acinus.

Glycogen metabolism has been related to this acinal model by histochemical techniques (Babcock and Cardell, 1974; Sasse, 1975). After an overnight fast, glycogen is restricted to zone III; glycogen synthase is slightly active in zone III but phosphorylase is active in

zones I and II. Feeding initially inactivates phosphorylase and activates glycogen synthase in zone II. Subsequently these processes are extended to zone I. In a well fed rat or hamster, glycogen is distributed throughout the liver; phosphorylase is active only in the immediately periportal region and glycogen synthase is active in those cells, mainly in zone I, that are actively forming glycogen. These enzyme patterns are reversed upon fasting when glycogen breakdown is initiated in the periportal cells and proceeds towards the pericentral cells.

The structure of glycogen can also influence glycogen turnover. Liver glycogen granules consist of large α-particles comprised of smaller β-particles (Drochmans, 1962). Glycogen is synthesized in the liver by the formation of additional β-particles and not by enlargement of pre-existing particles (Devos and Hers, 1979). Glycogenolysis degrades these newly formed β-particles, containing both core and outer chain glycosyl residues, rather than preferentially removing the outer chain residues from all β-particles.

Glycogen turnover in post-prandial meal-fed rats was estimated by comparing the rate of glycogen synthesis with the change in glycogen concentration. This analysis depends on there being no preferential addition and subsequent removal of identical tritiated glycosyl units. In the context of the whole liver, the last glycogen molecules synthesized are the first degraded (Devos and Hers, 1979). The inhibition of glycogen synthase by phosphorylase a (Stalmans et al, 1973) would, however, appear to preclude glycogen turnover within a single hepatocyte. Thus a significant rate of glycogen recycling, when glycogen was maintained at an elevated constant concentration, would be expected to be accompanied by substantial tritium incorporation from tritiated water. In control rats from +5h to +9h after feeding, no such stimulated rate of tritium incorporation was apparent (Fig. 31) and so the rate of hepatic glycogen recycling was considered to be low.

Conditions where hepatic glycogen turnover was apparently active included insulin-treatment of diabetic rats (Fig. 34) and foetal rats (Fig. 37). In both these groups of rats the tritium incorporation into hepatic glycogen was too great for the corresponding change in liver

glycogen concentration. The rationale and mechanism underlying the high glycogen re-cycling in insulin-treated diabetic rats at +7h and +8h after feeding are not clear. Interestingly, treatment of alloxan diabetic rats with insulin resulted in glycogen synthase and phosphory-lase both being active in periportal regions of the liver, with some hepatocytes apparently displaying active forms of both enzymes (Sasse, 1975). The dramatic increase in hepatic glycogen content of foetal animals towards the end of gestation has been known for many years (Bernard, 1859). Hepatic glycogen deposition begins between day 18 and day 19 of gestation in the foetal rat (Jacquot, 1959). These glycogen reserves are mobilized within 20h of birth (Shelley, 1961; Bittner et al, 1978). It is still uncertain whether hepatic glycogen in the foetus contributes to blood glucose homeostasis in late gestation. Such a role would require considerable glycogen recycling in the rat foetus. The results of the tritiated water incorporation into foetal hepatic glycogen support this hypothesis (Fig. 37). Previously, a rate of glycogen recycling of 5 mg/g of liver/day has been calculated for the foetal rat at term from the incorporation of deuterium from deuterated water (Goldwater and Stetten, 1947). A similar rapid turnover of liver glycogen was observed when $[1-^{14}C]$ glycerol was injected into the foetus (Gilbert and Bourbon, 1978). $[1-^{14}C]$ glycerol was incorporated for only 1h and the radioactivity thereafter rapidly declined. By contrast, there is also direct evidence against substantial glycogen recycling in foetal liver. $[6-^3H]$ glucose injected into the mother and incorporated into foetal liver glycogen was still present 38h later (Devos and Hers, 1974).

## 3.3.2. The Absolute Rate of Hepatic Glycogen Synthesis

The use of tritiated water overcame many of the problems encountered when glycogen synthesis is measured by incorporation of $[^{14}C]$-substrates. The complete equilibrium of body water, shown by the identical specific radioactivity of muscle and liver after tritiated water injection (Fig. 29), meant that dilution with endogenous unlabelled substrate could not occur. The lack of compartmentation of intra-cellular water enabled rates of tritium incorporation to be converted, by division with liver water specific radioactivity, into rates of total hydrogen atoms incorporated. The tritium isotope effect appeared to be slight, in agreement with

Fig.38. Tritium Redistribution during Glucose : Glucose-6-phosphate
Substrate Cycling
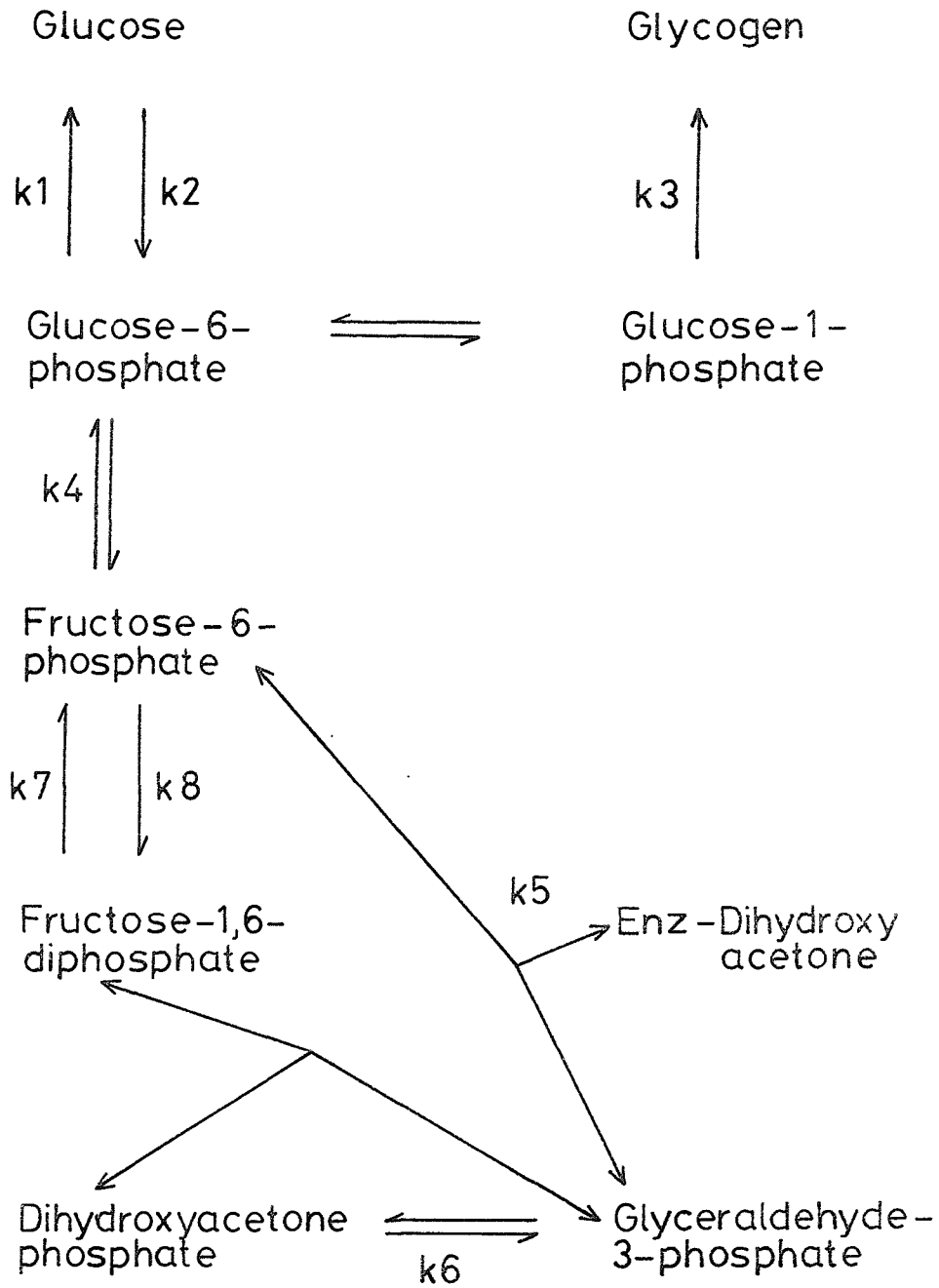
previous findings in hepatocytes (Rognstad and Katz, 1974). A large
tritium isotope effect would not have been consistent with the observed
incorporation at C-2 of glycogen glucose of between 0.77 and 0.92 tritium
atoms per molecule (Table 11).

Calculation of absolute synthetic rates from the glycogen tritium
incorporation could be affected by any glycogen turnover or by variability
of the source of glycogen precursor. Neither of these factors appeared
to be significant in control rats. Hepatic glycogen turnover was low
in the fed state (Section 3.3.1), and the ratio of total hydrogen incor-
porated to glycogen glucose formed was relatively constant (2.84±0.47).
Division by this ratio enabled the absolute rate of glycogen synthesis
to be calculated from the tritium incorporation.

### 3.3.3.  Distribution of Glycogen Tritium

A corrollary of the constant proportional incorporation of tritium
from tritiated water into glycogen is that it was not possible to use the
positional distribution of that incorporation to provide information
about which precursors were used in vivo for hepatic glycogen synthesis.
Glycogen synthesis in tritiated water in all experimental conditions
analyzed was accompanied by full tritiations at C-2 and C-5. There was
significantly more extensive incorporation of tritium only when rats were
fed the high fructose diet (Table 11). Trititiation at C-2 and C-5,
but not at C-3, probably resulted from the metabolism of glucose by
phosphoglucose isomerase transaldolase and triosephosphate isomerase
(Section 3.1.3.5, Fig. 23). However, it cannot be concluded from a
tritiation pattern exclusively at C-2 and C-5 that blood glucose is the
sole substrate for hepatic glycogen synthesis (Fig. 38). This can be
shown by consideration of a hypothetical case where there is no net up-
take or release of glucose by the liver and glycogen is synthesized
exclusively from triose substrates. Glucose-6-phosphate derived from
gluconeogenesis would be tritiated at C-2, C-3, C-4 and C-5 (Fig. 25).
This tritiated glucose-6-phosphate would either exchange with unlabelled
glucose or be converted to glycogen. If the rates of glucose-6-phos-
phatase (k1) and glucokinase (k2) exceeded that of glycogen synthase (k3),
then the glucose-6-phosphate pool would become less tritiated. The rates

of phosphoglucose isomerase (k4), transaldolase (k5) and triose phosphate
isomerase (k6) are greater than the rates k1, k2, k3, fructose-1,6-
bisphosphatase (k7) and phosphofructokinase (k8). The net result of
these reactions would be that tritiated glucose-6-phosphate would be
exchanged for unlabelled glucose which in turn would become tritiated
at C-2 and C-5. Such a labelling pattern of glycogen glucose would
therefore be expected whenever there was a substantial rate of substrate
recycling between glucose and glucose-6-phosphate. In support of this
concept, fructose decreased the rate of glucose recycling in perfused
liver (Clark et al, 1980) and fructose-feeding resulted in greater
tritium incorporation into glycogen than any other condition (Table 11).

### 3.3.4. Potential Applications of the Measurement of Glycogen Synthesis by Tritiated Water Incorporation

This method can be applied to measurements of absolute rates of
glycogen synthesis both in vivo and in vitro. It is possible that the
defined conditions of substrate supply in isolated hepatocytes or per-
fused liver could enable the tritiation pattern of glycogen glucose to
vary with the preferred glycogen precursor incorporated. Such a
correlation with precursor source was not observed in vivo because of
both glucose : glucose-6-phosphate recycling (Section 3.3.3) and the
effect of extra-hepatic metabolism on the presentation of substrates to
the liver.

The response to feeding can be separated on the basis of tritiated
water incorporation into two distinct phases. For the first 5h after
feeding, hepatic glycogen synthesis was active in control rats (Section
3.2.6). During this phase hepatic glycogen could not be a net precursor
for any other metabolic pathway, but it could serve such a function
during the subsequent phase from +5h to +9h after feeding. This dis-
tinction is relevant to the regulation of hepatic fatty acid biosynthesis.
Hepatic glycogen has been described as a significant lipogenic substrate
in the post-prandial rat (Wood and Krebs, 1971; Salmon et al, 1974;
Hems et al, 1975) and the inhibition of hepatic lipogenesis by glucagon
has been considered secondary to depletion of substrate glycogen (Ma et
al, 1978). However, this explanation for the action of glucagon cannot

apply during the first 5h after feeding when glycogen cannot be a lipogenic precursor. This example demonstrates how knowledge of the absolute rate of glycogen synthesis can provide information about the regulation of another hepatic pathway.

One experimental preparation where incorporation from tritiated water into glycogen could generate interesting results is isolated cell culture. One problem has been obtaining satisfactory rates of glycogen synthesis in isolated hepatocytes (Hue et al, 1976; Katz et al, 1979). Tritiated water incorporation could distinguish the condition where glycogen synthesis is preceded by a period of glycogenolysis from that where no change of glycogen concentration occurs. The use of deuterated water instead of tritiated water could also quantify the proportion of individual glycogen or glucose molecules synthesized by alternative pathways. For instance, the conversion of lactate to glucose would generate a molecule of $M + 5$ to $M + 7$, while glucose recycling would only generate a molecule of $M + 2$. This technique would not be applicable in vivo because of the high concentrations of deuterated water required.

Leucocytes are another cell type where glycogen synthesis measurement by tritiated water incorporation could prove useful. Leucocyte phagocytosis stimulates phosphorylase and glycogenolysis (Saugermann and Esmann, 1977; Petersen et al, 1978). Removal of the phagocytotic stimulus activates leucocyte glycogen synthase and replenishes the glycogen reserves, which can amount to 1% of the cellular wet weight. Incorporation of tritiated water would appear to be an ideal method to measure this glycogen re-synthesis which could provide potentially useful clinical information. A more general application to human patients in vivo would appear to be precluded by the large body load of tritiated water that would be necessary and by ethical considerations about obtaining liver biopsy samples for analysis.

CHAPTER 4
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HEPATIC FATTY ACID BIOSYNTHESIS - EFFECTS OF MEAL-FEEDING AND INSULIN

4.1. Introduction

The triglyceride fatty acid of adipose tissue is the major energy reserve of mammals. This fatty acid can be derived from three sources. These are directly from the diet or from de novo synthesis in liver or adipose tissue. The rate of de novo fatty acid biosynthesis is inversely proportional to the fat content of the diet (Romsos and Leveille, 1974); Numa and Yamashita, 1974). The diets fed to the rats in these studies had a low fat content (Table 2). Consequently fatty acid biosynthesis was active to convert dietary carbohydrate into storage triglyceride.

The liver is major site of fatty acid biosynthesis (Hems et al, 1975; Baker et al, 1978). However, some of the classical methods devised to analyse metabolic regulation (Newsholme and Start, 1973) have only limited applications to fatty acid metabolism. Despite extensive knowledge of the properties of lipogenic enzymes, the mechanism of the rapid regulation of the overall pathway of hepatic fatty acid biosynthesis is still largely uncertain. This introduction will briefly summarize possible approaches to this analysis and will outline the difficulties involved.

Two methods which rely on accurate concentration measurement of pathway intermediates are the calculation of mass action ratio and metabolite crossover analysis. The mass action ratio ( $\Gamma$ ) for the reaction

$$A + B \rightleftharpoons C + D$$
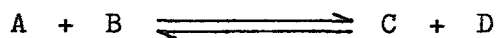
is calculated by substitution of in vivo reactant concentrations into the equation

$$\Gamma = \frac{(C)(D)}{(A)(B)}$$

The enzyme reaction is non-equilibrium, and hence potentially regulatory, if $\Gamma$ differs significantly from the apparent equilibrium constant $K'$.

A metabolite crossover plot is constructed by comparing the concentrations of all the intermediates in a pathway in conditions of high and low flux through that pathway. The possibility of an enzyme being regulatory is suggested by the relative concentrations of an adjacent pair of metabolites changing in an opposite direction to the overall flux. These methods have proved useful in the investigation of oxidative phosphorylation (Chance et al, 1959) and glycolysis (Newsholme and Start, 1973), where intermediates are confined largely to one sub-cellular compartment. However, where intermediates are distributed both in cytosolic and mitochondrial compartments, measurement of total hepatic concentration need not provide any information about the concentration in the relevant compartment necessary for the above calculations. For instance, total hepatic acetyl CoA concentration increases upon fasting due to increased mitochondrial $\beta$-oxidation (Guynn and Veech, 1973; Siess et al, 1977). This measurement cannot be used to calculate $\Gamma$ for acetyl CoA carboxylase, nor to construct a meaningful crossover plot. A further difficulty in the interpretation of in vitro concentration measurements is the extent that binding to sub-cellular fractions modifies the concentration in vivo.

Most information about the regulation of hepatic fatty acid biosynthesis has been provided by measurements of maximum catalytic activities and concentrations of various lipogenic enzymes (Section 1.3.4). These studies have demonstrated that many enzyme activities vary in the long term in concert with the rate of fatty acid biosynthesis and that acetyl CoA carboxylase possesses the lowest Vmax activity. Such enzyme adaptations are due to changes in enzyme synthesis or degradation (Section 1.2.1) and are too slow to account for the rapid stimulation of hepatic fatty acid biosynthesis caused by meal-feeding. Therefore, in vitro properties of purified enzymes have been used to construct theories for the in vivo regulation of hepatic lipogenesis. The studies presented in this chapter were designed principally to investigate whether the catalytic regulation of hepatic acetyl CoA carboxylase activity could account for the measured changes of hepatic fatty acid biosynthesis. A second question posed is under what conditions can substrate supply regulate hepatic fatty acid biosynthesis?

Fig.39. Weight-gain of Meal-fed Rats
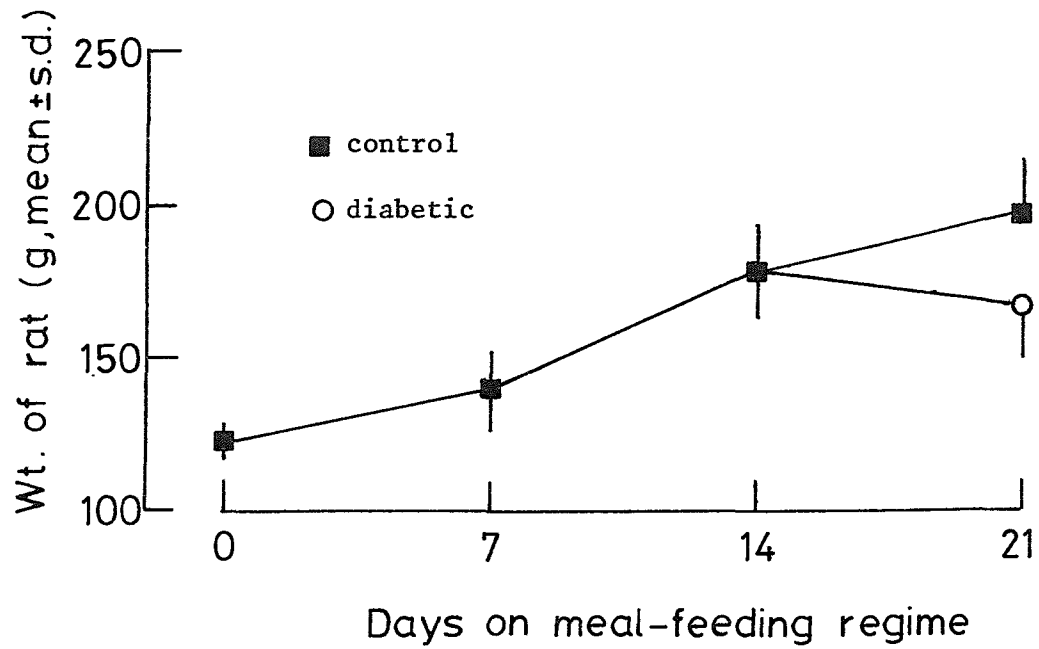


Fig.40. Food Intake of Meal-fed Rats
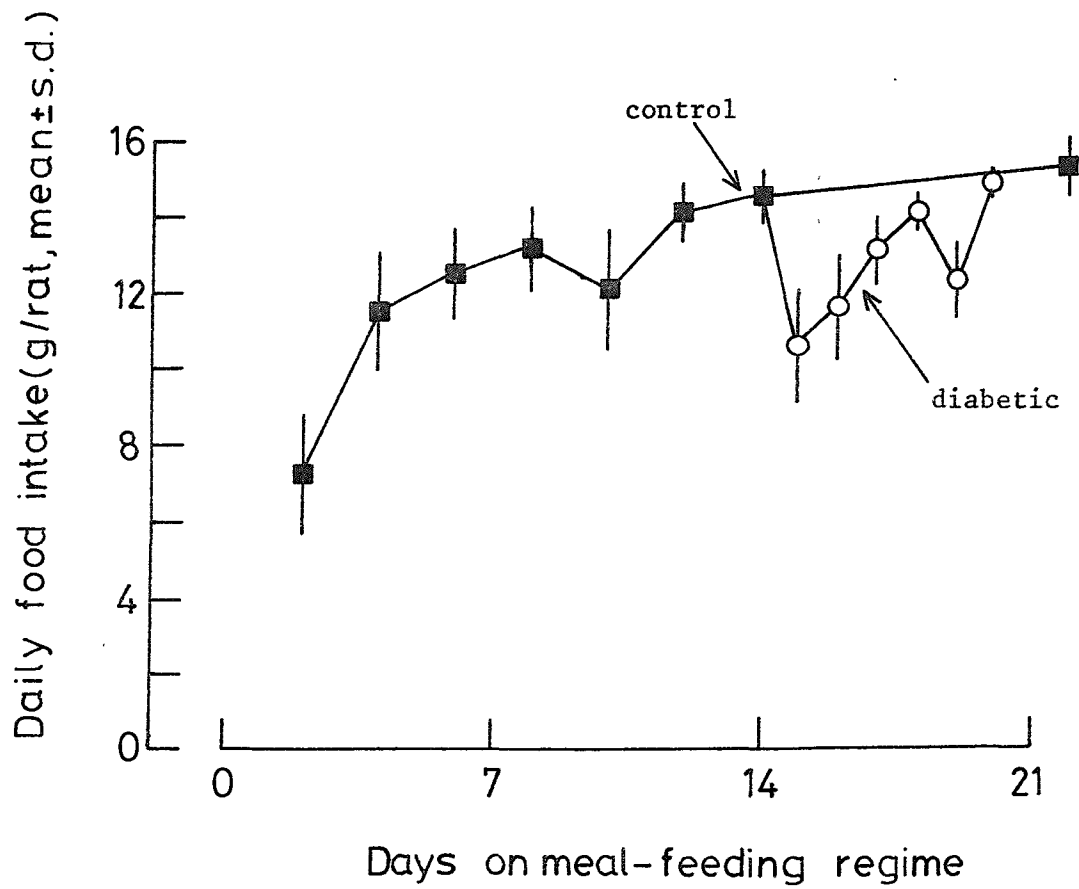
4.2. Results

4.2.1. Food Intake and Weight Gain

After 14 days on the meal-feeding regime control rats were gaining weight at a steady rate (Fig. 39) and the daily food consumption was approaching a plateau value (Fig. 40). On day 14 one group of rats was injected via the tail vein with streptozotocin (55 mg/kg) body weight); over the subsequent week food consumption initially fell but later returned to that of control rats (Fig. 40). While control rats continued to grow between days 14 and 21, diabetic rats decreased in weight over the same period by 7% (Fig. 39). The diabetic rats in Figs. 39 and 40 were mainted for 7 days after streptozotocin injection. The diabetic rats in subsequent experiments were used three days after streptozotocin injection. In all the figures in this chapter control rats are denoted by solid squares, untreated diabetic rats by open circles and insulin treated diabetic rats by closed circles.

The subsequent results are expressed in relation to liver weight or to the protein concentration of liver homogenate supernatant. Both these parameters varied on feeding, largely due to the considerable accumulation of glycogen. The liver represented 3.5% of the total body weight of control rats before feeding and 4.2% immediately post-prandially. The supernatant protein concentration fell from 184±7 to 168±15 mg protein/g of liver in response to feeding. Thus results calculated per unit weight of liver are underestimated by about 10% in fed, compared with fasted, rats.

4.2.2. Serum Insulin Concentration

Circulating insulin concentration increased in control rats in response to meal-feeding (Fig. 41). The maximum value was attained between +1h and +2h. Meal-feeding, compared with feeding ad libitum, exaggerates the circadian variation of insulin secretion and causes a significantly higher insulin : glucagon ratio for 8h after feeding (Ip et al, 1977). These facts are relevant to glucagon action in vivo (Chapter 5) as exogenous glucagon causes a maximum response when the insulin : glucagon ratio is high (Cook et al, 1977).

Fig.41.  Serum Insulin of Meal-fed Rats
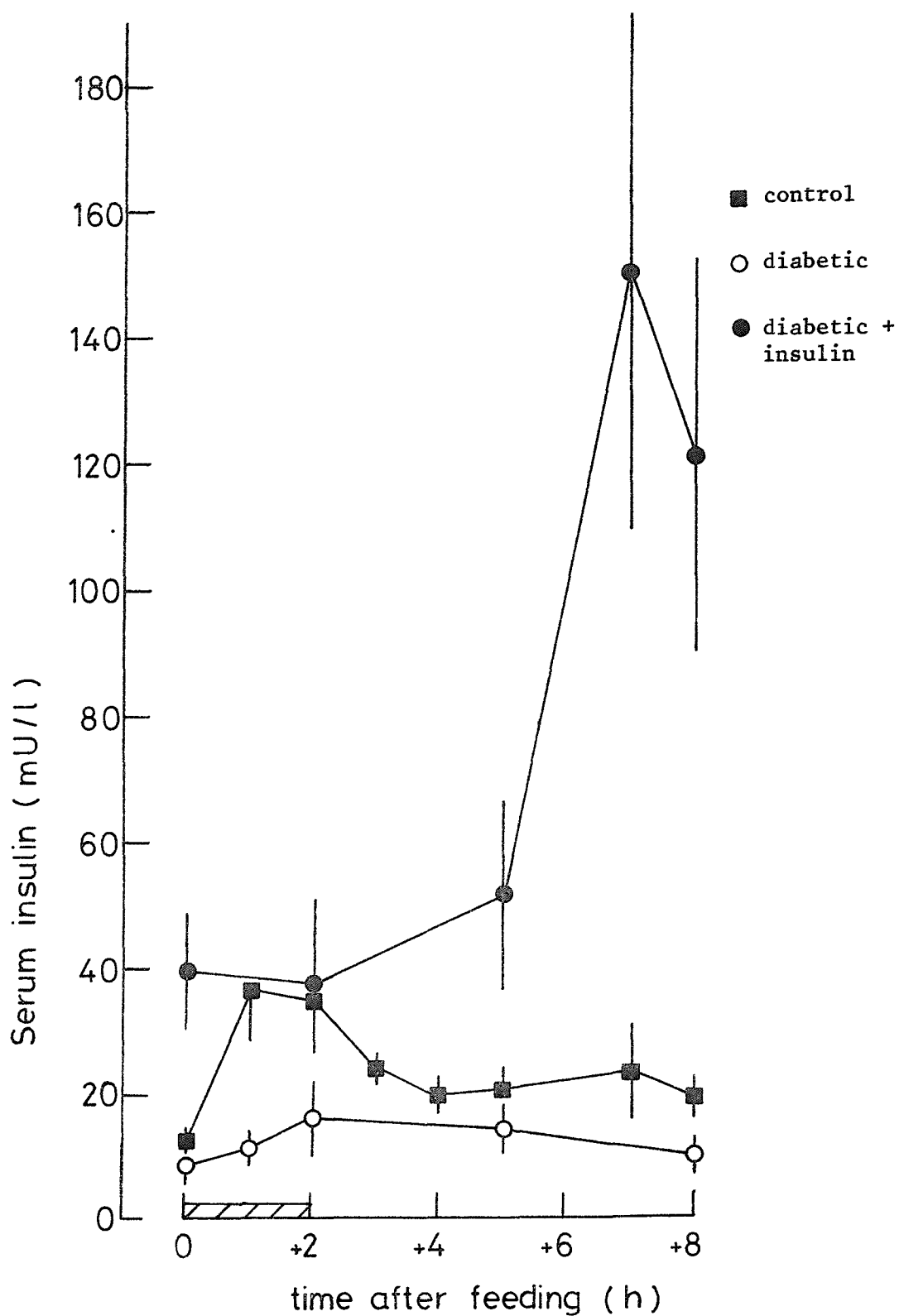


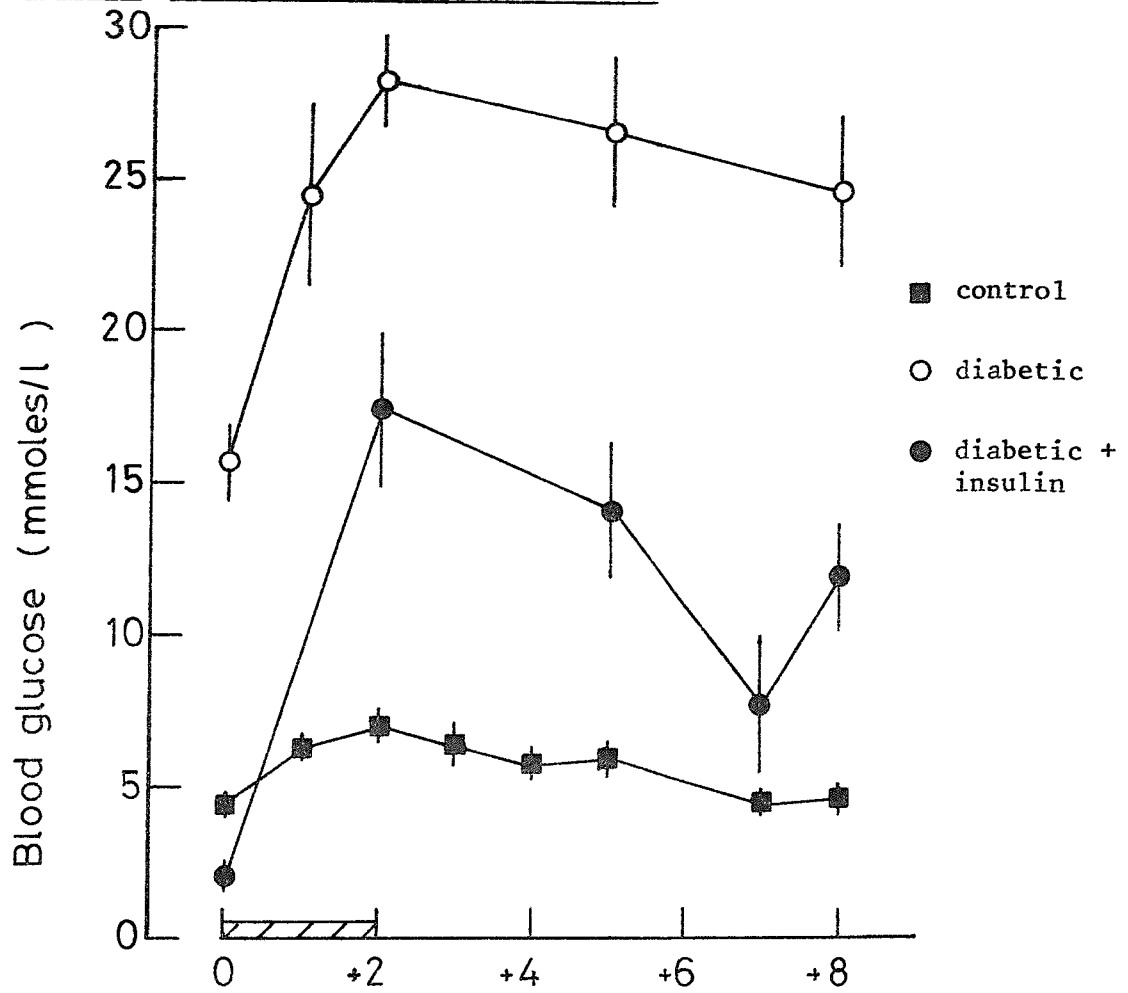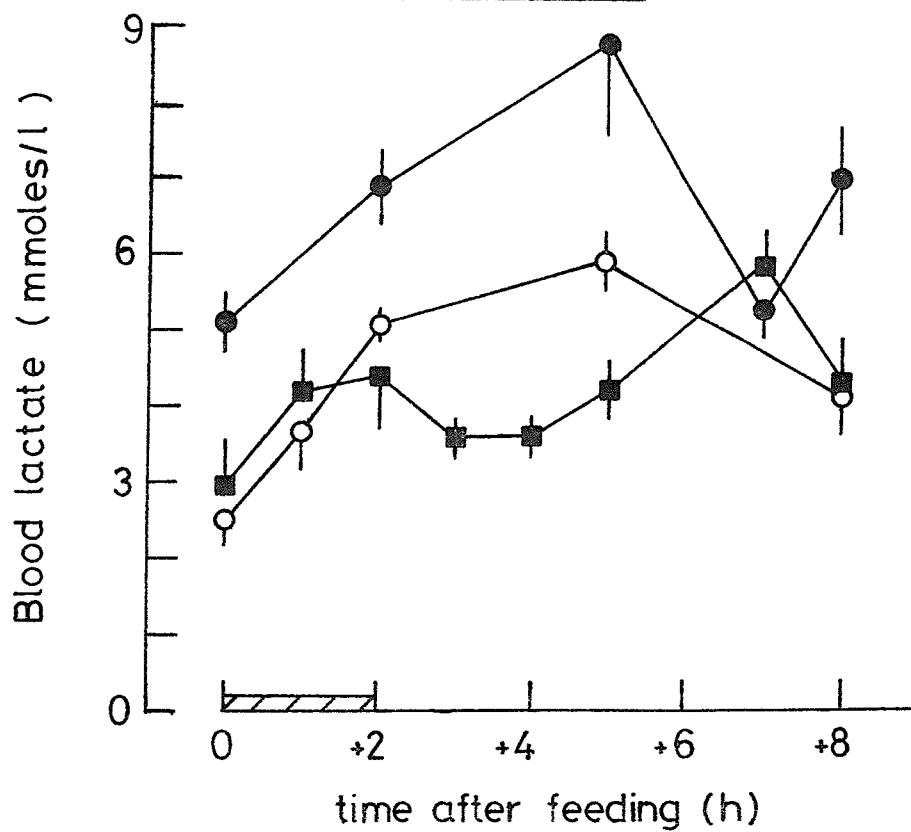The results of Figs. 41-48 & figs. 51-57 are
the mean ± S.E.M. of 4-8 rats.

Fig.42. Blood Glucose in Meal-fed Rats



- ■ control
- ○ diabetic
- ● diabetic + insulin

Fig.43. Blood Lactate in Meal-fed Rats


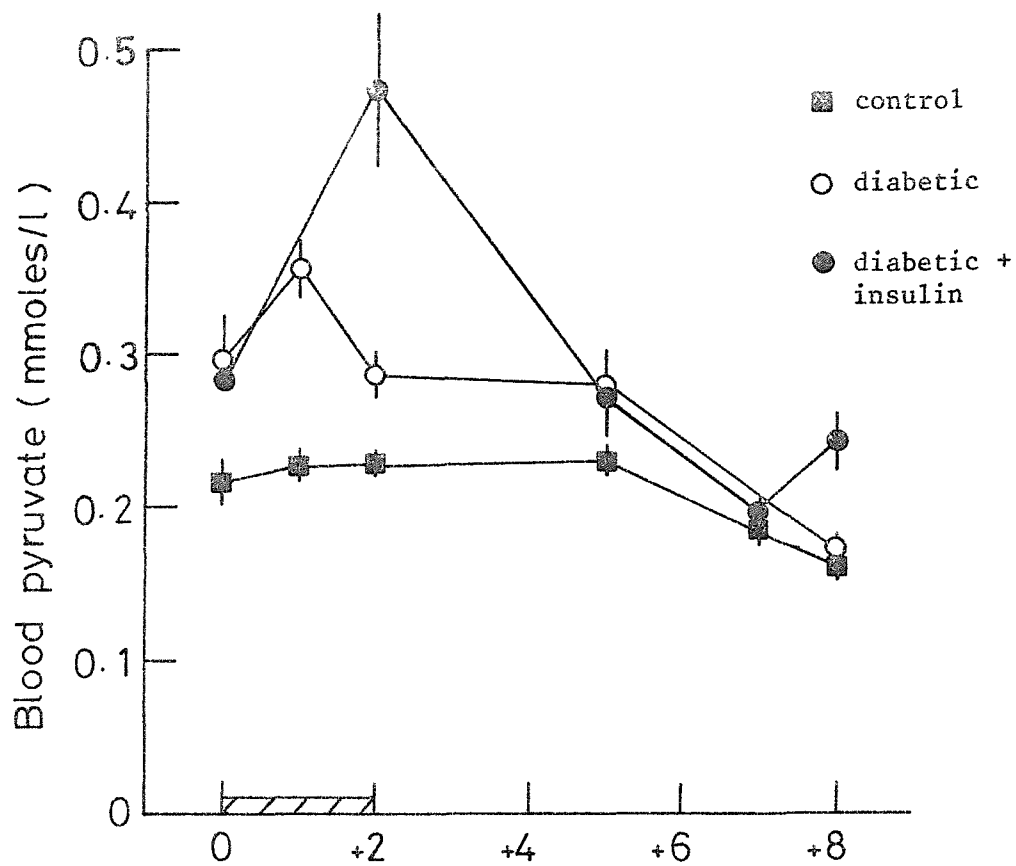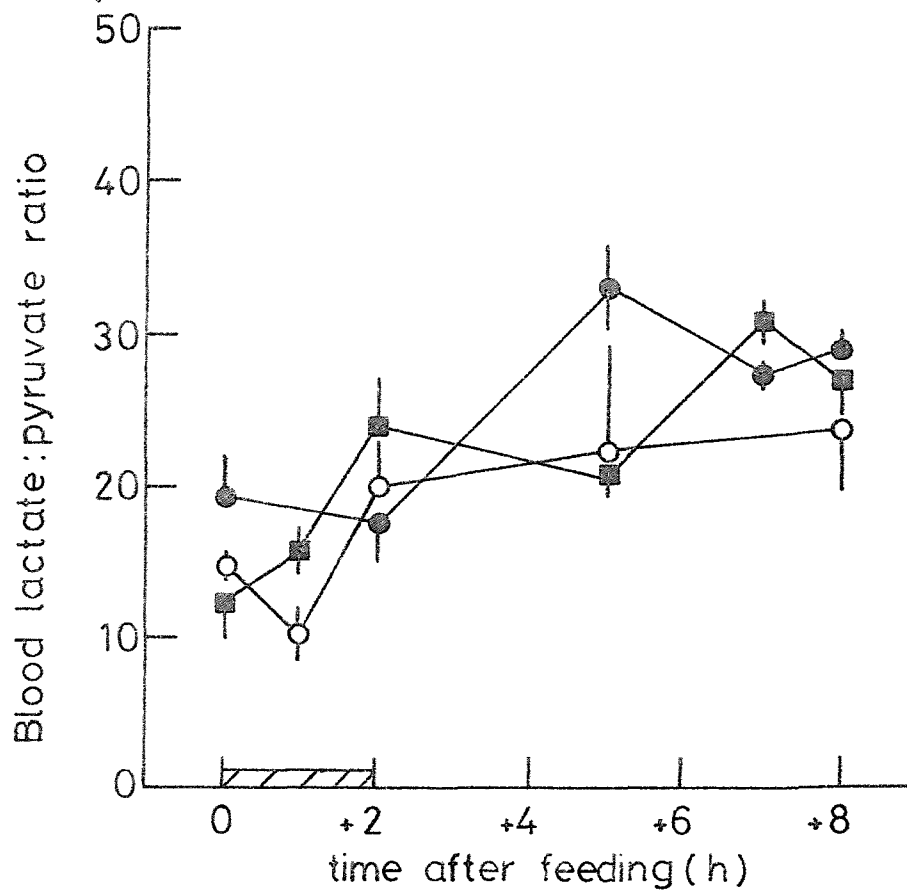
time after feeding (h)

Fig.44.  Blood Pyruvate in Meal-fed Rats



Fig.45.  Blood Lactate : Pyruvate Ratio in Meal-fed Rats

Serum insulin concentration was reduced by streptozotocin diabetes, both in the fasted and fed states (Fig. 41). The slight increase on feeding was not significant. Normal insulin concentrations were rapidly restored by injection of insulin. The high hormone concentrations at +7h and +8h after feeding appeared to have little physiological relevance and probably reflected the slow release and long duration of action of protamine zinc insulin. Many metabolic parameters responded to insulin treatment several hours earlier. For instance, diabetic rats injected with insulin became severely hypogycaemic before feeding (Fig. 42) and hepatic glycogen synthesis was restored to normal by +2h after feeding (Fig. 34).

### 4.2.3. Blood Metabolite Concentrations
Glucose (Fig. 42)

The whole blood glucose concentration in control rats increased by only 30% by the end of the meal-feeding period. From +5h onwards it was maintained near the pre-fed value of $4.7 \pm 0.4$ m moles/l. Glucose concentration of diabetic rats increased from $15.2 \pm 1.0$ m moles/l before feeding to about 25 m moles/l from +1h to +8h. Insulin treatment 2h before feeding caused severe hypoglycaemia, glucose concentration falling to $2.0 \pm 0.4$ m moles/l. Diabetic rats after insulin treatment were unable to maintain normo-glycaemia in response to feeding. However, they achieved a more rapid glucose clearance between +2h and +8h than did untreated diabetic rats.

Lactate (Fig. 43)

Blood lactate concentration increased in response to feeding in all three groups of rats. During the first 5h of feeding, insulin treatment of diabetic rats significantly elevated the lactate concentration above those of untreated diabetic or control rats.

Pyruvate (Fig. 44)

Blood pyruvate concentration tended to decrease after +5h in all three groups of rats. This decline was preceded in both diabetic groups by an increased concentration during the initial feeding response. The
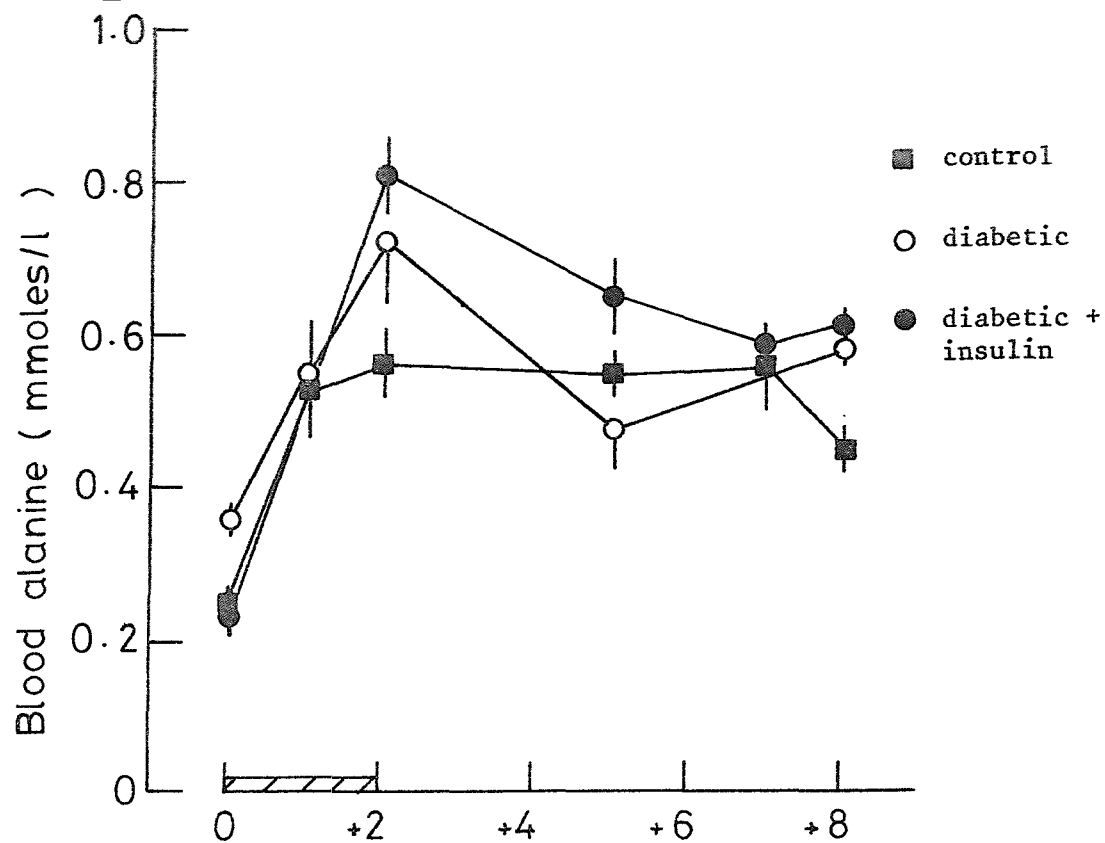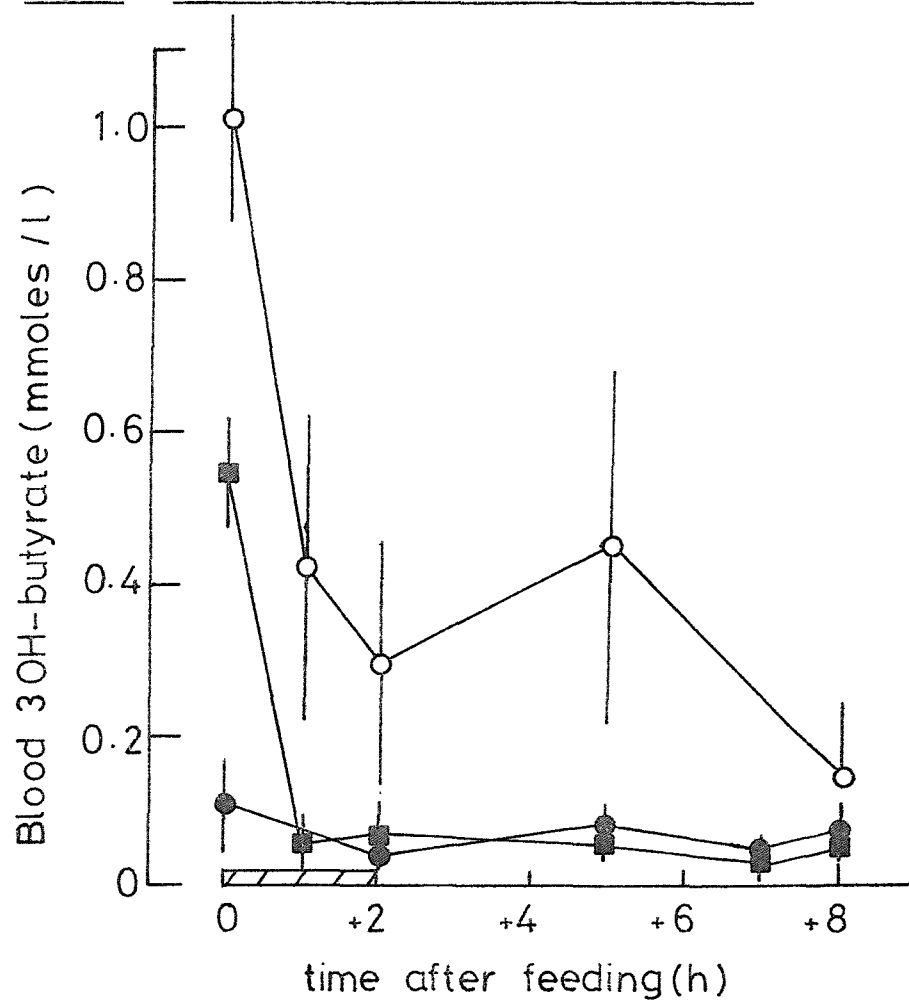
Fig.46. Blood Alanine in Meal-fed Rats



Fig.47. Blood 3-OH butyrate in Meal-fed Rats

combination of increasing lactate and decreasing pyruvate concentrations
caused the lactate : pyruvate concentration ratio to increase in response
to feeding (Fig. 45).

Alanine (Fig. 46)

Meal-feeding caused the blood alanine concentration to increase sig-
nificantly above the pre-fed concentration in all groups of rats. These
elevated concentrations were maintained for at least +8h after feeding.

3-Hydroxybutyrate (Fig. 47)

Blood 3-hydroxybutyrate concentration of diabetic rats before feeding
was double that of control rats, reflecting the diabetic stimulation of
lipolysis and β-oxidation (Section 1.3.6). When control rats meal-fed,
3-hydroxybutyrate concentration fell to a very low value which was main-
tained from +1h to +8h. Similar concentrations resulted from insulin
treatment of diabetic rats. By contrast, although 3-hydroxybutyrate
concentrations of untreated diabetic rats also fell in response to meal-
feeding, relatively elevated concentrations were maintained throughout
the post-prandial period.

4.2.4. Hepatic Fatty Acid Biosynthesis

The absolute rate of de novo fatty acid biosynthesis in liver was
measured in vivo by the incorporation of tritium from tritiated water.
Preliminary experiments established this technique in rats fed ad libitum,
which were housed under the reverse lighting schedule (Section 2.1.1).
Groups of 4 control rats were killed at 10.00h and 22.00h, in the mid-
dark and mid-light periods respectively. Fatty acid biosynthesis was
greatest at 10.00h but the circadian variation was not large, (Table 14).
Rats in another group were injected with streptozotocin (150 mg/kg body
weight) and maintained for 2 days with protamine zinc insulin (3U/rat/
day). Insulin was withheld the day before experiment. This very severe
diabetes effectively abolished all hepatic fatty acid biosynthesis at
either 10.00h or 22.00h (Table 14).

Meal-feeding caused a greater circadian variation than ad libitum
feeding (Fig. 48; Table 15). Rats fed ad libitum were effectively

Table 14

### Hepatic Fatty Acid Biosynthesis in Rats Fed ad libitum

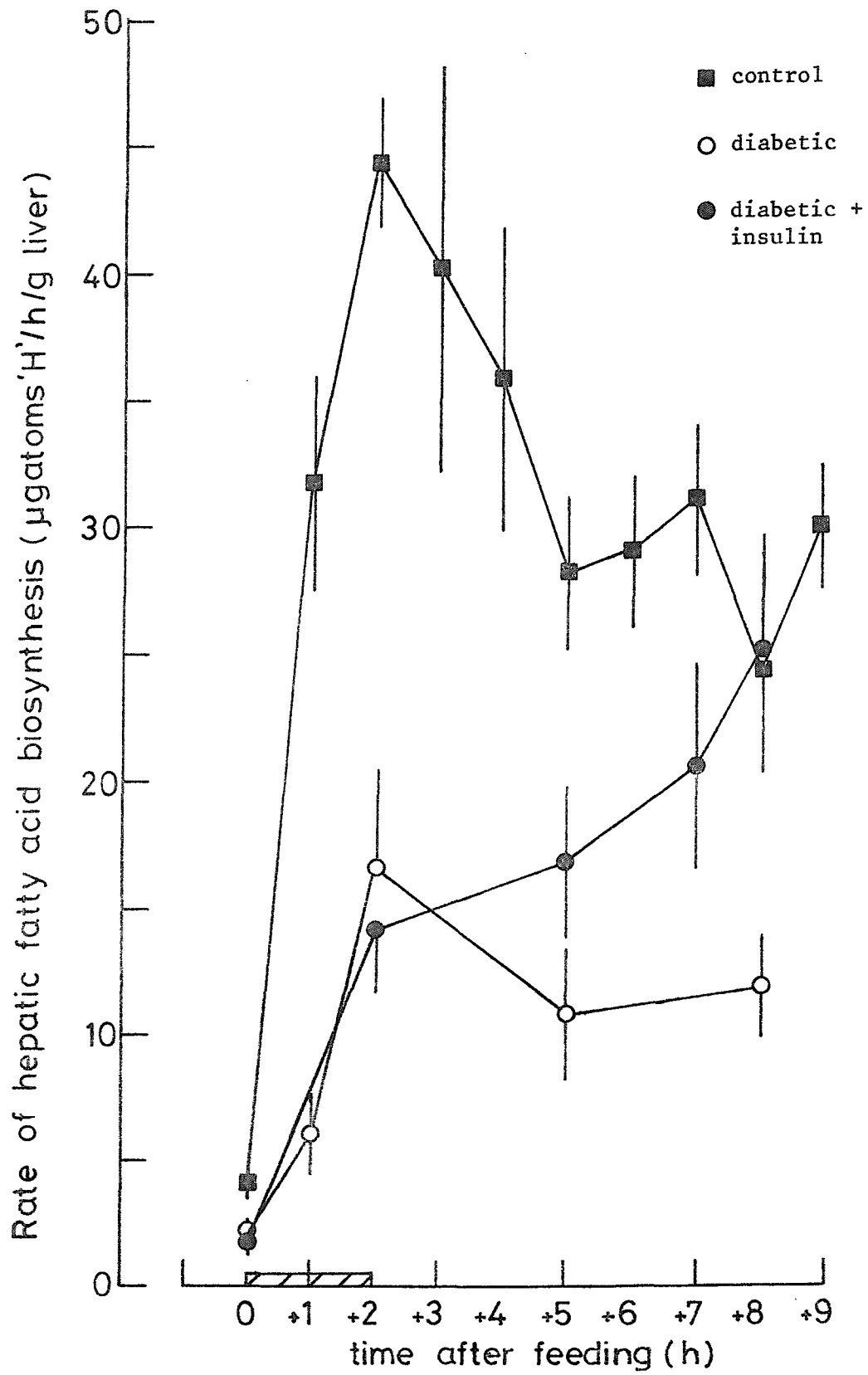| Time Killed (h) | Rate of Hepatic Fatty Acid Biosynthesis ( $\mu$gatoms 'H'/h/g of liver | |
| --- | --- | --- |
| | Control Rats | Streptozotocin Diabetic Rats (150mg/kg) |
| 10.00h (mid-dark) | 24.3±5.2 (4) | 2.5±0.6 (4) |
| 22.00h (mid-light) | 15.3±1.1 (4) | 2.1±0.7 (4) |

Results are expressed as mean ± S.E.M. (number of rats). For neither group was difference between 10.00h and 22.00h significant.

Table 15

### Hepatic Fatty Acid Biosynthesis in Meal-fed Rats

| Time Killed (h) | Rate of Hepatic Fatty Acid Biosynthesis ( $\mu$gatom 'H'/h/g of liver | | |
| --- | --- | --- | --- |
| | Control Rats | Streptozotocin Diabetic Rats | Insulin-treated Diabetic Rats |
| Minimum rate: | | | |
| Pre-fed | 3.8±0.6 (6) | 1.9±0.4 (6) | 1.6±0.4 (5) |
| Maximum rate: | | | |
| (+2h) | 44.4±2.6 (4) | ‡16.6±3.9 (6) | |
| (+8h) | | | †24.7±4.5 (4) |
| Range of values about mean maximum rate | 39.1-50.4 | 6.7-29.3 | 17.4-37.9 |

Results are expressed as mean ± S.E.M. (number of rats). Probability by Students' (t) test: † , 0.01 > p > 0.001; ‡ , p < 0.001 compared with control maximum rate.

Fig.48.    Hepatic Fatty Acid Biosynthesis in Meal-fed Rats

never fasting and, consequently, very low rates of hepatic fatty acid biosynthesis were not measured. For meal-fed rats, however, the synthetic rates after the 22h fast were comparable to the negligible rates measured in severe diabetic rats (compare Tables 14 and 15).

In all groups of rats, the low pre-fed rate of hepatic fatty acid biosynthesis increased in response to the 2h period of meal-feeding (Table 15). For control rats, this rate was maximal +2h after feeding (Fig. 48) and subsequently declined slightly to a plateau value of about 30 μ gatoms 'H'/h/g liver. Hepatic lipogenesis of untreated streptozotocin diabetic rats also increased to a maximum +2h after feeding, but at no time exceeded 37% the control rate (Fig. 48; Table 15). The streptozotocin dose administered (55 mg/kg) was chosen to impair but not abolish hepatic fatty acid biosynthesis.

Insulin treatment of diabetic rats did not restore the initial feeding response of hepatic lipogenesis (Fig. 48). The fatty acid synthetic rate was not stimulated by insulin +2h after feeding. This lipogenic rate subsequently increased steadily until at +8h it was comparable with the rate of control rats at the same time (24.7±4.5 v.s. 24.3±4.1 μgatoms 'H'/h/g of liver).

The analysis of results from diabetic rats was complicated by large individual variations (Table 15). At their respective maximum mean rates of hepatic lipogenesis the range of individual values about that mean was ±20.5% for control rats, ±138% for diabetic rats and ±82.6% for insulin-treated diabetic rats. This much greater spread of results presumably reflected the variability of the experimental diabetic lesion caused by streptozotocin.

### 4.2.5. Acetyl CoA Carboxylase Activity

Acetyl CoA carboxylase activity in liver homogenates was measured by the fixation of $[^{14}C]$-bicarbonate into malonyl CoA (Halestrap and Denton, 1973). The original method enabled the adipose tissue enzyme to be measured rapidly after centrifugation at 12,000 x g for 30 sec. Such a brief centrifugation proved impracticable for estimation of the liver enzyme. Liver acetyl CoA carboxylase was inactivated irreversibly

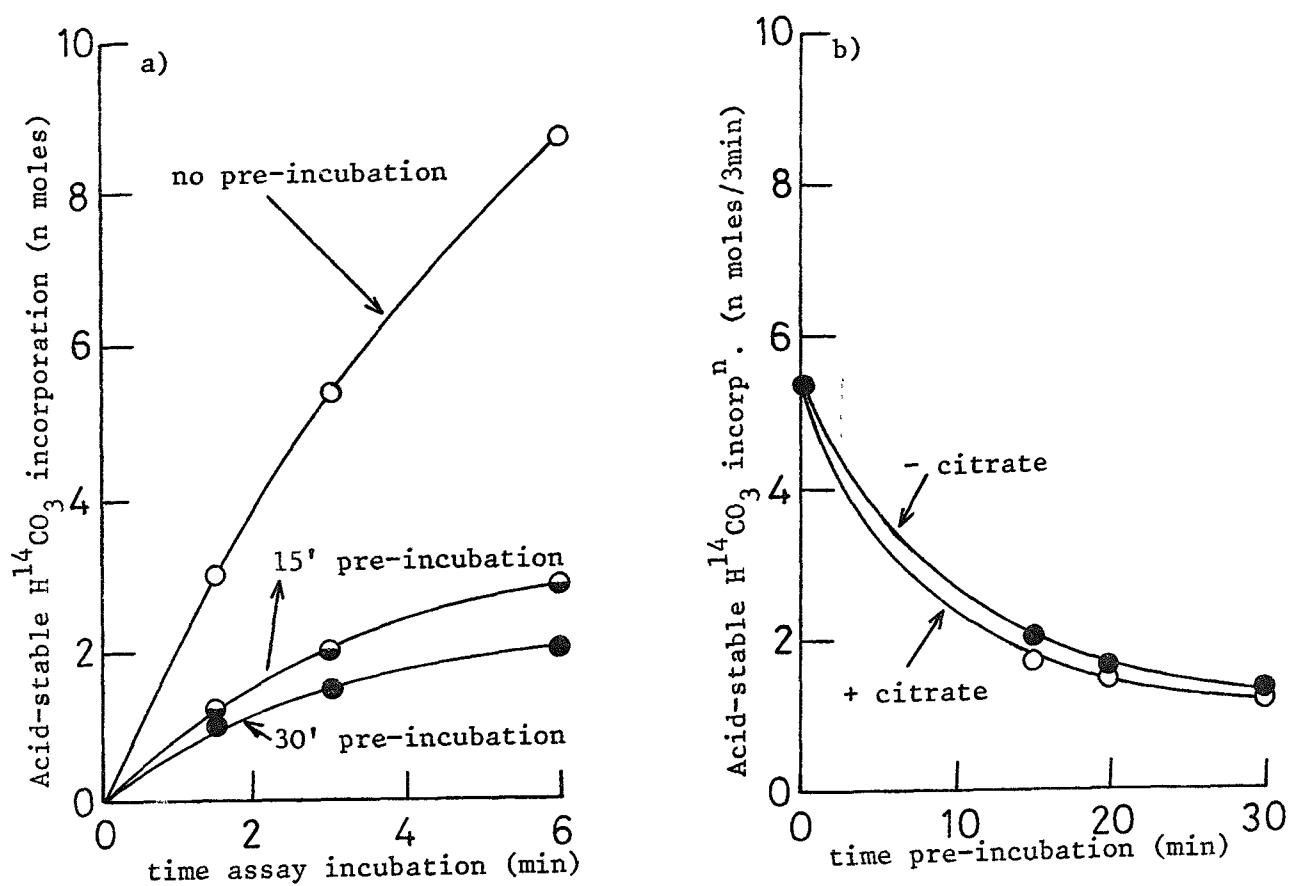## Fig.49. Acetyl CoA Carboxylase Assay after Low-speed Centrifugation



a) no pre-incubation

15' pre-incubation

30' pre-incubation

Acid-stable $H^{14}CO_3$ incorporation (n moles)

time assay incubation (min)

b) - citrate

+ citrate

Acid-stable $H^{14}CO_3$ incorp$^n$. (n moles/3min)

time pre-incubation (min)

## Fig.50. Acetyl CoA Carboxylase Assay after High-speed Centrifugation
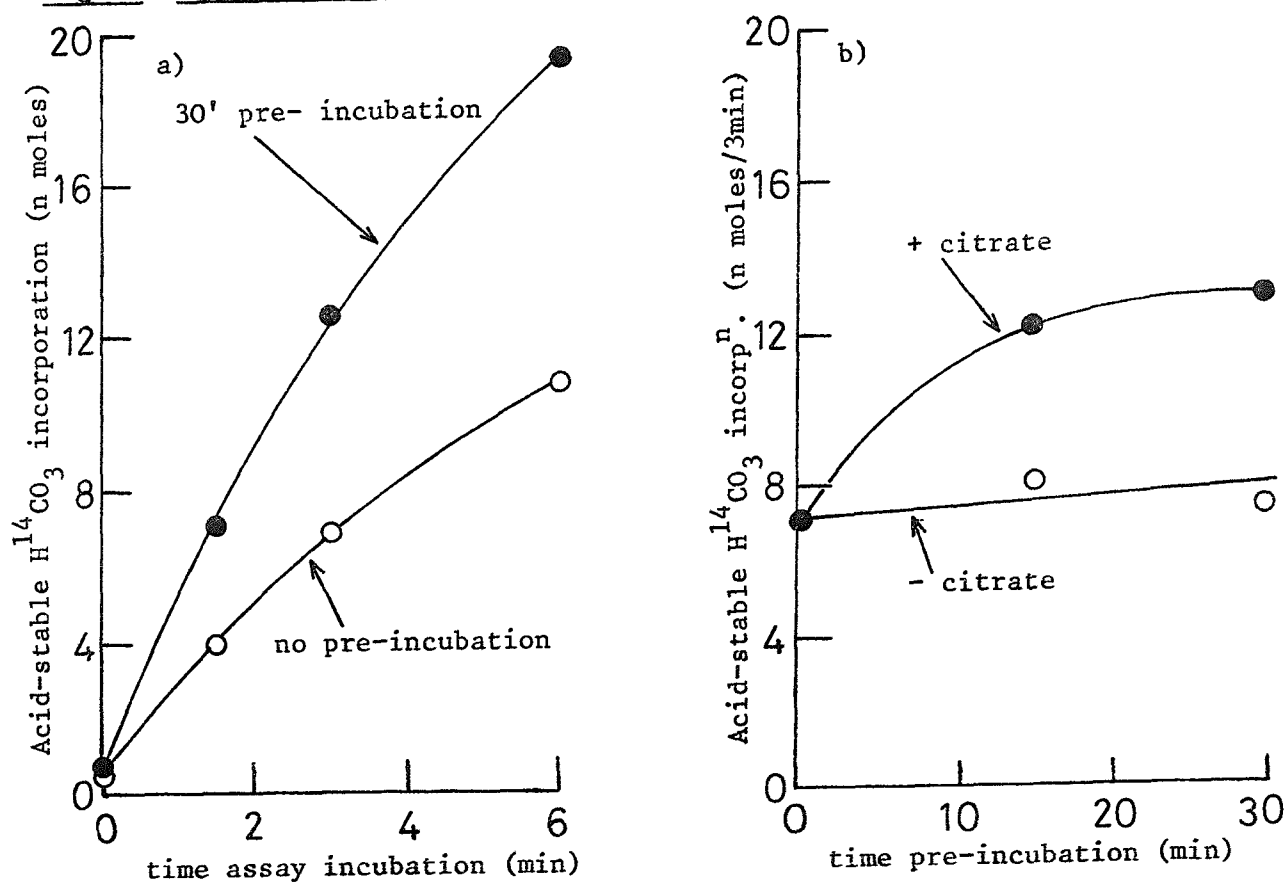


a) 30' pre- incubation

no pre-incubation

Acid-stable $H^{14}CO_3$ incorporation (n moles)

time assay incubation (min)

b) + citrate

- citrate

Acid-stable $H^{14}CO_3$ incorp$^n$. (n moles/3min)

time pre-incubation (min)

Fig.51 Variation of Hepatic Acetyl CoA Carboxylase Activity with
Homogenate Concentration



Fig.52 Variation of Hepatic Acetyl CoA Carboxylase Activity with
Substrate Concentration
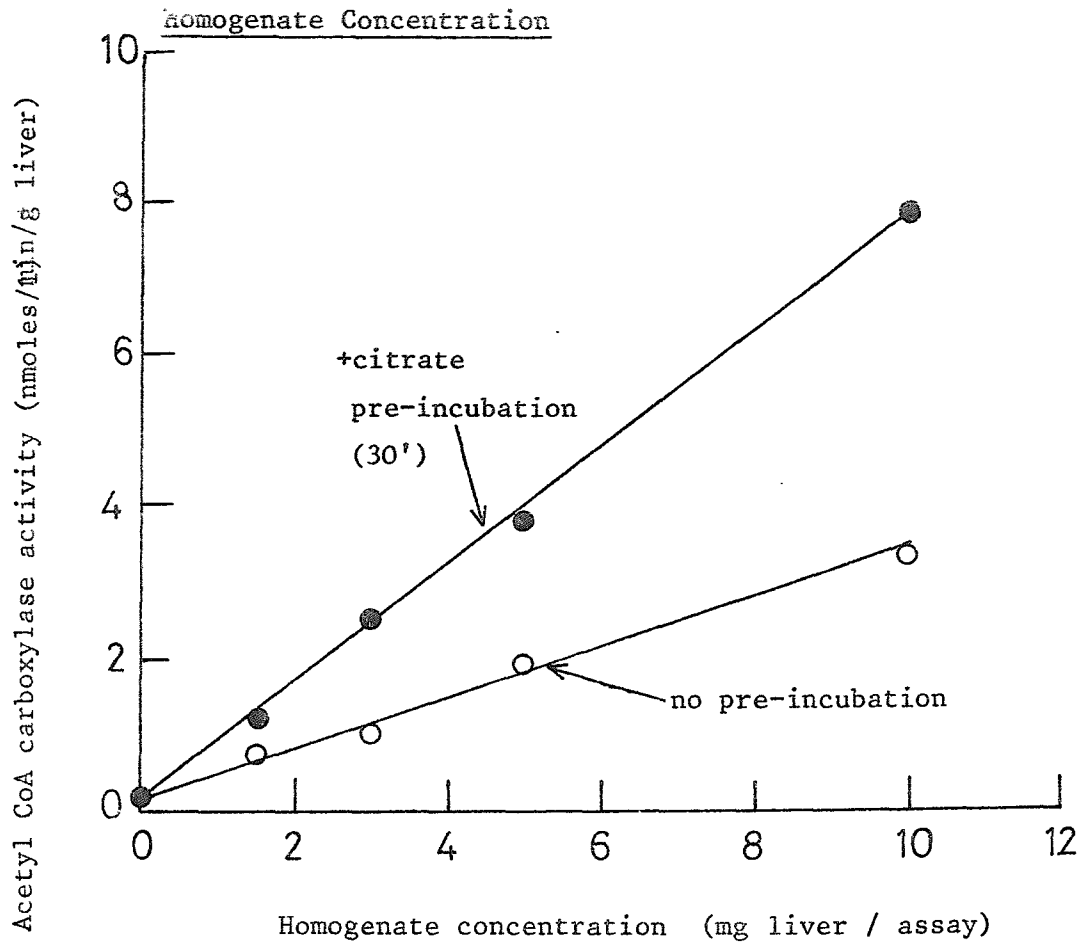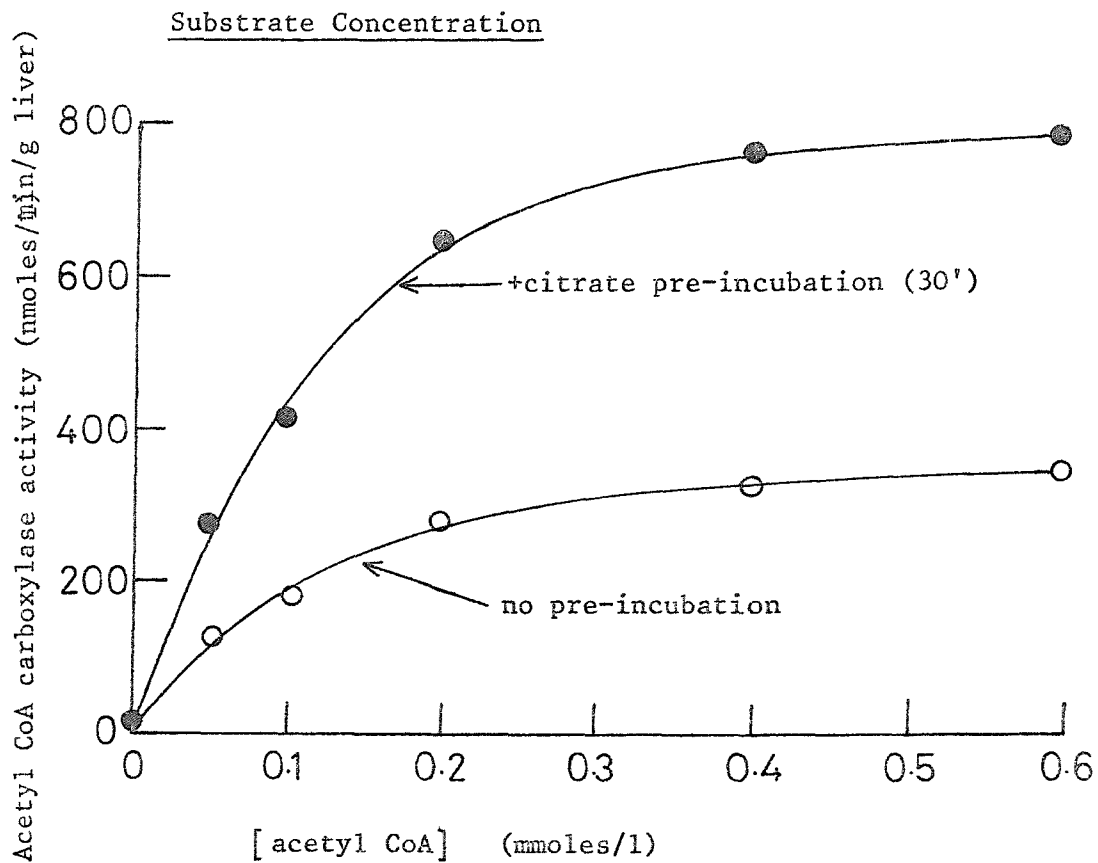
by incubation at $37^{\circ}C$ for 30 min. This inactivation was not opposed by 20 mM-citrate. The rate of fixation of $[^{14}C]$-bicarbonate in the enzyme assay declined throughout this incubation period (Fig. 49a). This effect was apparent for up to 6 min of $[^{14}C]$-bicarbonate fixation (Fig. 49b). This different behaviour between liver and adipose tissue could be due to the greater concentration of proteolytic enzymes in liver forming inactive fragments of acetyl CoA carboxylase (Tanake et al, 1975).

This problem was overcome by increasing the centrifugation velocity to 100,000 x g for 10 min. Active acetyl CoA carboxylase activity in this supernatant, measured without citrate preincubation, was similar to that after 12,000 x g for 30 sec (Fig. 50a). Preincubation of high speed supernatant with 20 mM-citrate resulted in enzyme activation to give a total enzyme activity. Both activities of acetyl CoA carboxylase were stable during the preincubation (Fig. 50b). This modification increased the total delay time in the enzyme assay from 10 to 30 min, and thus increased the possibility of in vitro modification of enzyme activity. 50 mM-sodium fluoride and 2 mM-EDTA were included in the homogenization buffer to help prevent such changes (Cook et al, 1977).

The incorporation of $[^{14}C]$ bicarbonate into acid-stable products was not linear with time (Fig. 50a). The incorporation over the first 3 min of incubation represented some 70% of the estimated true initial rate and was linear with increasing volume of homogenate supernatant (Fig. 51). Three minutes was therefore chosen as the standard assay incubation time. In agreement with previous reports (Inoue and Lowenstein, 1972; Section 1.3.4) citrate preincubation increased the Vmax but did not change the Km for acetyl CoA of the enzyme (Fig. 52). The concentration of ATP in the assay buffer was critical. At concentrations greater than 4 m moles/l, ATP inhibited the enzyme activity.

Total acetyl CoA carboxylase activity in control rats, measured after pre-incubation with 20 mM-sodium citrate, was not altered by feeding (Fig. 53). Streptozotocin diabetes reduced this control activity (mean$^{\pm}$ S.E.M. = 759 ± 20 n moles/min/g of liver) by 57.2% to a mean value of 325 ± 38 n moles/min/g of liver. This impaired enzyme activity in
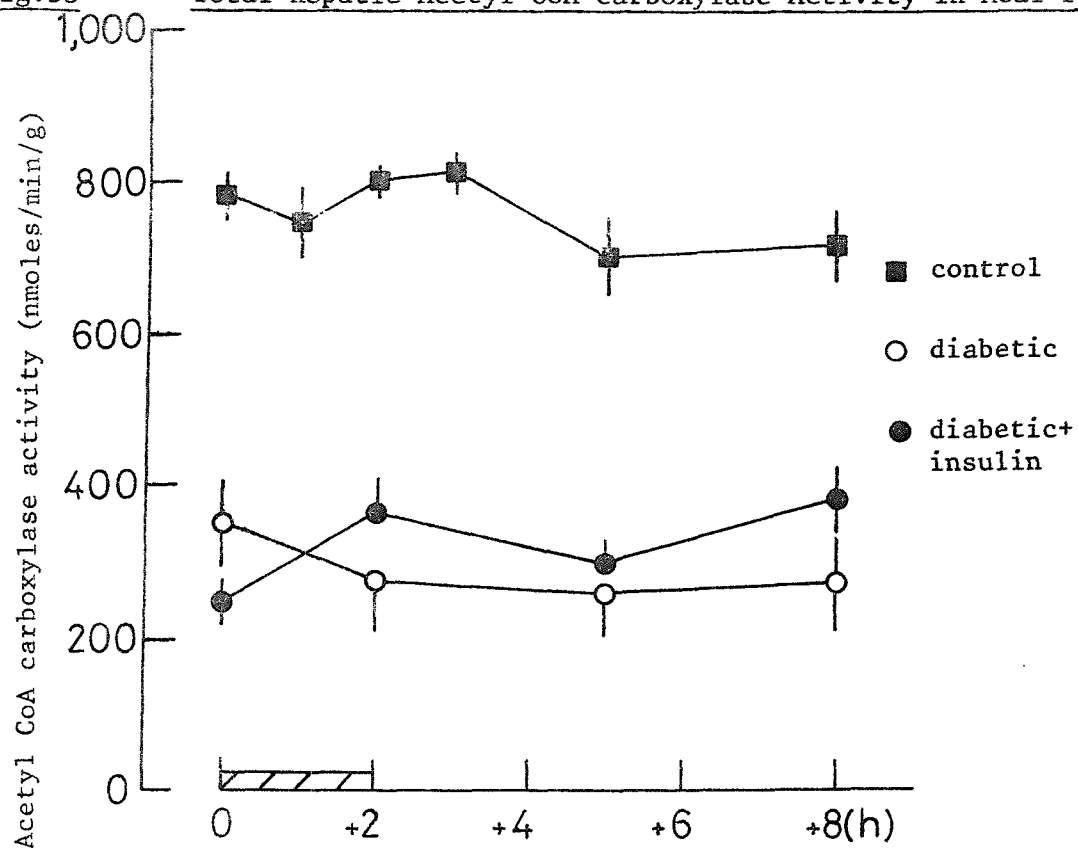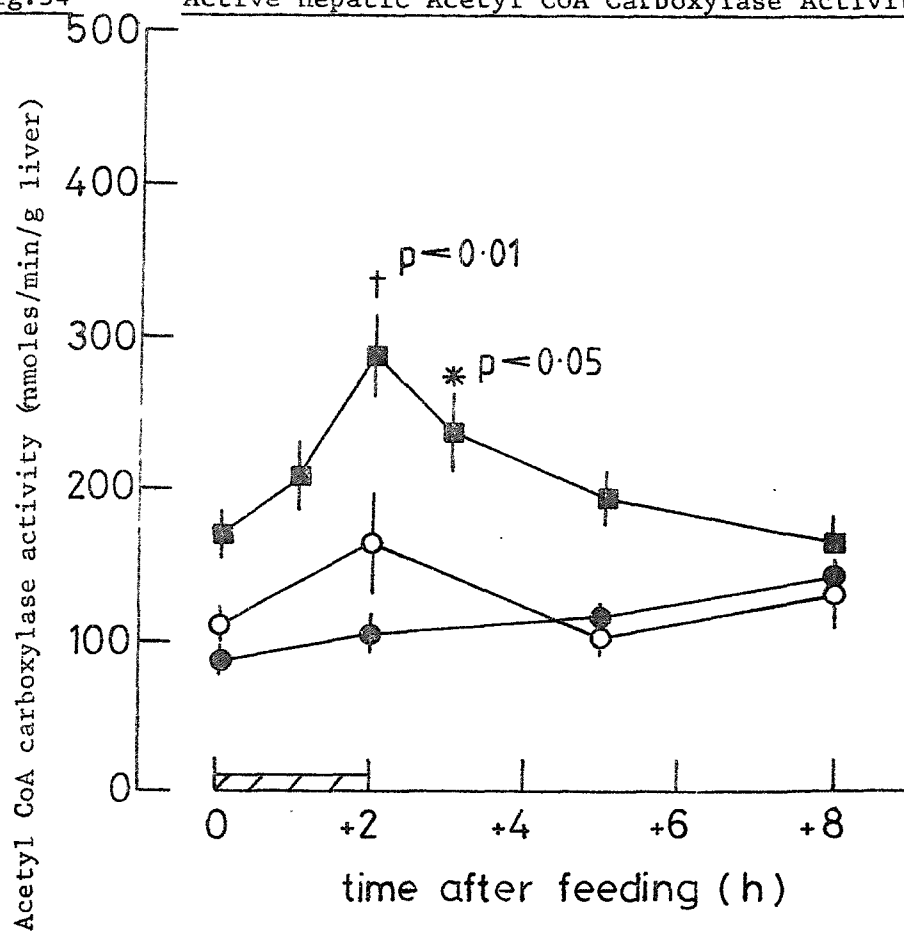
Fig.53    Total Hepatic Acetyl CoA carboxylase Activity in Meal-fed Rats



Fig.54    Active Hepatic Acetyl CoA Carboxylase Activity in Meal-fed Rats

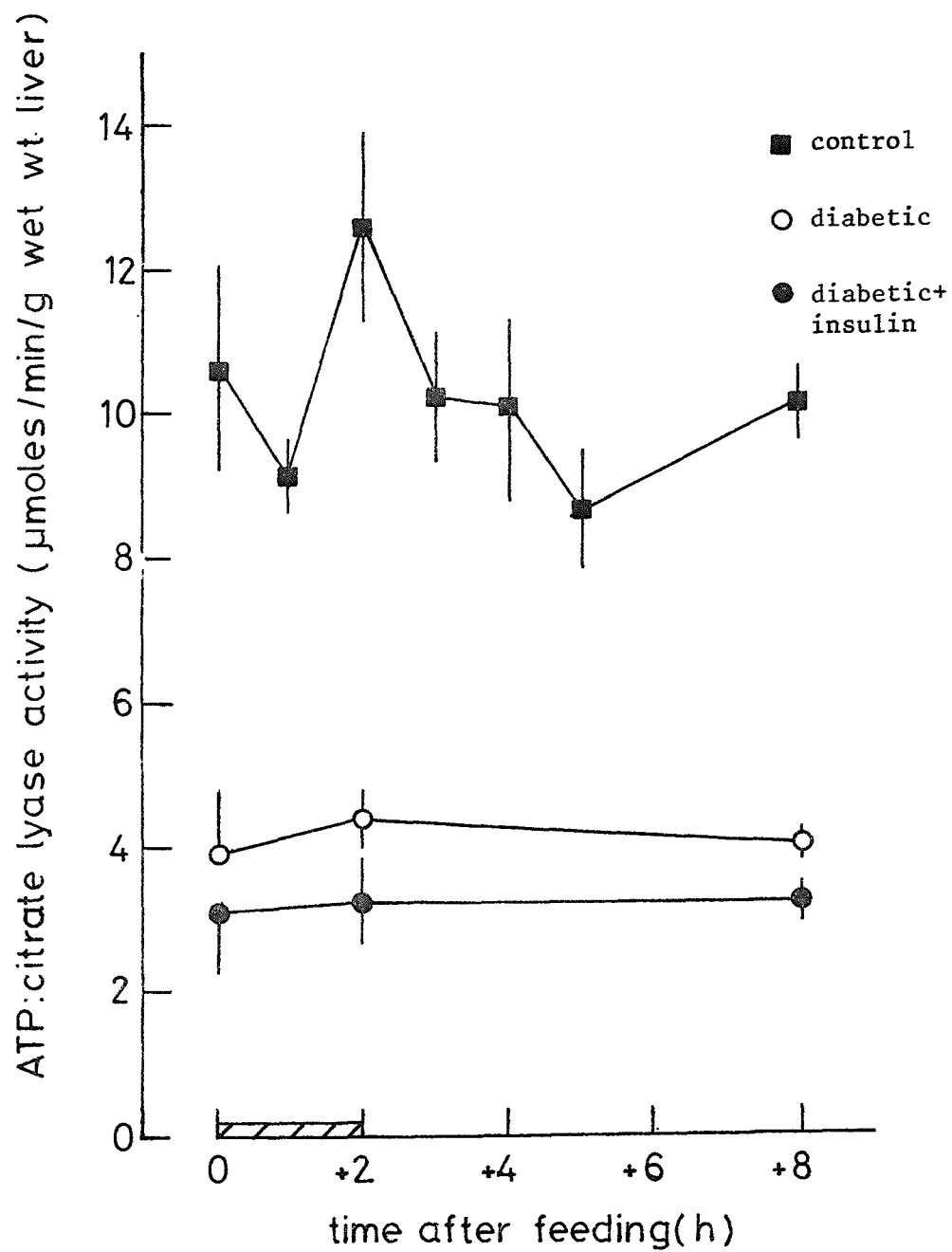Fig.55 Hepatic ATP:citrate Lyase in Meal-fed Rats

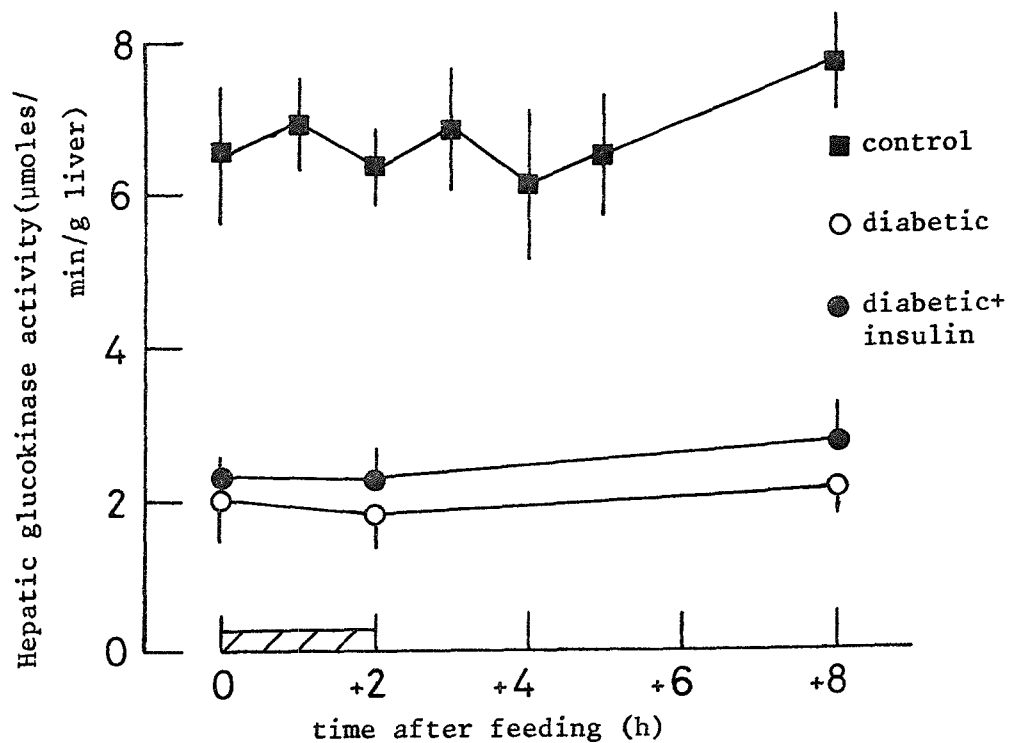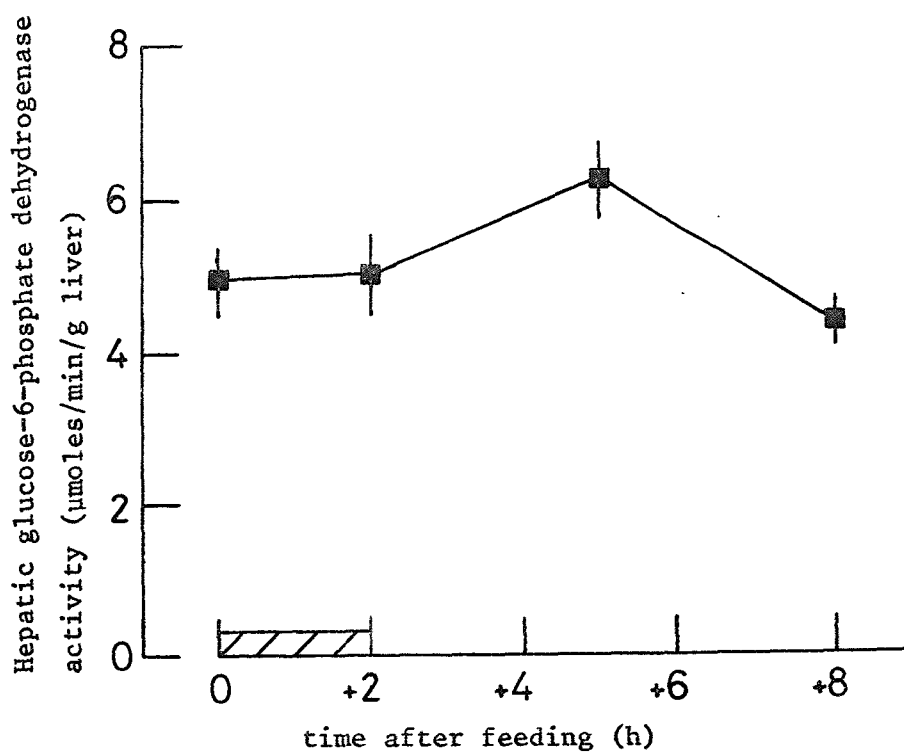Fig.56   Hepatic Glucokinase Activity in Meal-fed Rats



Fig.57   Hepatic Glucose-6-phosphate dH Activity in Meal-fed Rats

diabetic rats was not restored over the first 8h after feeding. The mean total acetyl CoA carboxylase activity of insulin-treated diabetic rats was $296\pm24$ n moles/min/g of liver.

Active acetyl CoA carboxylase activity in livers from control rats, measured without citrate preincubation, increased in response to feeding (Fig. 54). The maximum activation was 70% and this occurred at +2h after feeding. Subsequently the enzyme activity returned to the pre-fed rate by +5h after feeding. Active acetyl CoA carboxylase activity was depressed in diabetic, compared with control, rats before feeding. The slightly increased enzyme activity 2h after feeding was statistically insignificant. Insulin pre-treatment did not activate the impaired enzyme activity of diabetic rats. At no time did the active acetyl CoA carboxylase activity of insulin-treated rats significantly exceed that of untreated diabetic rats.

### ATP : Citrate Lyase, Glucokinase and Glucose-6-Phosphate Dehydrogenase

The hepatic activities of none of these three enzymes in control rats varied significantly in response to meal-feeding for up to 8h (Figs. 55, 56 and 57). Hepatic ATP : citrate lyase (Fig. 55) and hepatic glucokinase activities (Fig. 56) were decreased by about 70% by streptozotocin diabetes. Again, these activities did not change within 8h of meal-feeding. Moreover, within this time period, treatment of diabetic rats with insulin did not succeed in restoring the hepatic activities of either enzyme.

### 4.3. Discussion

The rats in these studies adapted rapidly to the feeding and lighting regime. The values for food consumption and body weight gain of control rats (Figs. 39 and 40) agreed closely with previously published data and represented 60 to 70% of those observed in ad libitum fed rats of a similar age (Carlson and Arnrich 1978; Cohn and Joseph, 1970; Leveille, 1970). Although initially after streptozotocin injection diabetic rats ate less food, 3 days after injection the food consumption of diabetic rats was approaching that of control rats (Fig. 39). Thus

Table 16

Half-lives of Enzymes of Rat Liver

| Enzyme | Half-life (h) | Reference |
|---|---|---|
| Acetyl CoA carboxylase | 18-59 | Nakanishi & Numa, 1970 Majerus & Kilburn, 1969 |
| Fatty acid synthase | 55-71 | Craig et al, 1972 Volpe et al, 1973 |
| ATP: citrate lyase | 14-27 | Gibson et al, 1972 |
| Glucokinase | 12-18 | Wicks, 1974b |
| L-pyruvate kinase | 47-75 | Cladaras & Cottam, 1980 Hopkirk & Bloxham, 1980 |
| Glucose-6-phosphate dehydrogenase | 15-69 | Rudack et al, 1971a |
| 6-phosphogluconate dehydrogenase | 14-81 | Rudack et al, 1971b |
| NADP: malate dehydrogenase | 34-63 | Gibson et al, 1972 |
| Phosphoenol pyruvate carboxykinase | 5.5 | Wicks, 1974b |
| Tyrosine aminotransferase | 1.5-2 | Wicks, 1974b |
| 3-hydroxy-3-methyl glutaryl CoA reductase | 0.5-3.5 | Higgins et al, 1974 Bell et al, 1976 |

the impaired metabolic processes and decrease in body weight observed
in diabetic rats were not simply a reflection of decreased appetite and
food consumption.

Meal-feeding increased the pre-fed rate of hepatic fatty acid bio-
synthesis of $3.8 \pm 0.6$ µgatoms 'H'/h/g to a value of $44.4 \pm 2.6$ µgatoms
'H'/h/g 2h later. Any factor proposed to regulate this pathway flux
must be shown to vary <u>in vivo</u> over a similar time span and to a sufficient
extent. The hepatic activities of total acetyl CoA carboxylase, ATP :
citrate lyase, glucokinase and glucose 6-phosphate dehydrogenase have
all been shown to vary in the long term in concert with the rate of
hepatic lipogenesis. (Nakashini and Numa, 1974). None of these activi-
ties changed significantly for up to 8h after feeding in control,
untreated diabetic or insulin treated diabetic rats (Figs. 53, 55 56 and
57). Thus changes in total enzyme concentration are unlikely to be
responsible for the meal-feeding stimulus of hepatic lipogenesis in
control rats or its restoration by insulin in diabetic rats (Fig. 48).
Consideration of the half lives of a variety of lipogenic enzymes (Table
16) also suggests that enzyme induction and repression is unlikely to
account for the rapid increase in hepatic fatty acid biosynthesis mea-
sured in meal-fed rats. For comparison, the half-lives of 3-hydroxy
3-methyl glutaryl CoA reductase and tyrosine amino transferase are
included in Table 16 . These are sufficiently rapid that enzyme induction
can largely account for the variations in flux of their respective path-
ways.

Hepatic glycogen is a good fatty acid precursor in the post-prandial
rat (Salmon et al, 1974; Clark et al, 1974). In perfused liver (Wood
and Krebs, 1971) and isolated hepatocytes (Clark et al, 1974), depletion
of liver glycogen has been correlated with fatty acid biosynthesis. It
is therefore possible that the stimulation of hepatic lipogenesis after
meal-feeding is a consequence of the increased deposition of liver glycogen.
This hypothesis was tested by the simultaneous measurements of hepatic
rates of glycogen and fatty acid biosynthesis at various times after
feeding. The incorporation of tritium from tritiated water into liver
glycogen of control rats (Fig. 31) indicated that glycogen synthesis was
active for +5h after meal-feeding. Evidence was presented that during

Fig.58. Comparison of the Rate of Hepatic Fatty Acid Biosynthesis with the Activity of "Active" Acetyl CoA Carboxylase
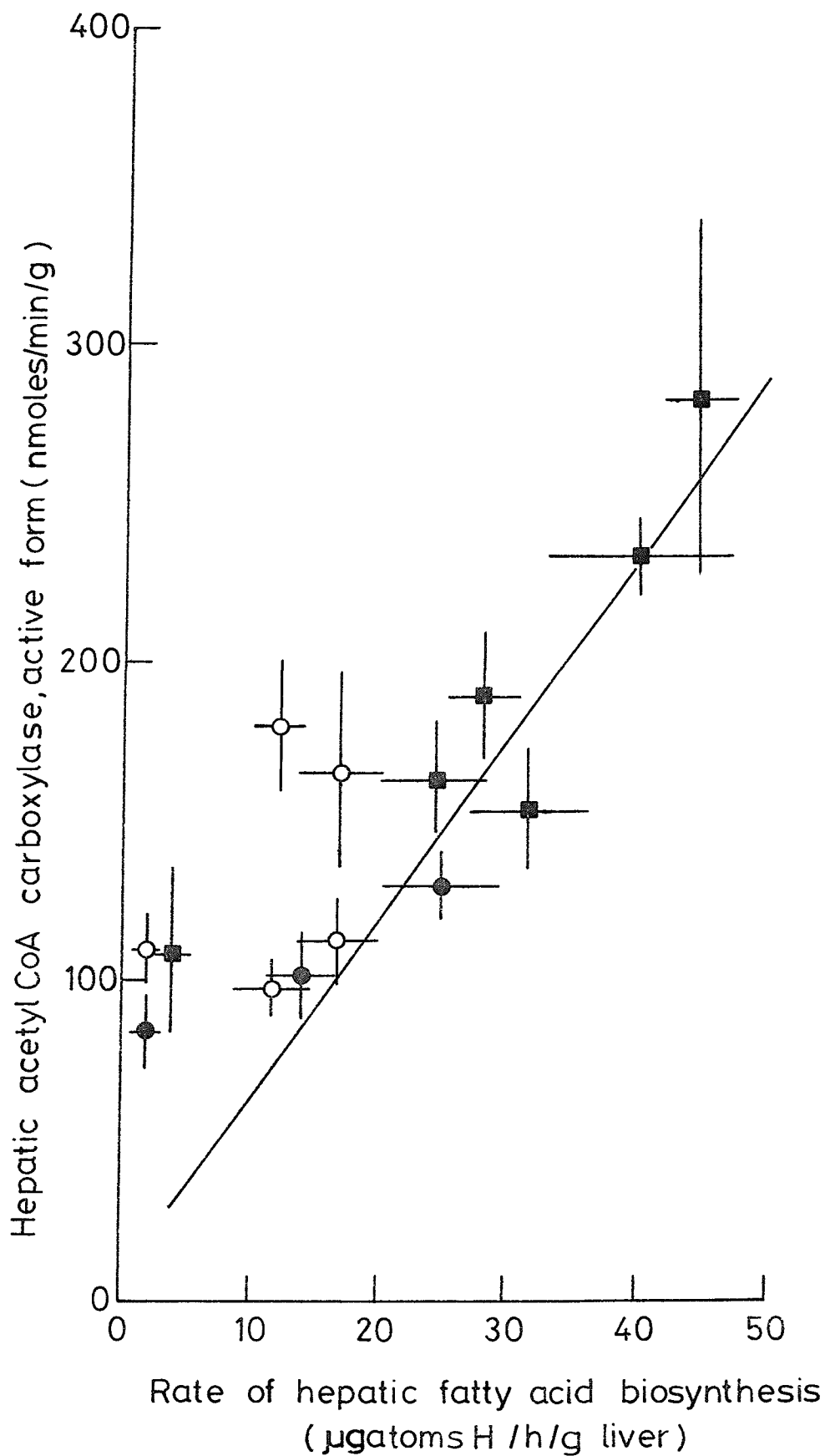
this phase the recycling of liver glycogen was probably slight (Section 3.3.1). These conditions of glycogen synthesis effectively preclude the net conversion of glycogen to other products (Ma et al, 1978). The maximum rate of hepatic fatty acid biosynthesis occurred during this phase of active glycogen synthesis. It is thus unlikely that the increased hepatic glycogen concentration was responsible for the elevated lipogenic rate after feeding. From +5h after feeding onwards hepatic glycogen concentration remained constant and the rate of glycogen synthesis was similar to the low rate measured before feeding (Fig. 31). During this second phase hepatic glycogen could have contributed to the maintenance of the elevated rate of hepatic lipogenesis in control rats (Fig. 48).

The active form of hepatic acetyl CoA carboxylase, measured without citrate pre-incubation, was taken as an index of the enzyme activity in vivo. This activity increased in control rats after feeding to reach a maximum at +2h after feeding (Fig. 54) which coincided with the maximum rate of hepatic fatty acid biosynthesis (Fig. 48). In fed control rats (Fig. 58, square symbols), the active form of hepatic acetyl CoA carboxylase correlated well with the rate of hepatic fatty acid biosynthesis ($r = 0.88$). These results suggest the possibility that, in fed control rats, activation of acetyl CoA carboxylase could be in part responsible for the stimulated lipogenesis.

Comparison of the absolute rates of hepatic fatty acid biosynthesis and acetyl CoA carboxylase provides evidence in support of this hypothesis. The maximum lipogenic rate at +2h after feeding of 45 $\mu$ gatoms 'H'/h/g was equivalent to a substrate flux to fatty acid of 420 nmoles of acetyl CoA/min/g (Jungas, 1968). The corresponding Vmax activity of active acetyl CoA carboxylase was 283±27 nmoles/min/g. This represents an underestimation by about 30% of the true initial enzyme rate due to the non-linear incorporation of $[^{14}C]$ bicarbonate in the enzyme assay (Fig. 50a). This close agreement suggests that, in control rats, active acetyl CoA carboxylase is the rate-limiting enzyme at the time of maximum hepatic fatty acid biosynthesis.

Further analysis of Fig. 58 shows that this correlation fails to

hold in fasted or diabetic rats. In these states, active acetyl CoA
carboxylase activity cannot be an accurate reflection of the substrate
flux through the enzyme in vivo. This conclusion suggests that either
the enzyme assay is erroneous or that acetyl CoA carboxylase is not
always saturated with substrate acetyl CoA. If the latter possibility
were true, then feeding could stimulate hepatic lipogenesis both by
activating acetyl CoA carboxylase Vmax activity and by increasing sub-
strate acetyl CoA carboxylase. Such a mechanism could help explain the
restoration by insulin of the impaired diabetic lipogenesis (Fig. 48)
that happens in the absence of acetyl CoA carboxylase activation (Fig. 54).

There are no published estimates of changes in cytosolic acetyl
CoA concentration in the liver in response to feeding. Total hepatic
concentration decreases, due to decreased mitochondrial fatty acid
oxidation. However, the digitonin fractionation method (Zurrendonk and
Tager, 1974) has enabled the cytosolic acetyl CoA concentration of isolated
hepatocytes to be measured (Siess et al, 1976). Hepatocytes from fasted
rats were incubated with 10mM-lactate, thus simulating the increased
lactate concentration characteristic of the fed rat (Fig. 43). Cyto-
solic acetyl CoA concentration increased from 0.05 to 0.13mM and, more-
over, decreased to unmeasurable amounts when cells were incubated with
oleate. If these results reflect the response of the intact liver to
feeding, they would support activation of acetyl CoA carboxylase by
increased substrate concentration. These acetyl CoA concentrations are
near the Km of the enzyme, reported from 0.03 to 0.05mM (Inoue and
Lowenstein, 1972; Miller and Levy, 1975).

Cholesterol biosynthesis and fatty acid biosynthesis employ the
same substrate pool of cytoplasmic acetyl CoA. The role of substrate
concentration in the regulation of hepatic lipogenesis has been discounted
on the grounds that similar changes should be observed in the rate of
cholesterogenesis (Cook et al, 1977, Watkins et al, 1977). However,
when fatty acid biosynthesis was inhibited by glucagon in vivo (Cook et
al, 1977), in perfused liver (Ma et al, 1978) or in isolated hepatocytes
(Watkins et al, 1977) the rate of sterol synthesis declined to a much
lesser extent. This argument can be refuted for at least two reasons.
Firstly, 3-hydroxy-3-methyl glutaryl CoA reductase is the rate limiting

enzyme of hepatic cholesterogenesis (Siperstein, 1970) and so changes in acetyl CoA concentration would not be expected to greatly alter the overall pathway flux. Secondly, the absolute rate of cholesterol synthesis of isolated hepatocytes could not be increased by incubation with 3mM-pyruvate, 3mM-lactate, 11mM-glucose or 10mM-acetate although 3mM [$^{14}$C] pyruvate could supply all the carbon atoms required for that synthesis (Gibbons and Pullinger, 1979). By contrast fatty acid biosynthesis of isolated hepatocytes increased with substrate concentration up to 25mM lactate (Clark et al, 1974). Also the 90% inhibition of hepatocyte lipogenesis caused by glucagon could be largely prevented by incubation of cells with lactate, pyruvate and glucose (McGarry and Foster, 1979).

Possible regulation of hepatic fatty acid biosynthesis by substrate concentration is provided by the insulin treatment of diabetic rats. Hepatic glycogen concentration increased rapidly on feeding in these rats (Fig. 34) but hepatic fatty acid biosynthesis was not restored for another 3 to 4h (Fig. 48). This delay could be interpreted as the time required for glycogen accumulation to be maximal, and thus provide the fatty acid precursor required under these conditions.

In brief summary, the experiments in this chapter provide evidence in support of two main hypotheses. Firstly, that synthesis of adequate hepatic glycogen was not a pre-requisite of stimulated hepatic fatty acid biosynthesis in the fed control rat although it could serve such a function in the insulin-treated diabetic rate. Secondly, that the stimulation of hepatic fatty acid biosynthesis which occurred when control rats were fed was due both to increased substrate concentration and to activation of acetyl CoA carboxylase. Hepatic lipogenesis in diabetic rats appeared to be stimulated only by increased substrate supply. These experiments did not investigate the mechanism of the activation of acetyl CoA carboxylase in vivo in control rats.

CHAPTER 5

HEPATIC FATTY ACID BIOSYNTHESIS - EFFECTS OF GLUCAGON AND GLUCOCORTICOID
HORMONES

5.1. Introduction

The results presented in Chapter 4 support the hypothesis that the
rate of hepatic fatty acid biosynthesis can be stimulated both by acti-
vation of acetyl CoA carboxylase and by increased acetyl CoA concen-
tration in the cytosol. It follows from this concept that it should be
possible to inhibit hepatic lipogenesis either by inactivation of acetyl
CoA carboxylase or by decreasing acetyl CoA supply. Glucagon inhibition
of hepatic fatty acid biosynthesis has been described many times since
the original measurements of $[^{14}C]$ acetate incorporation into fatty acid
in liver slices (Berthet, 1960). These include studies in vivo (Klain
and Weiser, 1973; Cook et al, 1977), in perfused liver (Raskin et al,
1974; Ma et al, 1978) and in isolated hepatocytes (Watkins et al, 1977;
McGarry and Foster, 1979; Witters et al, 1979; Beynen et al, 1979).
Mechanisms postulated for this inhibition range from inhibition of acetyl
CoA carboxylase (Klain and Weiser, 1973; Witters et al, 1979; Beynen et
al, 1979), through partial limitation of substrate supply (Watkins et al,
1977; McGarry and Foster, 1979), to the effect being an artefact of
depleted hepatic glycogen (Ma et al, 1978). The experiments described
in this chapter were designed to provide more information about the locus
for the response of hepatic fatty acid biosynthesis to glucagon.

The enzyme activities chosen to be compared with the rate of
hepatic lipogenesis were acetyl CoA carboxylase and L-pyruvate kinase.
Inhibition of acetyl CoA carboxylase would immediately limit the capacity
of the liver to synthesize fatty acid (Section 1.3.4). Inhibition of
L-pyruvate kinase would have a more indirect effect. Interruption of the
substrate cycle between pyruvate and phosphoenolpyruvate could result in
a reduction of supply of substrate acetyl CoA (Section 1.3.3). L-pyruvate
kinase can be considered a lipogenic enzyme and its activity increases
under conditions of enhanced fatty acid synthesis (Engström, 1978;
Hopkirk and Bloxham, 1979). Both enzymes are subject to cyclic AMP
dependent phosphorylation (Section 1.2.2) and are thus potential targets

for glucagon action.

Many other actions of glucagon have been shown to be dependent on the presence of glucocorticoid hormones (Section 1.4.4). The possibility was considered that the inhibition of hepatic fatty acid biosynthesis by glucagon might also be steroid-dependent. The action of glucagon in corticotropin-treated and adrenalectomized rats was therefore explored to investigate this hypothesis. Metabolite crossover analysis in perfused liver has located a site for the inhibition by glucagon of gluconeogenesis between pyruvate and phosphoenolpyruvate (Exton et al, 1972). This crossover was absent when livers from adrenalectomized rats were exposed to glucagon. It is possible that this defective response to glucagon after adrenalectomy could be caused by altered regulatory properties of L-pyruvate kinase. Any such changes could also affect hepatic fatty acid synthesis as well as gluconeogenesis. Thus the kinetic properties of L-pyruvate kinase in the various experimental animal models were studied in greater detail.

## 5.2.  Results

### 5.2.1.  Serum Corticosterone Concentration

Corticosterone was assayed to monitor the effectiveness of the corticotropin and adrenalectomy treatments. A specific radioimmunoassay was developed using a $[^{125}I]$ derivative of corticosterone (Section 2.4.5). The high concentration of tritium in the blood precluded using $[^{3}H]$ corticosterone. The method developed possessed significant advantages over alternative techniques. No organic extraction was required as corticosterone binding globulin was inactivated at the assay pH of 4.8. Bound and free radio-label could be readily separated by low speed centrifugation of the cellulose : antibody complex. The gamma label did not require tedious scintillation counting, and serum samples of 10 μl were sufficient to be measured.

Comparison of the binding of a variety of other steroid hormones indicated that there was little cross-reactivity of the antibody (Table 17). Results by this technique agreed reasonably with a fluorometric procedure (Silber, 1966) and an adapted commercial competitive protein binding kit

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Table 17

Cross Reactions of Steroid Hormones in
Corticosterone Radio-immunoassay

| Steroid | % Cross Reaction |
|---|---|
| Corticosterone | 100 |
| Cortisol | 0.05 |
| Compound S | < 0.01 |
| Progesterone | 0.07 |
| Testosterone | < 0.01 |
| Dexamethasone | < 0.01 |

Cross-reactivity was assessed by the concentration of steroid required to displace 50% of the bound counts. The results are expressed as a percentage of the value for corticosterone.

Table 18

A Comparison of Corticosterone Analysis by Different Methods

| Serum sample | Corticosterone Concentration ( $\mu$moles/l) | | |
|---|---|---|---|
| | Radioimmuno-assay | Fluorometric assay | C.P.B. assay |
| 1 | 2.22 | 2.80 | 2.22 |
| 2 | 2.90 | 2.20 | 2.80 |
| 3 | 4.00 | 5.65 | 4.65 |
| 4 | 4.40 | 5.35 | 34.65 |
| 5 | 4.70 | 4.95 | |
| 6 | 4.20 | 4.75 | 4.40 |
| 7 | 2.15 | 2.93 | |
| 8 | 1.90 | | 1.23 |
| 9 | 2.40 | | 1.36 |
| 10 | 2.80 | | 2.45 |

Results presented are means of duplicate samples.

The methods were the radioimmunoassay described above, the fluorometric assay of Silber (1966) and the competitive protein binding assay ('Cortipac', Amersham).

Table 19

The effect of experimental procedures on food eaten, weight gained and serum corticosterone concentrations. The food eaten was calculated from the daily consumption per cage of 4 rats. The weight gain was measured over the treatment period or the previous week (control rats). Probabilities v.s. control rats : $\dagger$ 0.01 > p > 0.001; $\ddagger$ p < 0.001

| Animals | Food eaten (g/day) | Wt change (g/day) | Serum corticosterone ( $\mu$moles/l) |
|---|---|---|---|
| Control | 14.6 | + 5.4 | 1.04±0.13 |
| Corticotropin-treated | 11.0 | − 0.9 | $\dagger$ 1.96±0.41 |
| Adrenalectomised | 6.2 | − 1.9 | $\ddagger$ 0.136±0.027 |
| Sham operated | 9.1 | − 1.1 | 1.28±0.43 |

for cortisol (Cortipac, Amersham) using a [$^{75}$Se]-cortisol derivative.
In these analyses, corticosterone standards were substituted for the
cortisol standards supplied with the kit. Up to ten samples of plasma
were assayed by the three procedures (Table 18).

Corticotropin treatment significantly elevated corticosterone con-
centration above the normal range (Table 19). Corticosterone concentration
was depressed by 90% after adrenalectomy. Two adrenalectomized rats
showed visible signs of adrenal regrowth and had normal hormone concen-
trations. These animals were omitted from all further analysis.

### 5.2.2. Food Consumption and Weight Gain

The amount of food eaten can have a profound influence on the rate
of hepatic fatty acid biosynthesis. The daily food consumptions of the
control and corticotropin-treated rats was calculated as the mean food
intake per day in a cage of 4 rats. Surgically treated rats were housed
singly and individual food intakes were measured. All experimental
treatments decreased the amount of food eaten (Table 19). This reduction
was most extreme for adrenalectomized rats and consequently a group of
intact rats was pair-fed to provide an additional control for these
animals.

The mean weight gain of control rats, measured over the previous
week, was 5.4 g/day/rat. By comparison, corticotropin-treated, adrenalecto-
mized and sham adrenalectomized rats all consistently lost weight
(Table 19).

### 5.2.3. Blood Metabolite Concentrations

Groups of rats were injected with glucagon (1mg/kg) or saline at
+1.5h after feeding. At +2h after feeding, these animals were killed and
the concentrations of blood metabolites and serum triglyceride were
measured (Table 20). In both control and corticotropin-treated rats,
glucagon increased the blood concentrations of glucose, lactate and
3-hydroxybutyrate. By contrast, none of these responses were apparent
in adrenalectomized rats. This result was probably due to the permissive
requirement for steroid for the stimulation by glucagon of gluconeogenesis

Table 20

The Effect of Glucagon on Blood Metabolite Concentrations at +2h

| Experimental condition | | Metabolite Concentration (m moles/l) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Glucose | Lactate | Pyruvate | Alanine | 3-OH butyrate | Triglyceride |
| Control rats | + saline | 7.1±0.6 | 3.9±0.5 | 0.204±0.02 | 0.602±0.08 | 0.073±0.008 | 0.95±0.14 |
| | + glucagon | *8.4±0.9 | *5.5±1.3 | †0.378±0.03 | 0.588±0.052 | *0.125±0.037 | 1.15±0.15 |
| Corticotropin treated rats | + saline | 5.6±0.6 | 4.7±0.9 | 0.244±0.057 | 0.659±0.044 | 0.023±0.010 | 1.27±0.14 |
| | + glucagon | †10.0±1.3 | 5.6±0.7 | 0.272±0.045 | 0.607±0.106 | *0.064±0.010 | 1.12±0.07 |
| Adrenalect- omized rats | + saline | 5.3±0.4 | 3.6±0.6 | 0.238±0.041 | 0.583±0.022 | 0.039±0.007 | 0.73±0.09 |
| | + glucagon | 6.1±0.4 | 3.4±0.6 | 0.246±0.063 | * 0.441±0.013 | 0.031±0.001 | 0.90±0.14 |

Results are expressed as mean $\pm$ S.E.M; n = 4 - 8.

Probabilities (saline v.s. glucagon) were calculated by Students' 't' test: * $0.05 > p > 0.01$; † $0.01 > p > 0.001$.

and ketogenesis (Section 1.4.4).

The major direct effects of corticotropin-treatment and adrenalectomy were to decrease the concentrations of blood glucose and 3-hydroxybutyrate in both cases. The hypoglycaemic effect of a high dose of corticotropin was unexpected. Both exogenous steroid administration and Cushing's syndrome are characterized by hyperglycaemia (Munck, 1971). The low concentration of 3-hydroxybutyrate after corticotropin treatment agrees with results from corticotropin injection into man (Johnston et al, 1979). Despite the lipolytic action of both cortisol and corticotropin, fasting concentrations of neither ketone bodies nor NEFA were increased.

### 5.2.4. Hepatic Glycogen Metabolism

The effects of corticotropin-treatment and adrenalectomy on the rate of hepatic glycogen synthesis and glycogen concentration were shown in Figs. 35 and 36 and summarized in Table 8. In the hypercorticoid rat, glycogen synthesis increased rapidly in response to feeding. Glycogen concentration was maximal in these animals at +2h. Glycogen synthesis and concentration in adrenalectomized rats increased in response to feeding in a manner comparable to control rats. These concentrations were not sustained, however, and by +6h glycogen synthesis was negligible and glycogen concentration had fallen by 50%. It can be inferred from the tritium incorporation into hepatic glycogen from tritiated water that hepatic glycogen could only have been a potential fatty acid precursor after +2h in corticotropin-treated rats and after +4h in adrenalecto-mized rats.

Neither corticotropin injection nor adrenalectomy significantly affected the hepatic glycogenolytic response to glucagon (Table 8). The rate of tritiated water incorporation into glycogen was inhibited by glucagon in all experimental groups.

### 5.2.5. Hepatic Fatty Acid Biosynthesis

Rates of hepatic fatty acid biosynthesis measured in control rats over 30 min were similar to previous rates measured over 60 min (compare Figs. 59 and 48). These rates were significantly elevated by corticotropin
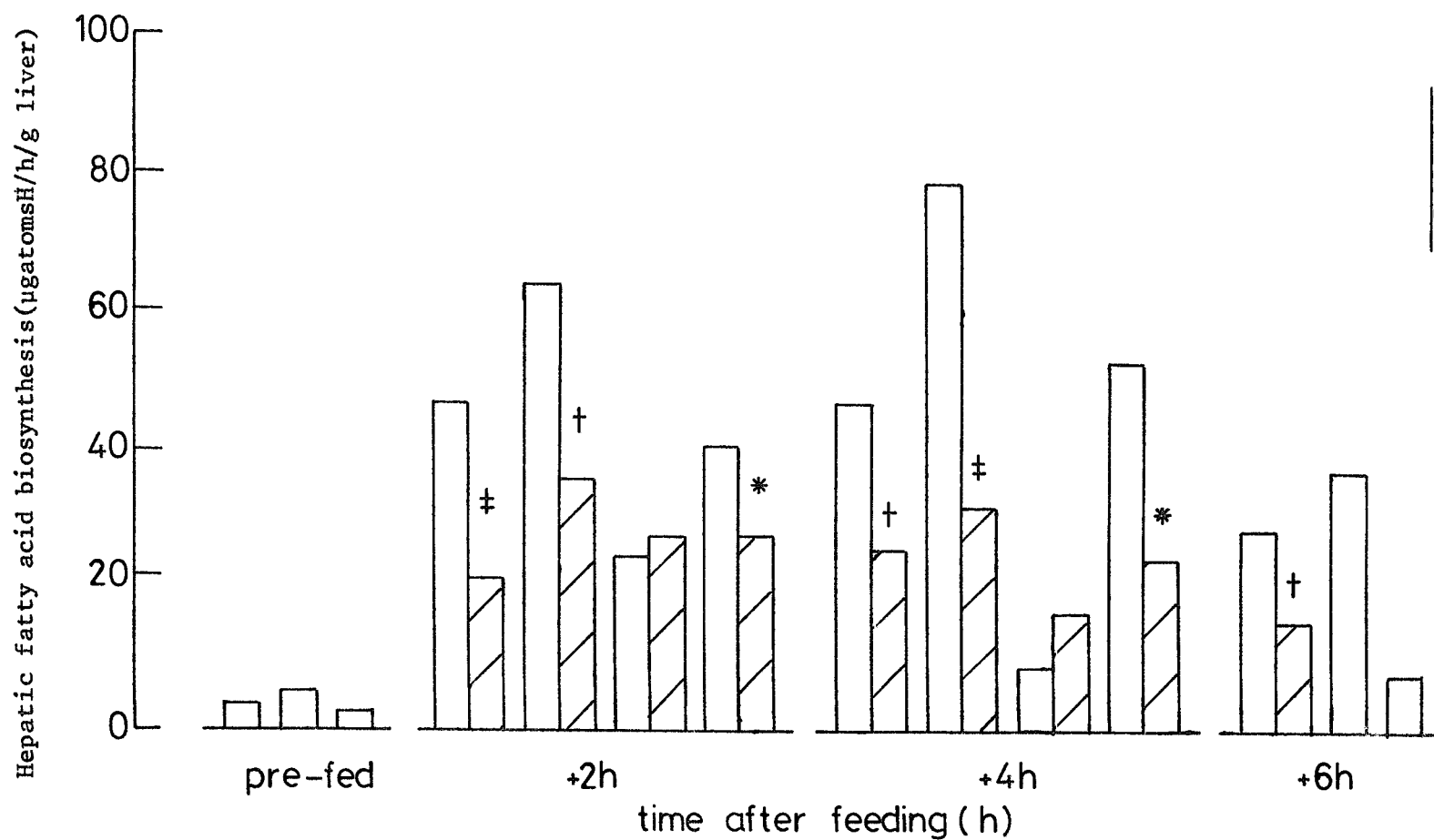
Table 21

<u>The Regulation of Hepatic Fatty Acid Biosynthesis by Glucagon</u>

Fatty Acid Biosynthesis ( μ gatoms 'H'/h/g liver wet wt.)

| Time after Feeding (h) | Experimental Injection | Control Rats | Corticotropin-treated Rats | Adrenalect-omized Rats | Sham Operated Rats | Pair-fed Rats |
|---|---|---|---|---|---|---|
| Pre-fed | | 3.8±0.6 | 5.4±1.1 | 2.8±1.7 | | |
| +2 | saline | 46.7±3.4 | 64.0±6.2 | 24.9±4.4 | 41.4±1.0 | |
| | glucagon | 22.1±1.5 | 36.3±3.5 | 28.9±4.8 | 28.2±6.6 | |
| | % inhibition | ‡52.7 | †43.3 | N.S. | *31.7 | |
| +4 | saline | 47.4±2.4 | 79.3±2.8 | 9.2±1.6 | 38.0±5.6 | 53.4±6.6 |
| | glucagon | 26.2±4.0 | 31.7±2.5 | 16.7±6.8 | | 24.0±6.6 |
| | % inhibition | †44.7 | ‡60.0 | N.S. | | *55.1 |
| +6 | saline | 29.0±1.7 | 36.7±2.5 | 8.4±2.1 | | |
| | glucagon | 15.9±2.5 | | | | |
| | % inhibition | †45.2 | | | | |

Results are mean ± S.E.M. of 4 - 8 rats. Probabilities of the glucagon inhibition, calculated by Students' 't' test were: * $0.05 > p > 0.01$; † $0.01 > p > 0.001$; ‡ $p < 0.001$

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Fig.59  Hepatic Fatty Acid Biosynthesis in Meal-fed Rats in Response to Glucagon

Open bars represent saline-injected rats ; closed bars represent glucagon-injected rats.

Probability by Students t-test : *,p<0.05 ;†, 0.01>p>0.001 ; ‡, p<0.001

treatment throughout the 6h period measured (Fig. 59; Table 21). This effect was not related to food intake, which was lower in these rates (Table 19). Conversely, adrenalectomy impaired the feeding response of hepatic lipogenesis. Even the low rates achieved by +2h were not maintained subsequently and had fallen to diabetic rates by +6h after feeding (Fig. 59; Table 21). The rates of hepatic fatty acid biosynthesis were not impaired to the same extent by sham adrenalectomy or pair-feeding with adrenalectomized rats.

Glucagon injection (1mg/kg) in vivo into control rats caused a 50% inhibition of hepatic fatty acid biosynthesis at +2h, +4h and +6h after feeding (Fig. 59; Table 21). This inhibition was maintained, but not exaggerated, when the incubation with tritiated water was extended to 60 min. Glucagon impairs hepatic fatty acid biosynthesis by a similar extent in corticotropin-treated rats. By contrast, neither at +2h nor +4h does glucagon inhibit hepatic fatty acid biosynthesis in adrenalectomized rats (Fig. 59; Table 21). The slight stimulation of lipogenesis at both times was in neither case significant. This lack of response to glucagon did not appear to be caused either by operational trauma or by reduced food intake. A control response to glucagon was apparent both in sham adrenalectomized and pair-fed rats (Table 21). Neither was this impaired response due solely to the low lipogenic rate in adrenalectomized rats. The rate of hepatic fatty acid biosynthesis in control rats at +6h (29.0±1.7 $\mu$gatoms 'H'/h/g) was comparable to that in adrenalectomized rats at +2h (24.9±4.4 $\mu$gatoms 'H'/h/g). Nevertheless, glucagon caused a 45% inhibition of hepatic lipogenesis in these control rats.

### 5.2.6. Acetyl CoA Carboxylase

Total acetyl CoA carboxylase activity in rat liver at +2h was increased by corticotropin treatment and decreased by adrenalectomy (Table 22). Both these effects were significant ($p < 0.05$). Total enzyme activity was not affected by sham adrenalectomy. This time point was chosen to investigate the response of hepatic acetyl CoA carboxylase to glucagon because feeding caused a maximum activation of the active enzyme form at +2h (Fig. 54).

Neither total nor active acetyl CoA carboxylase were altered in

Table 22

Effect of Glucagon on Hepatic Acetyl CoA Carboxylase Activity

| Experimental Condition | | Acetyl Co Carboxylase (n mol/min/g of liver) | |
|---|---|---|---|
| | | Active | Total |
| Control | + saline | 208 ± 25 | 556 ± 61 |
| | | n.s. | n.s. |
| | + glucagon | 245 ± 39 | 527 ± 50 |
| Corticotropin-treated rats | + saline | 410 ± 44 | 706 ± 51 |
| | + glucagon | †207 ± 30 | * 512 ± 42 |
| Adrenalecto-mized rats | + saline | 103 ± 11 | 443 ± 63 |
| | | n.s. | n.s. |
| | + glucagon | 97 ± 9 | 367 ± 11 |
| Sham adrenalecto-mized rats | + saline | 142 ± 16 | 524 ± 15 |

Probabilities of glucagon v.s. saline injection were calculated by Students' 't' test:    * $0.05 > p > 0.01$;    † $0.01 > p > 0.001$; n.s. - not significant.

response to glucagon in control, adrenalectomized or sham adrenalecto-
mized rats (Table 22). Thus acetyl CoA carboxylase activity was not
inhibited in control rats under conditions where glucagon inhibited
hepatic lipogenesis by 50%. Glucagon injection in corticotropin-treated
rats, however, diminished both total and active acetyl CoA carboxylase
activity. Also, the proportion of enzyme in the active form decreased
from 72.5 to 50.5% after glucagon administration. The mechanism behind
this inhibition is unclear as polymer to protomer enzyme conversion would
have been expected to be expressed in only the active and not in the
total enzyme form. One possibility is that increased hepatic concentrations
of fatty acyl CoA could have impaired the assay for total acetyl CoA
carboxylase. Hepatic longchain fatty acyl CoA concentrations were not
measured directly but were inferred indirectly from hepatic triglyceride
concentrations. Hepatic triglyceride concentrations of control rats
($3.01\pm0.23$ $\mu$moles/g) and adrenalectomized rats ($3.0\pm0.35$ $\mu$moles/g) were
greatly increased by corticotropin treatment ($33.0\pm6.8$ $\mu$moles/g). Glucagon
did not deplete hepatic triglyceride in control or adrenalectomized rats
but caused a decrease to $19.1\pm9.6$ $\mu$moles/g in corticotropin-treated rats.
Possibly the fatty acyl CoA generated affected the measurement of acetyl
CoA carboxylase activity. However, the proportion of enzyme in the
active form in corticotropin-treated rats after glucagon (50.5%) was
still higher than the fractional active form in control rats (42.1%).

5.2.7. Pyruvate Kinase

Hepatic pyruvate kinase Vmax activity at either +2h or +4h, measured
at 10mM-phosphoenol pyruvate, was not significantly changed by cortico-
tropin-treatment, adrenalectomy or glucagon injection (Table 23). This
maximum activity could not be measured reliably after activation with
fructose-1,6-bisphosphate. There was a rapid background utilization of
NADH which was independent of pyruvate kinase activity. The automated
enzyme assay (Section 2.3.2) could not cope with such a large blank rate.

The reaction velocity was measured at concentrations of substrate
phosphoenol pyruvate from 0.125 to 10mM. This relationship was sigmoidal
for control rats, displaying positive co-operativity (Fig. 60). The
degree of co-operativity was increased by corticotropin (Fig. 61) and

Table 23

The Regulation of Hepatic Pyruvate Kinase by Glucagon

| Animals | Hepatic Pyruvate Kinase ( μmoles/min/100 mg cytosol protein) | | | Vmax : V 0.5mM-PEP | | |
|---|---|---|---|---|---|---|
| | Saline in vivo | Glucagon in vivo | Cyclic AMP in vitro | Saline in vivo | Glucagon in vivo | Cyclic AMP in vitro |
| Control rats | 42.7±6 (6) | 43.8±1.3 (6) | 40.5±1.2 (6) | 13.8±0.3 | *16.6±1.0 | †22.0±1.2 |
| Corticotropin-treated rats | 45.4±1.4 (5) | 45.8±4.2 (3) | 43.9±2.1 (3) | 15.7±1.4 | *21.8±0.8 | n.s. 28.6±3.0 |
| Adrenalect-omized rats | 36.0±3.7 (6) | 47.6±6.6 (4) | 38.0±3.8 (4) | 9.4±1.3 | n.s. 8.9±1.0 | †14.4±1.8 |

Results are expressed as mean ± S.E.M. (number of rats).

Probabilities calculated by Students' 't' test were: * $p < 0.05$ saline v.s. glucagon; †    0.05 glucagon v.s. cyclic AMP.

decreased by adrenalectomy (Fig. 62). Co-operativity can be quantified
by comparison of the reaction velocity at high and low substrate con-
centrations (Engström, 1978). The ratio Vmax :   0.5mM-phosphoenol
pyruvate (Vmax :   0.05mM-PEP) has been used as an indirect index of
the phosphorylation state of L-pyruvate kinase (Hopkirk and Bloxham,
1979). This ratio was significantly (p< 0.05) increased by corticotropin
and decreased by adrenalectomy (Table 23).

Glucagon inhibits L-pyruvate kinase by increasing the substrate co-
operativity (Engström, 1978). This response to glucagon injection in vivo
can be seen in control (Fig. 60) and corticotropin-treated rats (Fig.61).
The substrate : velocity graph shifted in each case to the right and the
Vmax :   0.5mM-PEP ratios increased (Table 23). Such a response was
absent after glucagon treatment of adrenalectomized rats (Fig. 62).
The reaction velocity at low concentrations of phosphoenol pyruvate
remained unchanged, as did the Vmax :   0.5mM-PEP ratio (Table 23).
Results within each group were similar at +2h and +4h and have thus been
combined in these figures. An additional presentation of results, which
contrasted the different response to glucagon in adrenalectomized rats
with that in the other groups, was to calculate the reaction velocity
± glucagon. This was then plotted against the relevant phosphoenol
pyruvate concentration (Fig. 64). A decrease in this activity ratio
denotes an inhibition of pyruvate kinase activity at that substrate con-
centration. In contrast to the inhibition of L-pyruvate kinase in control
and corticotropin-treated rats, glucagon caused a slight enzyme activation
below 0.5mM-phosphoenol pyruvate. This effect was, of course, small in
absolute terms (Fig. 62).

The permissive role for corticosterone in the inhibition of pyruvate
kinase by glucagon was examined further by phosphorylation in vitro using
cyclic AMP and endogenous protein kinase. Supernatant samples (100 μl)
of liver homogenates were incubated for 30 min at $37^{\circ}C$ with 10 M cyclic
AMP and 10mM $Mg^{2+}$.ATP in 1 ml of assay buffer. The reaction velocity
was then measured at concentrations of phosphoenol pyruvate from 0.125
to 10mM. In control (Fig. 60) and corticotropin-treated rats (Fig. 61),
this treatment caused a significant inhibition of hepatic pyruvate kinase
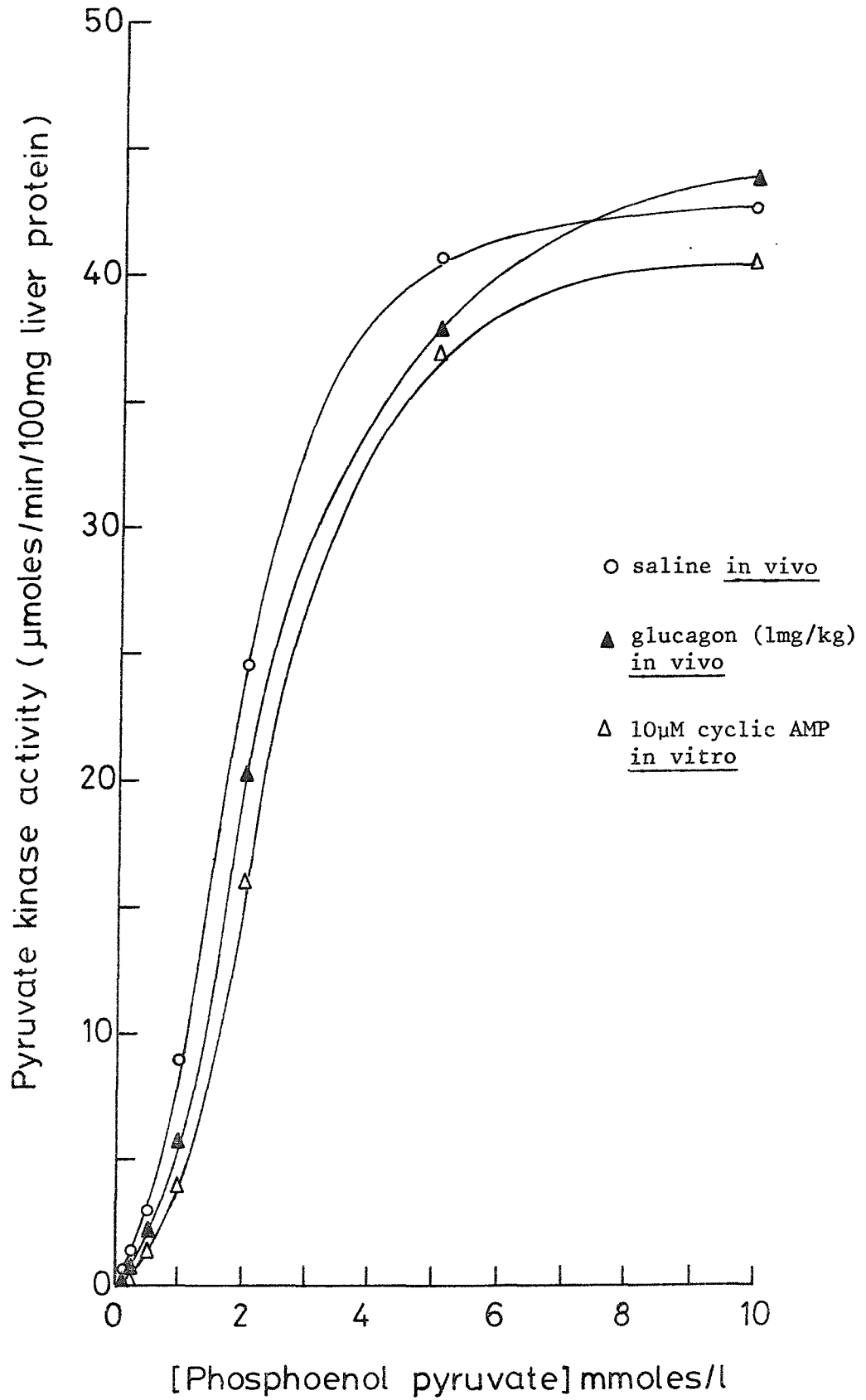activity. The Vmax :   0.5mM-PEP ratio increased in both cases to a

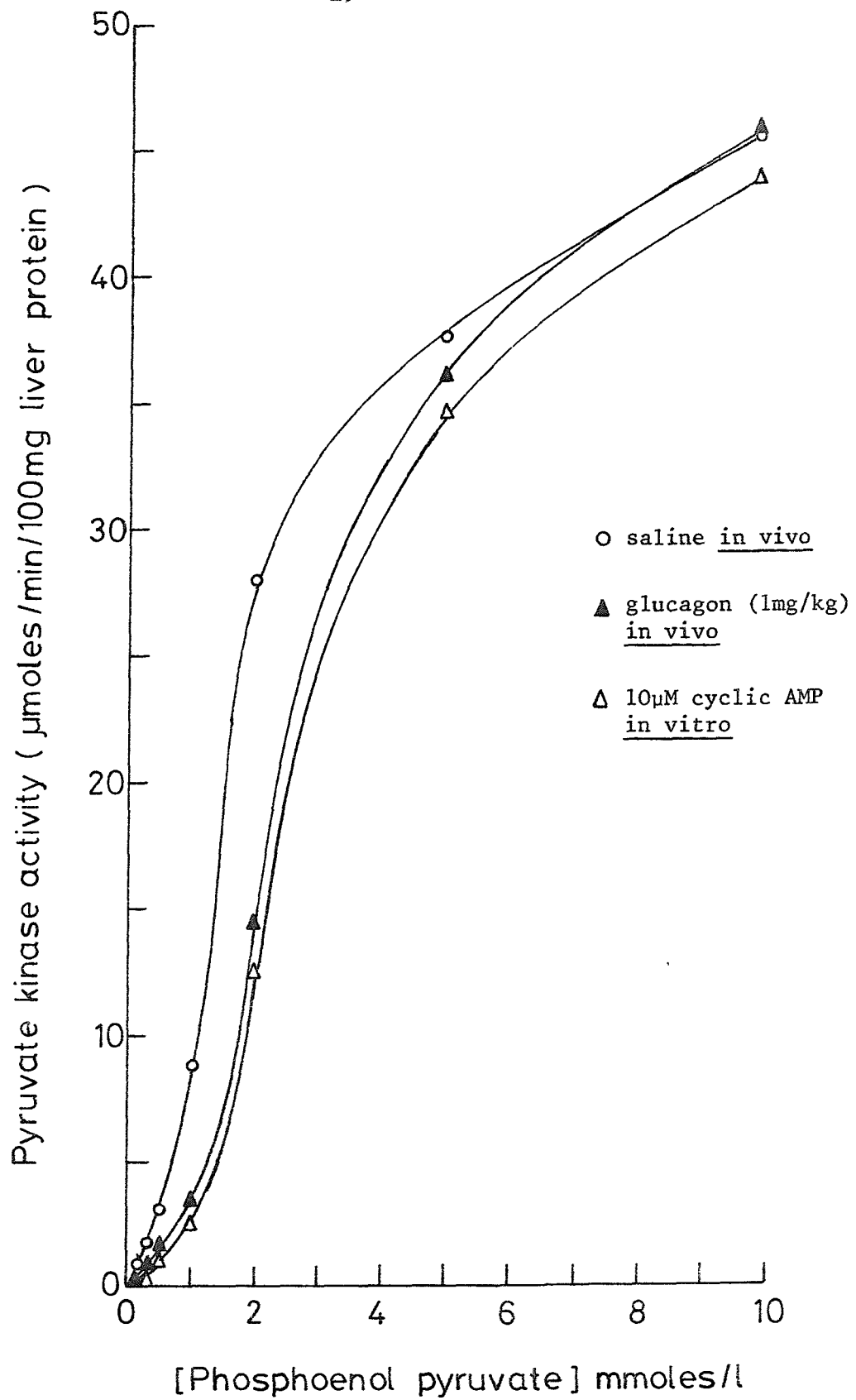Fig.60. Hepatic Pyruvate Kinase in Control Rats +2h-+4h after Meal-feeding

Fig.61. Hepatic Pyruvate Kinase in Corticotropin-treated Rats
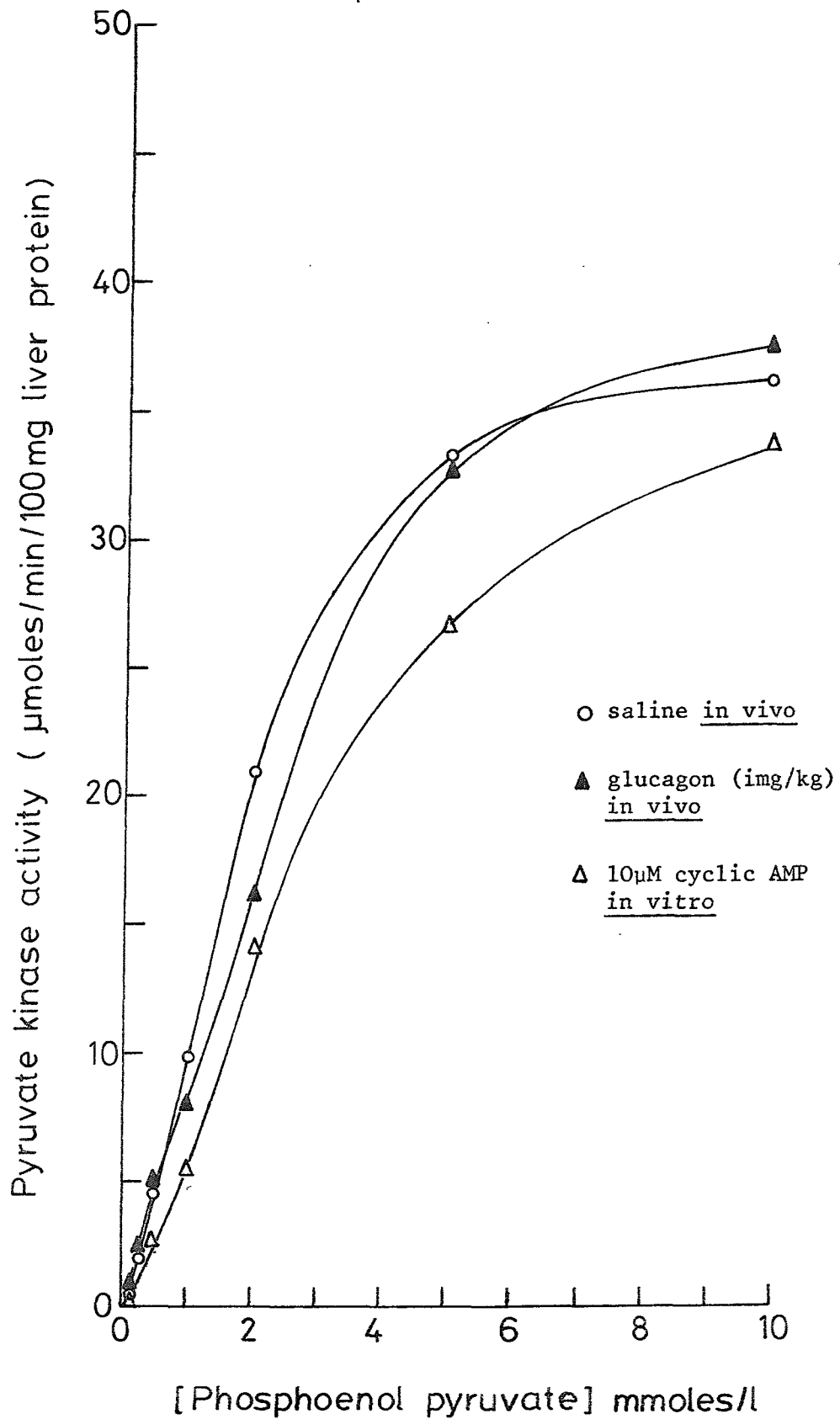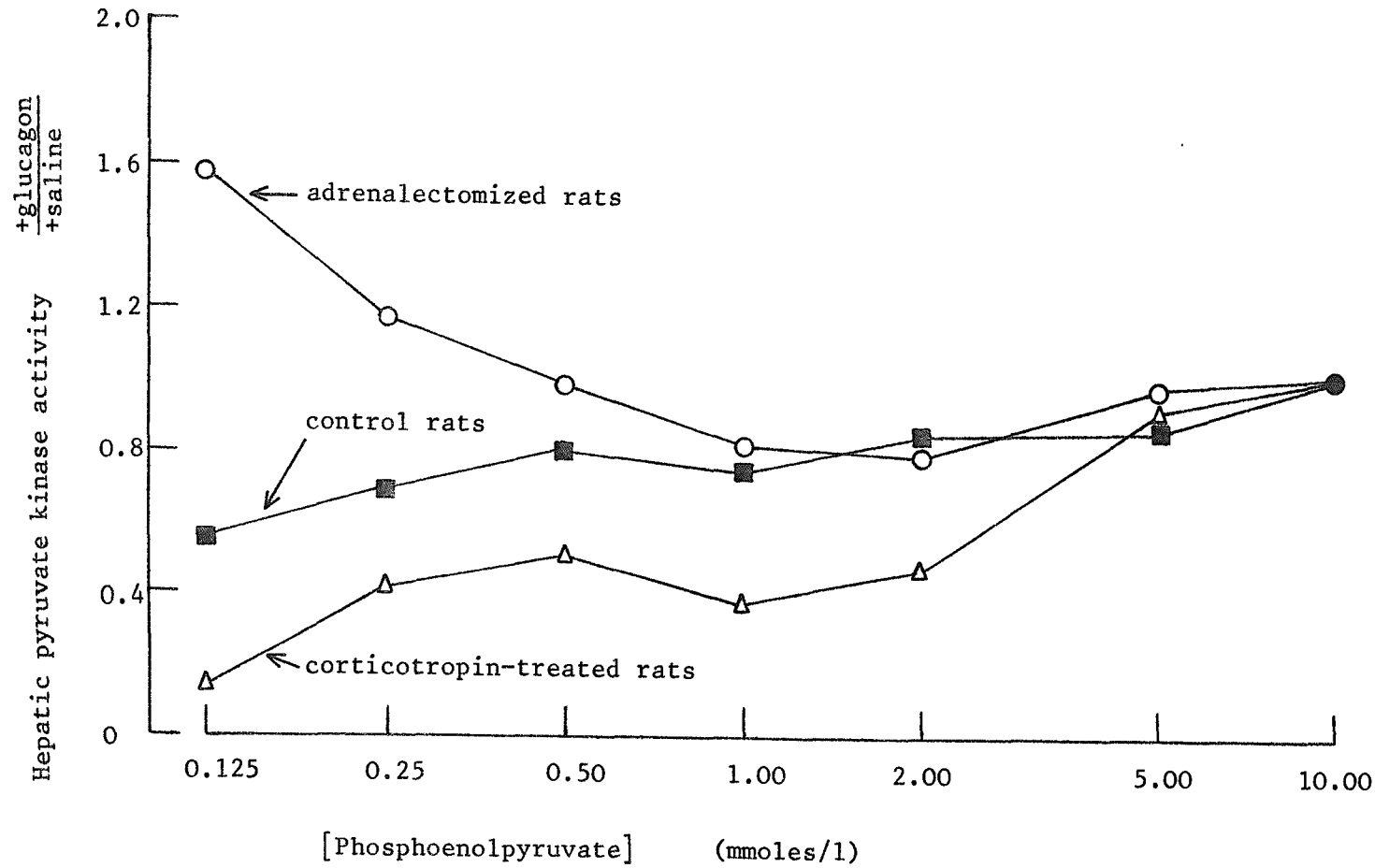+2h-+4h after Meal-feeding

Fig.62. Hepatic Pyruvate Kinase in Adrenalectomized Rats +2h-+4h
after Meal-feeding

Fig.63   The Response of Hepatic Pyruvate Kinase to Glucagon

greater extent than in response to glycagon in vivo (Table 23). Samples from saline and glucagon injected animals were inhibited to the same degree. The results presented for cyclic AMP action are thus the means of both groups. This in vitro inhibition of L-pyruvate kinase in fed control rats has been described previously (Van Berkel et al, 1979).

Hepatic pyruvate kinase from adrenalectomized rats also responded to cyclic AMP in vitro (Fig. 62). This was a marked contrast to the lack of response to glucagon in vivo. The Vmax : 0.5mM-PEP ratio, which was unchanged by glucagon, was significantly increased by incubation with cyclic AMP (Table 23). This result suggests that there was no absolute impairment of the endogenous hepatic protein kinase in adrenalectomized rats.

## 5.3. Discussion

These results provided additional insight into the mechanism behind the action of glucagon upon hepatic fatty acid biosynthesis. Proposed explanations for this inhibition include depletion of hepatic glycogen, inhibition of acetyl CoA carboxylase and limitation of the conversion of pyruvate to acetyl CoA.

Hems and his colleagues suggested that the inhibition of lipogenesis by glucagon was not a direct action but was secondary to the depletion of substrate glycogen (Ma et al, 1978). They argued that the magnitude of hormonal response increased with duration of exposure (Raskin et al, 1974) and that stimulation of glycogenolysis was much more sensitive to glucagon than was inhibition of lipogenesis. The incorporation of tritium from tritiated water into liver glycogen provided a technique to evaluate this hypothesis. The response of glycogen metabolism to feeding can be divided into two phases by the incorporation of tritium into glycogen (Section 3.3.2). During the first 5h after feeding, the rate of glycogen synthesis was elevated (Fig. 31); during this phase hepatic glycogen could not have been a fatty acid precursor. Glucagon administration within this period, however, halved the rate of hepatic fatty acid biosynthesis in control rats (Table 21). Similarly, in corticotropin-treated rats at +2h, glucagon inhibited hepatic lipogenesis (Table 21)

while the rate of hepatic glycogen synthesis was still elevated (Table 8). Under these conditions, when glycogen could not have a significant net precursor for fatty acid, it is unlikely that depletion of glycogen could have been the primary determinant of the inhibition of lipogenesis. This argument does not deny any role for glycogen in the regulation of hepatic fatty acid biosynthesis. For instance, a high glycogen content is required to maintain maximum fatty acid biosynthetic rats in perfused liver (Ma et al, 1978) and isolated hepatocytes (Cook et al, 1977).

Evidence for a direct inhibition of hepatic acetyl CoA carboxylase by glucagon has been sought many times, with varying results. Such an inhibition has been detected (Klain and Weiser, 1973; Stansbie et al, 1976; Beynen et al, 1980), but has also proved elusive (Cook et al, 1977; Watkins et al, 1977; Ma et al, 1978). No inhibition by glucagon of hepatic acetyl CoA carboxylase was apparent in the studies presented above (Table 22). Two arguments suggest that this result might be valid. Firstly, the precautions taken against in vitro enzyme changes (Section 4.2.5) were sufficient for the activation in response to feeding to be demonstrated (Fig. 54). Secondly, when liver homogenates were incubated at $37^\circ$C for 30 min in the absence of citrate, acetyl CoA carboxylase activity did not change (Fig. 49a). The results do not prove that acetyl CoA carboxylase in liver does not respond directly to glucagon. They do suggest, however, that any such response could be relatively minor in the overall inhibition of hepatic lipogenesis.

The adaptation of hepatic acetyl CoA carboxylase, by changes in protein synthesis (Section 1.3.4), was well demonstrated in corticotropin-treated and adrenalectomized rats. Such activity changes were probably responses to the circulating concentration of insulin, which was increased by glucocorticoid treatment (Malaisse and Malaisse-Lagae, 1967; Van Lan et al, 1974) and decreased after adrenalectomy (Kawai & Kuzuya, 1977). The stimulation of total acetyl CoA carboxylase activity by corticotropin (Table 22) agreed with previous studies where rats were chronically ex-posed to dexamethasone (Diamant and Shafrir, 1975). No enzyme activation was observed when alloxam diabetic rats were treated with steroid.

The apparent steroid dependence of the inhibition of hepatic

lipogenesis by glucagon (Fig. 59) provided an additional model to
investigate this process. A permissive role for glucocorticoid has
become accepted for the stimulation by glucagon of gluconeogenesis
(Exton et al, 1972) and ketogenesis (Chernick et al, 1972). This was
reflected in adrenalectomized rats by the blood concentrations of glucose
and 3-hydroxybutyrate not being increased by glucagon (Table 20). It
is possible that this glucocorticoid effect on the regulation of all
three pathways could reside in a common mechanism. For instance, in
control rats, stimulation of gluconeogenesis by glucagon could inhibit
lipogenesis partially by limitation of acetyl CoA. The associated
decrease of malonyl CoA concentration would relieve the inhibition of
acyl carnitine transferase II and thus stimulate ketogenesis. In
adrenalectomized rats, a lack of response of gluconeogenesis to glucagon
could be reflected in the responses of the other two pathways.

L-type pyruvate kinase is one target enzyme for the stimulation of
gluconeogenesis by glucagon (Engström, 1978; Section 1.3.2). Also,
L-pyruvate kinase activity is essential for the efficient conversion of
pyruvate to acetyl CoA (Fig. 3; Section 1.3.3). The inhibition of
hepatic pyruvate kinase by glucagon in control and corticotropin-treated
rats, shown by the increased ratio of Vmax : 0.5mM-PEP (Table 23), was
in agreement with previous observations (Engström et al, 1978; Van
Berkel et al, 1979). The lack of inhibition in adrenalectomized rats
(Fig. 62; Table 23) provided an explanation for observations in perfused
liver (Exton et al, 1972). The metabolite crossover between pyruvate and
phosphoenolpyruvate in response to glucagon was absent in livers from
adrenalectomized rats.

The lack of change in the Vmax activity of pyruvate kinase in
livers from control or adrenalectomized rats was taken as evidence
against a defect of pyruvate kinase regulation. This result would be
expected as adrenalectomy only abolished the effect of glucagon on
pyruvate kinase activity at low phosphoenol pyruvate concentrations
(Fig. 63.)

The nature of the permissive role for corticosterone in the regu-
lation of L-pyruvate kinase was uncertain. The invitro inhibition by

cyclic AMP of the hepatic enzyme from adrenalectomized rats suggested
that the enzyme phosphorylation mechanism remained intact. This experi-
ment used the endogenous protein kinase. If this enzyme was rendered
defective by adrenalectomy, no inactivation by glucagon of pyruvate kinase
should have been evident in these rats. Previous studies have shown
that adrenalectomy did not alter the hepatic accumulation of cyclic AMP
in response to glucagon (Exton et al, 1972;  Wicks, 1974;  Section 1.4.4).
It is possible that corticosterone could change the relative sensitivities
of these various hepatic pathways to glucagon. Thus the steroid-
dependence for the glucagon stimulation of hepatic glycogenolysis in
perfused liver could be overcome by increasing the glucagon concentration
in the perfusate (Exton et al, 1972). The experiments presented in this
chapter used a fixed, maximal concentration of glucagon (1mg/kg). The
in vivo response to glucagon would also have been affected by the glucagon
stimulation of insulin secretion (Section 1.4.2). Any analysis of the
effect of glucagon concentration on the inhibition of hepatic lipo-
genesis in adrenalectomized rats should ideally be performed in perfused
liver or isolated hepatocytes. In these preparations, the metabolic
responses to defined concentrations of single hormones can be studied.

The lack of pyruvate kinase inhibition by glucagon after adrenal-
ectomy suggests a possible cause for the different kinetic properties of
pyruvate kinase in these rats. The activity and phosphorylation state
of L-type pyruvate kinase in control rats is the resultant of the balance
between insulin and glucagon. For instance, even the enzyme from control
fed rats was partially phosphorylated (Pilkis et al, 1980), despite the
elevated insulin : glucagon ratio. If L-type pyruvate kinase from
adrenalectomized rats did not respond to glucagon, then the enzyme would
be activated due to the action of insulin. A result similar to that in
Table 23 might be expected, where the Vmax :   0.5mM-PEP ratio of con-
trol rats was $13.8\pm0.3$ and that for adrenalectomized rats was $9.4\pm1.3$.

In summary, these results suggest that the major action of glucagon
in the inhibition of hepatic fatty acid biosynthesis was neither by
depletion of hepatic glycogen nor by inhibition of hepatic acetyl CoA
carboxylase. The inhibition by glucagon of hepatic pyruvate kinase was

one possible mechanism contributing to the limitation of lipogenesis.
This inference was supported by the observation that corticosterone
was essential for the glucagon inhibition of both hepatic fatty acid
biosynthesis and pyruvate kinase activity. L-type pyruvate kinase
could be the reaction which confers a permissive role for glucocorticoid
upon the regulation of gluconeogenesis, ketogenesis and lipogenesis in
the liver. This enzyme is a potential site for the integrated regulation
of all three hepatic pathways.

CHAPTER 6

SUMMARY AND PERSPECTIVE

The studies presented in this thesis demonstrated that hepatic glycogen synthesis could be quantified by the incorporation of tritium from tritiated water _in vivo_. Substantial tritium incorporation into glycogen was only observed during periods of active glycogen deposition in both control and diabetic rats. The extent of glycogen recycling in rat liver was analysed by comparing the tritium incorporation with the change of glycogen concentration over the same 1h period. The results indicated that the rate of any such recycling was low in control rats after +5h. The ratio of tritium incorporated : glycogen glycosyl moeities formed was relatively constant (2.87). This ratio was not greatly affected by feeding diets with a high content of glucose or fructose, or by streptozotocin diabetes. Positional analysis showed that, under all conditions measured, there were effectively 2 full tritiations per glycosyl moeity incorporated, one each at glycosyl carbon numbers 2 and 5. The combination of a lack of glycogen recycling and the constant proportional tritium incorporation suggested that this method provided valid estimates of hepatic glycogen synthesis.

The feeding response of control rats was divided into 2 phases on the basis of tritium incorporation into glycogen from tritiated water. During the first 5h after meal-feeding, hepatic glycogen synthesis was active and thus hepatic glycogen could not have been a net precursor for any other pathway (Ma et al, 1978). From +5h onwards, however, hepatic glycogen concentration was high and the rate of tritium incorporation low. These conditions would allow glycogen to be a substrate for other pathways.

Hepatic fatty acid biosynthesis was measured in the same liver samples, also by incorporation of tritium from tritiated water. This simultaneous measurement permitted certain deductions to be formulated about the role of hepatic glycogen as a potential fatty acid precursor. Such a net conversion would not have been possible during the first 5h of feeding. A corollary of this argument is that depletion of hepatic

glycogen by glucagon within this 5h period could not have directly inhibited hepatic lipogenesis. One condition where hepatic glycogen reserves appeared to be necessary for lipogenic stimulation was insulin treatment of streptozotocin diabetic rats. The depressed fed rate of hepatic fatty acid biosynthesis was only restored by insulin in these rats after glycogen synthesis was normal.

Evidence was provided that hepatic fatty acid biosynthesis was stimulated on feeding by a mechanism involving increased supply of substrate acetyl CoA and by activation of acetyl CoA carboxylase. The stimulation of active acetyl CoA carboxylase in control rats at +2h coincided with the maximum rate of lipogenesis. The rate of active acetyl CoA carboxylase was too great to account for the low lipogenic rates measured in fasted rats. Further evidence that substrate limitation might limit flux through this enzyme in vivo was provided by diabetic rats. In diabetic rats, either untreated or insulin-treated, the feeding stimulus of hepatic lipogenesis did not involve activation of acetyl CoA carboxylase.

The mechanism of glucagon inhibition of lipogenesis was investigated during the initial response to feeding. The responses of a variety of hepatic pathways are dependent on the presence of glucocorticoid hormones. Such a "permissive" role for corticosterone was indicated in the regulation of hepatic fatty acid biosynthesis. The glucagon inhibition of hepatic lipogenesis was abolished by adrenalectomy but not potentiated by elevated corticosterone, caused by corticotropin treatment.

No conclusive evidence was obtained for in vivo inhibition of acetyl CoA carboxylase by glucagon. Such a response was observed in corticotropin-treated rats but not in control rats. By contrast, hepatic pyruvate kinase activity was inhibited by glucagon in both control and corticotropin-treated rats. L-type pyruvate kinase was stimulated under meal-feeding conditions of elevated hepatic lipogenesis (Hopkirk and Bloxham, 1979). The combined inactivations of L-pyruvate kinase and pyruvate dehydrogenase would be expected to divert substrate pyruvate away from fatty acid synthesis towards gluconeogenesis. Results from adrenalectomized rats strengthened the concept that glucagon partially

inhibits lipogenesis by decreasing the supply of substrate acetyl CoA. Glucagon caused no inhibition of hepatic pyruvate kinase in adrenal-ectomized rats at in vivo concentrations of phosphoenol pyruvate. Thus the normal hepatic responses to glucagon of both lipogenesis and pyruvate kinase were abolished by adrenalectomy. The mechanism behind the lack of glucagon response was unclear as hepatic pyruvate kinase from adrenalectomized rats could be inactivated by in vitro incubation of liver homogenate with cyclic AMP.

The regulation of hepatic pyruvate kinase could provide a common mechanism for the integrated regulation and steroid-dependence of the pathways of fatty acid synthesis, gluconeogenesis and ketogenesis. Glucagon administration to intact rats would, by inhibiting L-pyruvate kinase, decrease the phosphoenol pyruvate : pyruvate substrate cycling. Gluconeogenesis would be stimulated and fatty acid synthesis inhibited. Ketogenesis would be stimulated by increased fatty acid concentration presented to the liver and by decreased malonyl CoA concentration. The absence of L-pyruvate kinase inhibition by glucagon after adrenalectomy would tend to decrease all these subsequent responses.

There are a number of further experiments which could provide additional evidence concerning the permissive steroid dependence of glucagon action. It is possible that the steroid effect would be most notable at low glucagon concentrations. For instance, although adrenal-ectomy impaired the glycogenolytic response to glucagon in perfused liver, this inhibition was overcome by increasing the hormone concentration (Exton et al, 1972). The dose-response relationship for glucagon inhi-bition of fatty acid synthesis and pyruvate kinase activity would be most suitably studied in replicate cultures of hepatocytes prepared from either control or adrenalectomized rats. Such a model system would also be required to study the mechanisms behind the defective regulation of pyruvate kinase after adrenalectomy. Incorporation of $^{32}$Pi into the enzyme in hepatocytes, followed by precipitation with a specific anti-body, could help distinguish between different possible mechanisms. For instance, lack of inhibition of pyruvate kinase could result from either defective stimulation of protein kinase or by altered inhibition of phosphoprotein phosphatase 1.

Previous attempts to study glycogen synthesis in isolated hepatocytes have been hindered by the relative insensitivity of this model. Appreciable rates of glycogen synthesis have only been achieved using high substrate concentrations or glutamine supplements (Katz et al, 1979). Hepatocytes should be able to synthesize glycogen in tritiated water from a variety of single substrates. In the absence of exogenous glucose, the rate of substrate cycling between glucose and glucose-6-phosphate should be low. Under these conditions, incorporation of tritium into glycosyl positions other than C-2 and C-5 should be preserved in the glycogen molecule. Other potential applications of this method are to measure rates of glycogen synthesis in leucocyte preparations and, by using deuterated water, to evaluate the proportion of glycogen synthesized via different metabolic pathways.

## REFERENCES

Abraham, S., Katz, J., Bartley, J., & Chaikoff, I.L. (1963) Biochim. Biophys. Acta 70, 690.

Alberts, A.W., Gordon, S.G., & Vagelos, P.R. (1971) Proc. Nat. Acad. Sci. (USA) 68, 1259.

Alberty, R.W., Miller, W., & Fisher, H. (1957) J.A.C.S. 79, 3973.

Allred, J.B., & Roehrig, K.L. (1973) J. Biol. Chem. 248, 4131.

Andrews, P., Hough, L., & Picken, J.M. (1965) Biochem. J. 94, 75.

Antinow, J.F., Nimmo, H.G., Yeaman, S.J., and Cohen, P. (1977) Biochem. J. 162, 423.

Atkinson, D.E. (1970) Curr. Top. Cell. Reg. 1, 29.

Babcock, M.B., & Cardell, R.R. (1974) Am. J. Anat. 140, 299.

Baker, N., Learn, D.B., & Bruckdorfer, K.R. (1978) J. Lip. Res. 19, 879.

Barritt, G., Zander, G.L., & Utter, M.F. (1976) in "Gluconeogenesis: Its Regulation in Mammalian Species" (ed. Hanson, R.W., & Mehlman, M.L.) p. 3, Wiley, New York.

Beale, E.G., Dedman, J.R., Means, A.R. (1977) J. Biol. Chem. 252, 6322.

Beavo, J.A., Bechtel, P.J., & Krebs, E.G. (1974) Proc. Nat. Acad. Sci. 71, 3580.

Bell, J.J., Sargeant, T.E., & Watson, J.A. (1976) J. Biol. Chem. 251, 1745.

Bergman, H.C., & Klein, D. (1943) Endocrinol 33, 174.

Bernard, C. (1859) Compt. Rend. 48, 77.

Berthet, J. (1960) Proc. 4th Int. Congr. Biochem. 17, 107.

Berthet, J., Jacques, P., Hers, H.G., & de Duve, C. (1956) Biochim. Biophys. Acta 20, 190.

Beynan, A.C., Vaartjes, W.J., & Geelan, M.J.H. (1979) Diabetes 28, 825.

Bittner, R., Böhme, H.J., Didt, L., Goltzsch, W., Hoffmann, E., Levin, M.J., & Sparmann, G. (1978) Adv. Enz. Reg. 17, 37.

Blackmore, P.F., Assimacoupolos-Jeannet, F., Chan, T.M., & Exton, J.H. (1979) J. Biol. Chem. 254, 2828.

Blackmore, P.F., Dehaye, J.P., & Exton, J.H. (1979) J. Biol. Chem. 254, 6945.

Bloom, B. (1962) Anal. Biochem. 3, 85.

Bloxham, D.P., Clark, M.G., Holland, P.C., & Lardy, H.A. (1973a) Biochem. J. 134, 581.

Bloxham, D.P., Clark, M.G., Goldberg, D.M., Holland, P.C., & Lardy, H.A. (1973b) Biochem. J. 134, 586.

Bloxham, D.P., Fitzsimmons, J.T.P., & York, D.A. (1977) Horm. Metab. Res. 9, 304.

Bloxham, D.P., & Lardy, H.A. (1973) in "The Enzymes" (ed. Boyer, P.) Vol. 8, p. 240, Academic Press, New York.

Bohmer, J. (1967) Biochim. Biophys. Acta 144, 259.

Borner, K., & Mattenheimer, H. (1959) Biochim. Biophys. Acta 34, 592.

Bortz, W.M., & Lynen, F. (1963) Biochem. Z. 339, 77.

Bouillon, D.J., & Bendanier, C.D. (1980) J. Nutr. 110, 286.

Boxer, G.E., & Stetten, D. (1944) J. Biol. Chem. 155, 237.

Brady, R.O., & Gurin, S. (1952) J. Biol. Chem. 199, 421.

Brandt, H., Capulong, Z.L., & Lee E.Y.C. (1975) J. Biol. Chem. 250, 8038.

Brandt, H., Killilea, S.D., & Lee, E.Y.C. (1974) Biochem. Biophys. Res. Comm. 68, 45.

Bremer, J., Christiansen, R.Z., & Borrebæk, B. (1977) in "Regulatory Mechanisms of Carbohydrate Metabolism" (ed. Esmann, V.) p. 161, Pergamon, Oxford.

Brosnan, J.T., Kopec, B., & Fritz, I.B. (1973) J. Biol. Chem. 248, 4075.

Brown, P.C., & Papaconstantinou, J. (1979) J. Biol. Chem. 254, 5177.

Brunengraber, H., Boutry, M., & Lowenstein, J.M. (1973) J. Biol. Chem. 248, 2656.

Bücher, T., & Pfleiderer, G. (1955) Methods in Enzymology 1, 435.

Burton, O.N., Haavik, A.G., & Porter, J.W. (1968) Arch. Biochem. Biophys. 126, 141.

Cahill, G.F., Ashmore, J., Earle, A.S., & Zottu, S. (1958) Am. J. Physiol. 192, 491.

Carlson, S.E., & Arnrich, L. (1978) J. Nutr. 108, 1162.

Carlson, C.A., & Kim, K.H. (1973) J. Biol. Chem. 248, 378.

Carlson, C.A., & Kim, K.H. (1974) Arch. Biochem. Biophys. 164, 478.

Carlson, C.A., & Kim, K.H. (1974) Arch. Biochem. Biophys. 164, 490.

Chan, T.M., Blackmore, P.F., Steiner, P.F., & Exton, J.H. (1979) J. Biol. Chem. 254, 2428.

Chance, B., Holmes, W., Higgins, J.J., & Connelly, C.M. (1958) Nature, Lond. 182, 1190.

Chernick, S.S., Clark, C.M., Gardiner, R.J. & Scow, R.O. (1972) Diabetes 21, 946.

Chrisman, T.D. & Exton, J.H. (1980) J. Biol. Chem. 255, 3270.

Cladaras, C., & Cottam, G.L. (1980) Arch. Biochem. Biophys. 200, 426.

Clark, D.G., Rognstad, R., & Katz, J. (1974) J. Biol. Chem. 249, 2048.

Clark, D.G., Storer, G.B., & Topping, D.L. (1980) Biochem. Biophys. Res. Comm. 93, 155.

Clark, M.G., Boxham, D.P., Holland, P.C., & Lardy, H.A. (1974) J. Biol. Chem. 249, 279.

Clarke, S.D., Watkins, P.A., & Lane, M.D. (1980) J. Lip. Res. 20, 974.

Cohen, P. (1973) Eur. J. Biochem. 34, 1.

Cohen, P. (1978) Curr. Top. Cell. Reg. 14, 118.

Cohen, P., Burchell, A., Foulkes, G., Cohen, P.T.W., Vanaman, T.C., & Nairn, A.C. (1978) FEBS Lett 92, 287.

Cohen, P., Rylatt, D.B., & Nimmo, G.A. (1977) FEBS Lett 76, 182.

Cohn, C., & Joseph D. (1970) J. Nutr. 100, 78.

Cook, G.A., King, M.T., & Veech, R.L. (1978) J. Biol. Chem. 253, 2529.

Cook, G.A., Nielson, R.C., Hawkins, R.A., Mehlman, M.A., Lakshmanan, M.R., & Veech, R.L. (1977) J. Biol. Chem. 252, 4421.

Corbin, J.D., Keely, S.L., & Park, C.R. (1975) J. Biol. Chem. 250, 218.

Corbin, J.D., & Lincoln, T.M. (1977) Adv. Cyclic Neucl. Res. 9, 159.

Craig, M.C., Neprokroeff, C.M., Lakshmanan, M.R., & Porter, J.W. (1972) Arch. Biochem. Biophys. 152, 619.

Cuatrecasas, P., & Hollenberg, M.D. (1976) Adv. Prot. Chem. 30, 251.

D'Adamo, A.F., Hoberman, H.D., & Haft, D. (1961) Fed. Proc. 20, 274.

Das, M., & Fox, F.C. (1978) Proceed. Nat. Acad. Sci. (USA) 75, 2644.

Decker, K. (1974) in "Methods of Enzymatic Analysis" (ed. Bergmeyer, H.U.) p. 1988, Academic Press, New York.

Dedman, J.R., Potter, J.D., & Means, A.R. (1977) J. Biol. Chem. 252, 2437.

Demaille, J.G., Peters, K.A., & Fischer, E.H. (1977) Biochemistry 16, 3080.

Denton, R.M., Randle, P.J., Bridges, B.J., Cooper, R.H., Kerbey, A.L., Pask, H.T., Severson, D.L., Stansbie, D., & Whitehouse, S. (1975) Mol. Cell. Biochem. 9, 27.

DiMarco, J.P., & Hoppel, C. (1975) J. Clin. Invest. 55, 1237.

Devos, P., & Hers, H.G. (1974) Biochem. J. 140, 331.

Devos, P., & Hers, H.G. (1979) Eur. J. Biochem. 99, 161.

De Wulf, H., & Hers, H.G. (1967) Eur. J. Biochem. 2, 50.

De Wulf, H., & Hers, H.G. (1968a) Eur. J. Biochem. 6, 545.

De Wulf, H., & Hers, H.G. (1968b) Eur. J. Biochem. 6, 558.

Diamant, S., & Shafrir, E. (1975) Eur. J. Biochem. 53, 541.

Dimroth, P., Guchait, R.B., Stoll, E., & Lane, M.D. (1970) Proc. Nat. Acad. Sci. (USA) 67, 1353.

Dinovo, E., & Boyer, P. (1971) J. Biol. Chem. 246, 4580.

Dopère, F., Vanstapel, F., & Stalmans, W. (1980) Eur. J. Biochem. 104, 137.

Dorsey, T.A., & Porter, J.W. (1968) J. Biol. Chem. 243, 3572.

Drochmans, P. (1962) J. Ultrastructure Res. 6, 141.

Edwards, P.A., Lemongello, D., Kane, J., Shector, I., & Fogelman, A.M. (1980) J. Biol. Chem. 255, 3715.

El-Maghrabi, M., Haston, W.S., Flockhart, D.A., Claus, T.H., & Pilkis, S.J. (1980) J. Biol. Chem. 255, 668.

Embi, N., Rylatt, D.B., & Cohen, P. (1979) Eur. J. Biochem. 100, 339.

Engström, L. (1978) Curr. Top. Cell. Reg. 13, 29.

Exton, J.H., Lewis, S.B., Ho, R.J., Robinson, G.A., & Park, C.R. (1971) Ann. N.Y. Acad. Sci. 185, 85.

Exton, J.H., Miller, T.B., Harper, S.C., & Park, C.R. (1976) Am. J. Physiol. 230, 163.

Exton, J.H., & Park, C.R. (1967) J. Biol. Chem. 242, 2622.

Exton, J.H., & Park, C.R. (1972) in "Handbook of Physiology" (ed. Steiner, D., & Freinkel, N.) 7, Vol. 1, p. 437 Am. Phys. Soc., Washington, D.C.

Exton, J.H., Friedmann, N., Wong, E.H.A., Brineaux, J.P., Corbin, J.P., & Park, C.R. (1972) J. Biol. Chem. 247, 3579.

Fahmy, D., Read, G.F., & Hillier, S.G. (1975) Steroids, 26, 175.

Fain, J.N. (1974) in "Biochemitry of Hormones" (ed. Rickenberg, H.G.) p. 1, M.T.P. & Butterworth, London.

Fain, J.N. (1967) Adv. Enz. Reg. 5, 39.

Fain, J.N., & Czech, M.P. (1975) in "Handbook of Physiology" (ed. Green, R.O., & Astwood, E.B.) 7, Vol. 6, p. 169 Am. Phys. Soc., Washington D.C.

Fall, R.R., & Vagelos, P.R. (1972) J. Biol. Chem. 247, 8005.

Flatt, J.P., & Ball, E.G. (1966) J. Biol. Chem. 241, 2862.

Florey, W., Peczon, B.D., Koeppe, R.E. & Spivey, H.O. (1974) Biochem. J. 141, 127.

Foster, D.W., & Bloom, B. (1963) J. Biol. Chem. 238, 888.

Foster, D.W., & Srere, P.A. (1968) J. Biol. Chem. 243, 1926.

Foulkes, J.G., Jefferson, L.S., & Cohen, P. (1980) FEBS Lett. 112, 21.

Friedmann, N., Exton, J.H., & Park, C.R. (1967) Biochem. Biophys. Res. Comm. 29, 113.

Friedmann, N., & Wertheimer, H.E. (1966) Metabolism 15, 225.

Fritz, I.B. in "Biochemical Actions of Hormones" (ed. Litwack, G.) p. 166, Academic Press, New York.

Fritz, I.B., & Lee, P.K. (1972) in "Handbook of Physiology" (ed. Steiner, D., & Freinkel, N.) 7, Vol. 1, p. 579 Am. Phys. Soc., Washington D.C.

Geelan, M.J.H., Beynan, A.C., Christiansen, R.J., & Gibson, D.M. (1978) FEBS Lett. 95, 326.

Gibbons, G.F., & Pullinger, C.R. (1979) Biochem. J. 177, 255.

Gibson, D.M., Lyons, R.T., Scott, D.F., & Muto, Y. (1972) Adv. Enz. Reg. 10, 187.

Gilbert, M., & Bourbon, J. (1978) Biochem. J. 176, 785.

Gill, G.N., Holdy, K.E., Walton, G.M., Komstein, C.B. (1976) Proc. Nat. Acad. Sci. 73, 3918.

Gill, G.N., & McCune, R.W. (1979) Curr. Top. Cell. Reg. 15, 1.

Glenny, H.P., & Brindley, D.N. (1978) Biochem. J. 176, 777.

Glinsmann, W.H., & Mortimore, G.E. (1968) Am. J. Physiol. 215, 553.

Goldhammer, A.R., & Paradies, H.H. (1979) Curr. Top. Cell. Reg. 15, 109.

Goldwater, W.H., & Stetten, D. (1947) J. Biol. Chem. 169, 723.

Goodridge, A.G. (1972) J. Biol. Chem. 247, 6946.

Goris, J., Kalala, L.R., Merlevede, W. (1974) Arch. Int. Physiol. Biochem. 82, 598.

Granner, D.K., Tompson, E.B., & Tompkins, G.M. (1970) J. Biol. Chem. 245, 1472.

Greenbaum, A.L., Gumaa, K.A., & McLean, P. (1970) Arch. Biochem. Biophys. 143, 617.

Grim, E. (1963) in "Handbook of Physiology" (ed. Hamilton, W.F.) 2, Vol. 2, p. 1439 Am. Phys. Soc., Washington, D.C.

Guder, W.G., Schmidt, U., Funk, B., Weis, J., & Pürschel, S. (1976) Z. Physiol. Chem. 357, 1793.

Gunn, J.M., Hanson, R.W., Meyuhlas, O., Reshef, L., & Ballard, F.J. (1975) Biochem. J. 150, 195.

Guynn, R.W., Veloso, D., & Veech, R.L. (1972) J. Biol. Chem. 247, 7325.

Halestrap, P. (1978) Biochem. J. 172, 377.

Halestrap, A.P., & Denton, R.M. (1973) Biochem. J. 132, 509.

Halperin, M.L., Robinson, B.H., & Fritz, I.B. (1972) Proc. Nat. Acad. Sci. (USA) 69, 1003.

Hansen, J., Dinovo, E., & Boyer, P. (1969) J. Biol. Chem. 244, 6270.

Hardie, D.G., & Cohen, P. (1978) Eur. J. Biochem. 92, 25.

Hardie, D.G., & Cohen, P. (1978) FEBS Lett. 91, 1.


Heimberg, M., Wilcox, H.G., Dunn, D.G., Woodside, W.F., Breen, K.J., & Soler-Argilaga, S. (1974) in "Regulation of Hepatic Metabolism" (ed. Lundquist, F., & Tygstrup, N.) p. 119 Munksgaard, Copenhagen.

Hems, D.A., Whitton, P.D., & Taylor, E.A. (1972) Biochem. J. 129, 529.

Hems, D.A., Rath, E.A., & Verrinder, T.R. (1975) Biochem. J. 150, 167.

Hers, H.G. (1957) "Le Metabolisme du Fructose" Editions Arscia, Bruxelles.

Hers, H.G. (1976) Ann. Rev. Biochem. 45, 167.

Hers, H.G., De Wulf, H., & Stalmans, W. (1970) FEBS Lett. 12, 73.

Higgins, M.J.P., & Rudney, H. (1973) Nature (New Biol.) 246, 60.

Higgins, M.J.P., Brady, D., & Rudney, H. (1974) Arch. Biochem. Biophys. 163, 271.

Hopkins, H.A., Bonney, R.J., Walker, P.R., Yager, J.D., & Potter, V.R. (1972) Adv. Enz. Reg. 11, 169.

Hopkirk, T.J., & Bloxham, D.P. (1979) Biochem. J. 182, 383.

Horecker, B.L., Tsolas, O., & Lai, C.Y. (1972) in "The Enzymes" (ed. Boyer, P.) Vol. 7, 213  Academic Press, New York.

Hornbrook, K.R., Burch, H.B., & Lowry, O.H. (1966) Mol. Pharmacol. 2, 106.

Huang, F.L., & Glinsmann, W.H. (1976) FEBS Lett. 62, 326.

Huang, K.P., Lee, S.H., & Huang, F.L. (1979) J. Biol. Chem. 254, 9867.

Hue, L., Bontemps, F., & Hers, H.G. (1975) Biochem. J. 152, 105.

Hue, L., & Hers, H.G. (1974) Biochem. Biophys. Res. Comm. 58, 532.

Illiano, G., & Cuatrecasas, P. (1972) Science 175, 906.

Illiano, G., Tell, G.P.E., Siegel, M.J., & Cuatrecasas, P. (1973) Proc. Nat. Acad. Sci. (USA) 70, 2443.

Ingebritsen, T.S., Lee, H.S., Parker, R.A., & Gibson, D.M. (1978) Biochem. Biophys. Res. Comm. 81, 1268.

Ingle, D.J. (1951) Recent Prog. Horm. Res. 6, 159.

Inoue, H., Casper, C.B., & Pitot, H.C. (1971) J. Biol. Chem. 246, 2626.

Inoue, H., & Lowenstein, J.M. (1972) J. Biol. Chem. 247, 4825.

Ip, M., Ip, C., Tepperman, H., & Tepperman, J. (1977) J. Nutr. 107, 746.

Issekutz, B. (1977) Metabolism 26, 157.

Itarte, E., Robinson, J.C., & Huang, K.P. (1977) J. Biol. Chem. 252, 1231

Iyendjian, P.B., Kioussis, D., Garcia-Ruiz, J.P., & Hanson, R.W. (1977) in "Regulatory Mechanisms of Carbohydrate Metabolism" (ed. Esmann, V) p. 83, Pergamon, Oxford.

Jacquot, R. (1959) J. Physiol. (Paris) 51, 655.

Janoski, A.H., Shulman, F.C., & Wright, G.E. (1974) Steroids 23, 49.

Jefferson, L.S., Exton, J.H., Butcher, R.W., Sutherland, E.W., & Park, C.R. (1968) J. Biol. Chem. 243, 1031.

Jett, M.F., & Soderling, T. (1979) J. Biol. Chem. 254, 6739.

Johnston, D.G., Postle, A.D., Barnes, A.J., & Alberti, K.G.M.M. (1979) in "Lipoprotein Metabolism and Endocrine Regulation" (ed. Hessel, L.W., & Krans, H.M.J.) p. 117, Elsevier, Amsterdam.

Jungas, R.L. (1968) Biochemistry 7, 3708.

Kaplan, N.O. (1960) in "The Enzymes" (ed. Boyer, P.D., Lardy, H., & Myrback, K.) Vol. 3, p. 105, Academic Press, New York.

Karlson, P. (1963) Perspect. Biol. Med. 6, 203.

Katz, J., Golden, S., & Wals, P.A. (1976) Proc. Nat. Acad. Sci. 73, 3433.

Katz, J., Golden, S., & Wals, P.A. (1979) Biochem. J. 180, 389.

Katz, J., & Rognstad, R. (1976) Curr. Top. Cell. Reg. 10, 237.

Kawai, A., & Kuzuya N. (1977) Horm. Metab. Res. 9, 361.

Keppler, D., & Decker, K. (1974) in "Methods of Enzymatic Analysis" (ed. Bergmeyer, H.U.) p. 1127, Academic Press, New York.

Khandelwal, R.L., Vanderheede, J.R., & Krebs, E.G. (1976) J. Biol. Chem. 251, 4850.

Khoo, J.C., Sperry, P.J., Gill, G.N., & Steinberg, D. (1977) Proc. Nat. Acad. Sci. (USA) 74, 4843.

Kirk, C.J., Verrinder, T.R., & Hems, D.A. (1976) Biochem. J. 156, 593.

Klain, G.J., & Weiser, P.C. (1973) Biochem. Biophys. Res. Comm. 55, 76.

Kneer, N.M., Wagner, M.J., & Lardy, H.A. (1979) J. Biol. Chem. 254, 12160.

Knowles, J.R., Leadley, P.F., & Maister, S.G. (1971) Cold Spring Harbour Quant. Biol. 36, 157.

Kohl, E.A., & Cottam, G.L. (1977) Biochim. Biophys. Acta, 484, 49.

Koshland, D.E., Nemethy, G., & Filmer, D. (1966) Biochemistry, 5, 365.

Krebs, E.G. (1972) Curr. Top. Cell. Reg. 5, 99.

Krebs, E.G., & Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923.

Krebs, H.A. (1966) Adv. Enz. Reg. 4, 339.

Krebs, H.A. (1972) Adv. Enz. Reg. 10, 397.

Krotkiewski, M., Krotkiewski, J., & Björntorp, P. (1970) Acta Endocrinol 63, 185.

L'Age, M., Fechner, W., Langholz, J., & Salzmann, H. (1974) Diabetologia 10, 131.

Lakshmanan, M.R., & Veech, R.L. (1977) J. Biol. Chem. 252, 4667.

Laloux, M., & Hers, H.G. (1979) FEBS Lett. 105, 239.

Landau, B.R., Leonards, J.R., & Barry, F.M. (1961) Am. J. Physiol. 201, 41.

Lederer, B., Van Hoof, F., Van den Berghe, G., & Hers, H.G. (1975) Biochem. J. 147, 23.

Lee, K.L., Reel, J., & Kenney, F.T. (1970) J. Biol. Chem. 245, 5806.

Lent, B.A., Lee, K.H., & Kim, K.H. (1978) J. Biol. Chem. 253, 8149.

Le Febre, P.J., & Unger, R.H. (1972) "Glucagon, molecular physiology, chemical and theraputic implications" Elmsford, N.Y.

Leveille, G.A. (1970) Fed. Proc. 29, 1294.

Levine, R., & Fritz, I.B. (1956) Diabetes 2, 209.

Levitan, I.B., & Webb, T.E. (1970) J. Mol. Biol. 48, 339.

Lin, D.C., & Segal H.L. (1973) J. Biol. Chem. 248, 7007.

Lincoln, T.M., & Corbin, J.D. (1977) Proc. Nat. Acad. Sci. (USA) 74, 3239.

Linder, C.L., & Migliorini R.H. (1974) Diabetes 23, 143.

Linn, T.C., Pettit, F.H., Hucho, F., & Reed, L.J. (1969) Proc. Nat. Acad. Sci. (USA) 64, 227.

Litwack, G., & Singer, S. (1972) in "Biochemical Actions of Hormones" (ed. Litwack, G.) Vol. 2, p. 113, Academic Press, New York.

Lloyd, B., Burrin, J., Smythe, P., & Alberti, K.G.M.M. (1978) Clin. Chem. 24, 1724.

Long, C.N.H., Katzin, B., & Fry, E.G. (1940) Endocrinology 26, 309.

Longshaw, I.D., Bowen, N.L., & Pogson, C.I. (1972) Eur. J. Biochem. 25, 366.

Loten, E.G., Assimacopoulis-Jeannet, F.D., Exton, J.H., & Park, C.R. (1978) J. Biol. Chem. 253, 746.

Loten, E.G., Sneyd, J.G.T. (1970) Biochem. J. 120, 187.

Lowenstein, J.M. (1971) J. Biol. Chem. 246, 629.

Lowry, O H., Rosborough, N.J., Farr, A.L., & Randall, R.J. (1951) J. Biol. Chem. 193, 265.

Lunzer, M.A., Manning, J.A., & Ockner, R.K. (1977) J. Biol. Chem. 252, 5483.

Ma, G.A., Gove, C.D., & Hems, D.A. (1978) Biochem. J. 174, 761.

Macdonald, D.W.R., & Saggerson E.D. (1978) Biochem. J. 174, 641.

McDonough, M., & Wood, W. (1961) J. Biol. Chem. 236, 1220.

McGarry, J.D., & Foster, D.W. (1974) J. Biol. Chem. 249, 7984.

McGarry, J.D., & Foster, D.W. (1977) Arch. Int. Med. 137, 495.

McGarry, J.D., & Foster, D.W. (1979) J. Biol. Chem. 254, 8163.

McGarry, J.D., Mannaerts, G.P., & Foster, D.W. (1977) J. Clin. Invest. 60, 265.

McGarry, J.D., Takabayashi, Y., & Foster, D.W. (1978) J. Biol. Chem. 253, 8294.

McGarry, J.D., Wright, P.H., & Foster, D.W. (1975) J. Clin. Invest. 55, 1202.

McVerry, P.H., & Kim, K.H. (1974) Biochemistry 13, 3505.

Madvig, P., & Abraham, S. (1980) J. Nutr. 110, 90.

Majerus, P.W., Alberts, A.W., & Vagelos, P.R. (1965) Proc. Nat. Acad. Sci. (USA) 53, 410.

Majerus, P.W., & Kilburn, E. (1969) J. Biol. Chem. 244, 6254.

Malaisse, W.J., Malaisse-Lagae, F., & Mayhew, D. (1967) J. Clin. Invest. 46, 1724.

Martial, J.A., Seeburg, P.H., Guenzi, D., Goodman, H.M., & Baxter, J.D. (1977) Proc. Nat. Acad. Sci. (USA) 74, 4293.

Martin, D.B., Horning, M.G., & Vagelos, P.R. (1961) J. Biol. Chem. 236, 663.

Mersmann, H.J., & Segal, H.L. (1969) J. Biol. Chem. 244, 1701.

Meyer, W.L., Fischer, E.H., & Krebs, E.G. (1964) Biochemistry 3, 1033.

Miller, J.B., Exton, J.H., & Park, C.R. (1971) J. Biol. Chem. 246, 3672

Miller, A.L., & Levy, H.R. (1975) Methods in Enzymology 35, 11.

Monod, J., Wyman, J., & Changeux, J.P., (1965) J. Mol. Biol. 12, 88.

Munck, A. (1971) Perspect. Biol. Med. 14, 265.

Nakanishi, S., & Numa, S. (1970) Eur. J. Biochem. 16, 161.

Newsholme, E.A., & Crabtree, B. (1977) in "Regulatory Mechanisms of Carbohydrate Metabolism" (ed. Esmann, V.) p. 285, Pergamon, Oxford.

Newsholme, E.A., & Geves, W. (1967) Vitamins & Hormones, 25, 1.

Newsholme, E.A., & Start, C. (1973) "Regulation in Metabolism", Wiley, London & New York.

Newsholme, E.A., & Underwood, A.H. (1966) Biochem. J. 99, 24c.

Nichols, W.K., & Goldberg, N.D. (1972) Biochim. Biophys. Acta, 279, 245

Niemeyer, H., Clark-Turri, L., Perez, N., & Rabajille (1965) Arch. Biochem. Biophys. 109, 634.

Nieto, A., & Castaño, J.G. (1980) Biochem. J. 186, 953.

Nimmo, G.A., & Cohen, P. (1978) Eur. J. Biochem. 87, 341.

Nordlie, R.C. (1974) Curr. Top. Cell. Reg. 8, 33.

Numa, S., Bortz, W.M., & Lynen, F. (1965) Adv. Enz. Reg. 3, 407.

Numa, S., Ringelman, E., & Lynen, F. (1964) Biochem. Z 340, 228.

Numa, S., & Yamashita, S. (1974) Curr. Top. Cell. Reg. 8, 197.

Nyfeler, F., & Walter, P. (1979) FEBS Lett. 108, 197.

Ochs, R.S., & Harris, R.A. (1978) Arch. Biochem. Biophys. 190, 193.

Ogiwara, H., Tanaka, T., Nikawa, J., & Numa, S. (1978) Eur. J. Biochem. 89, 37.

Owen, O.E., Felig, P., Morgan, A.P., Wahren, J., & Cahill, G.F. (1969) J. Clin. Invest. 48, 574.

Owen, O.E., & Reichard, G.A. (1971) Progr. Biochem. Pharmacol. 6, 177.

Pabst, M.L., Sheppard, R., & Kuizenga, M.H. (1947) Endocrinol. 41, 55.

Pekala, P.H., Meredith, M.J., Tarlow, D.M., & Lane, M.D. (1977) J. Biol. Chem. 253, 5267.

Petersen, C.S., Herlin, T., & Esmann, V. (1978) Biochim. Biophys. Acta, 542, 77.

Phillipens, K.M.H., Von Mayersbach, H., & Schevig, L.E. (1977) J. Nutr. 107, 176.

Pilkis, S.J. (1970) Biochim. Biophys. Acta, 215, 461.

Pilkis, S.J. (1975) Methods in Enzymology, 42, 31.

Pilkis, S.J., El-Maghrabi, M.R., Coven, B., Claus, T.H., Tager, H.S., Steiner, D.F., Keim, P.S., & Heinrikson, R.L. (1980) J. Biol. Chem. 255, 2770.

Pires, E., Perry, S.V., & Thomas, M.A. (1974) FEBS Lett. 41, 292.

Postle, A.D., & Goodland, F.C. (1978) Anal. Clin. Biochem. 15, 18.

Proud, C.G., Rylatt, D.B., Yeaman, S.J., & Cohen, P. (1977) FEBS Lett. 80, 435.

Qureshi, A.A. (1975) Biochem. Biophys. Res. Comm. 64, 836.

Qureshi, A.A. (1975) Biochem. Biophys. Res. Comm. 66, 344.

Qureshi, A.A., Solomon, J.K., & Eichelman, B. (1978) Biochem. Biophys. Res. Comm. 83, 1191.

Rahim, Z.H.A., Lutaya, G., & Griffiths, J.R. (1979) Biochem. J. 184, 173.

Rakieten, N., Rakieten, M.L., & Nadkarni, M.V. (1963) Cancer Chemother. Rep. 29, 91.

Rall, T.W., Sutherland, E.W., & Wosilait, W.D. (1956) J. Biol. Chem. 218, 483.

Rappaport, A.M. (1960) Klin. Wschr. 38, 561.

Raskin, P., McGarry, J.G., & Foster, D.W. (1974) J. Biol. Chem. 249, 6029.

Reed, W.D., Ozand, P.T., Tildon, J.T., & Cornblath, M. (1977) Biochem. J. 164, 27.

Rémésey, C., Demigné, C., & Aufrère, J. (1978) Biochem. J. 170, 321.

Renner, P., Kemmler, W., & Hepp, D. (1974) Eur. J. Biochem. 49, 129.

Rétey, J., & Lynen, F. (1965) Biochem. Z. 342, 256.

Richards, . (1970) in "The Enzymes" (ed. P. Boyer) Vol. 2, p. 321. Academic Press, New York.

Rieder, S.V., & Rose, I.A. (1959) J. Biol. Chem. 234, 1007.

Riou, J.P., Claus, T.H., Flockhart, D.A., Corbin, J.D., & Pilkis, S.J. (1977) Proc. Nat. Acad. Sci. (USA) 74, 4615.

Rittenberg, D., & Schoenheimer, R. (1937) J. Biol. Chem. 121, 235.

Seth, J., & Brown, L.M. (1978) Clin. Chem. Acta, 86, 109.

Shafrir, E., & Kerpel, S. (1964) Arch. Biochem. Biophys. 105, 237.

Shafrir, E., & Ruderman, N.B. (1974) Diabetologia, 1, 731.

Sharma, C., Manjeshwar, R., & Weinhouse, S. (1964) Adv. Enz. Reg. 2, 189.

Shelley, H.J. (1961) Br. Med. Bull. 17, 143.

Sherwin, R.S., Kramer, K.J., Tobin, J.D., Insel, P.A., Lilenquist, J.E., Berman, M., & Andres, R. (1974) J. Clin. Invest. 53, 1481.

Shrago, E., Lardy, H.A., Nordlie, R.C., & Foster, D.O. (1963) J. Biol. Chem. 238, 3188.

Siess, E.A., Brocks, D.G., Lattke, H.K., & Wieland, O.H. (1977) Biochem. J. 166, 225.

Siess, E.A., Brocks, D.G., & Wieland, O.H. (1976) FEBS Lett. 69, 265.

Siess, E.A., & Wieland, O.H. (1975) FEBS Lett. 52, 226.

Silber, R.H. (1966) Methods of Biochemical Analysis, 14, 63.

Simon, E.J., & Shemin, D. (1953) J. Am. Chem. Soc. 75, 2520.

Siperstein, M.D. (1970) Curr. Top. Cell. Reg. 2, 65.

Smith, C.M., & Savage, C.R. (1980) Biochem. J. 188, 175.

Skuster, J.R., & Graves, D.J., Procs. FEBS Meet 11th, 1977 Abstract A1-6, 060.

Soderling, T.R., Srivastava, A.K., Bass, M.A., & Khatra, B.S. (1979) Proc. Nat. Acad. Sci. (USA) 76, 2536.

Soeldner, J.S., & Stone, D. (1965) Diabetes 14, 771.

Söling, H.D., Bernhard, G., Kuhn, H.J., & Luck, H.J. (1977) Arch. Biochem. Biophys. 182, 563.

Soskin, S., Essex, H.E., Herrick, J.F., & Mann, F.C. (1938) Am. J. Physiol. 124, 558.

Spelsberg, T.C., Steggles, A.W., & O'Malley, B.W. (1971) J. Biol. Chem. 246, 4186.

Stalmans, W. (1976) Curr. Top. Cell. Reg. 11, 51.

Stalmans, W., & Hers, H.G. (1975) Eur. J. Biochem. 54, 341.

Stalmans, W., De Wulf, H., Hue, L., & Hers, H.G. (1974) Eur. J. Biochem. 41, 127.

Stansbie, D., Brownsey, R.W., Crettaz, M., & Denton, R.M. (1976) Biochem. J. 160, 413.

Start, C., & Newsholme, E.A. (1977) Biochem. J. 107, 411.

Steele, R. (1975) in "Handbook of Physiology" (ed. Green, R.O., & Astwood, E.B.), 7, Vol. 6, p. 135. Am. Phys. Soc., Washington D.C.

Steiner, D.F., & King, J. (1964) J. Biol. Chem. 239, 1292.

Stetten, D., & Boxer, G.E. (1944) J. Biol. Chem. 155, 231.

Sturton, R.G., Pritchard, P.H., Ham, L.Y., & Brindley, D.N. (1978) Biochem. J. 174, 667.

Szmigielski, A., Guidotti, A., & Costa, E. (1977) J. Biol. Chem. 252, 3848

Taketa, K., & Pogell, B.M. (1966) J. Biol. Chem. 241, 720.

Tanake, T., Wanda, K., Okazaki, T., & Numa, S. (1975) Eur. J. Biochem. 57, 15.

Taylor, C.B., & Bailey, E. (1967) Biochem. J. 102, 32c.

Tilghman, S.M., Hanson, R.W., & Ballard, F.J. (1976) in "Gluconeogenesis: Its Regulation in Mammalian Species" (ed. Hanson, R.W., & Mehlman, M.A.) p. 47, Wiley, New York.

Titani, K., Cohen, P., Walsh, K.A., & Neurath, H. (1975) FEBS Lett. 55, 120

Tompkins, G.M., Tompson, E.B., Hayashi, S., Gelehrter, T., Granner, D.K., & Peterkofsky, B. (1967) Cold Spring Harbour Symp. Quant. Biol. 31, 349.

Tonnies, G., & Kolb, J.J. (1951) Anal. Chem. 23, 823.

Topping, D.L., & Mayes, P.A. (1976) Biochem. Soc. Trans. 4, 717.

Unger, R.H. (1972) in "Impact of Insulin on Metabolic Pathways" (ed. Shafrir, E.) p. 78, Academic Press, New York.

Ussing, H.H. (1937) Skand. Arch. Physiol. 77, 85.

Utter, M.F., & Kolenbrander, H.M. (1972) in "The Enzymes" (ed. Boyer, P.) Vol. 6, p. 117. Academic Press, New York.

Vagelos, P.R. (1974) in "Biochemistry of Lipids" (ed. Goodwin, T.W.) p. 99, M.T.P., & Butterworth, London.

Vagelos, P.R., Alberts, A.W., & Martin, D.B. (1963) J. Biol. Chem. 238, 533.

Van Berkel, T.J.C., Kruijt, J.K., Van Den Berg, G.B., & Foster, J.F. (1979) Eur. J. Biochem. 92, 553.

Van den Berg, G.B., Van Berkel, T.J.C., & Koster, J.F. (1979) FEBS Lett. 101, 289.

Van den Berghe, G., De Wulf, H., & Hers, H.G. (1970) Eur. J. Biochem. 16, 358.

Vandenheede, J.R., De Wulf, H., Merlevede W. (1979) Eur. J. Biochem. 101, 51.

Vandenheede, J.R., Khandelwal, R.L., & Krebs, E.G. (1977) J. Biol. Chem. 252, 7488.

Van de Werve, G., Van den Berghe, G., & Hers, H.G. (1974) Eur. J. Biochem. 41, 97.

Van Harken, D.R., Dixon, C.W., & Heimberg, M. (1969) J. Biol. Chem. 244, 2278.

Van Lan, V., Yamaguchi, N., Garcia, M.J., Ramey, E.R., & Penbos, J.C. (1974) Endocrinology 94, 671.

Vennesland, B., Solomon, A.K., Buchanan, J.M., & Hastings, A.B. (1942) J. Biol. Chem. 142, 379.

Villar-Palasi, C., & Larner, J. (1960) Biochim. Biophys. Acta 39, 171.

Volpe, J.J., Lyles, T.O., Roncari, D.A.K., & Vagelos, P.R. (1973) J. Biol. Chem. 248, 2502.

Walker, D.G., & Rao, S. (1964) Biochem. J. 90, 360.

Walker, P.R., & Potter, V.R. (1974) in "Chronobiology" (ed. Scheving, L.E., Halberg, F., & Pauly, J.E.) p. 17, Igaku Shoin Ltd., Tokyo.

Walsh, D.A., Perkins, J.P., & Krebs, E.G. (1968) J. Biol. Chem. 243, 3763.

Wang, J.H., Stull, J.T., Huang, T.S., & Krebs, E.G. (1976) J. Biol. Chem. 251, 4521.

Wang, J.H., & Waisman, D.M. (1979) Curr. Top. Cell. Reg. 15, 47.

Watkins, P.A., Tarlow, D.M., & Lane, M.D. (1977) Proc. Nat. Acad. Sci. (USA) 74, 1497.

Watson, J.A., & Lowenstein, J.M. (1970) J. Biol. Chem. 245, 5993.

Weinhouse, S. (1976) Curr. Top. Cell. Reg. 11, 1.

Whiting, P.H., Bowley, M., Sturton, R.G., Pritchard, P.H., Brindley, D.N., & Hawthorne, J.N. (1977) Biochem. J. 168, 147.

Whitton, P.D., & Hems, D.A. (1976) Biochem. J. 156, 585.

Wicks, W.D. (1974a) in "Biochemistry of Hormones" (ed. Rickenberg, H.V.) p. 211, M.T.P. & Butterworth, London.

Wicks, W.D. (1974b) Adv. Cyclic. Nuc. Res. 4, 335.

Wicks, W.D., Kenney, F.T., & Lee, K.L. (1969) J. Biol. Chem. 244, 6008.

Wicks, W.D., Van Wijk, R., & McKibbin, J.B. (1973) Adv. Enz. Reg. 11. 117.

Wide, L. (1969) Acta Endocrinol 63 Suppl. 142, 207.

Wieland, O.H., Patzelt, C., & Loffler, G. (1972) Eur. J. Biochem. 26, 426.

Williamson, J.R. (1976) in "Gluconeogenesis: Its Regulation in Mammalian Species" (ed. Hanson, R.W., & Mehlman, M.A.) p. 165, Wiley, New York.

Williamson, J.R., Browning, E.T., Scholz, R.A., Kreisberg, R.A., & Fritz, I.B. (1968) Diabetes, 17, 194.

Windmueller, H.G., & Spaeth, A.E. (1966) J. Biol. Chem. 241, 2891.

Wititsurwannakul, D., & Kim, K.H. (1977) J. Biol. Chem. 282, 7812.

Witters, L.A., Kowaloff, E.M., & Avruch, J. (1979) J. Biol. Chem. 254, 245.

Witters, L.A., Moriarity, D., & Martin, D.B. (1979) J. Biol. Chem. 254, 6644.

Wood, H.F., & Krebs, H.A. (1971) Biochem. J. 125, 129.

Yazawa, M., & Yagi, K. (1977) J. Biochem. 82, 287.

Yeh, L.A., Lee, K.H., & Kim, K.H. (1980) J. Biol. Chem. 255, 2308.

Young, V.R. (1970) in "Mammalian Protein Metabolism" (ed. Munro, H.N.) 4, p. 585, Academic Press, New York.

Zammit, V.A., Beis, A., & Newsholme, E.A. (1979) FEBS Lett. 103, 212.

Zinman, B., & Hollenberg, C.H. (1974) J. Biol. Chem. 249, 2182.

Zurrendonk, P.F., & Tager, J.M. (1974) Biochim. Biophys. Acta 333, 393.