REGULATION OF PYRUVATE KINASE IN ISOLATED HEPATOCYTES BY METABOLITES ARISING FROM THE GLYCOLYSIS OF FRUCTOSE AND OTHER RELATED SUBSTRATES

A Thesis submitted by

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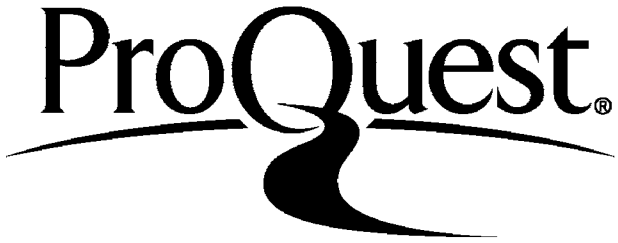

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Dedicated to

Mr. & Mrs. E.Mapungwana

my first teachers

ABSTRACT

It has been reported that fructose is rapidly converted to lactate by the liver. The only regulatory enzyme involved in this conversion is L-type pyruvate kinase. Thus the effects of fructose and other related substrates which enter the glycolytic sequence at the triose phosphate level are of physiological importance.

Incubation of isolated hepatocytes with fructose at high concentrations and with glycerol caused an apparent inhibition of pyruvate kinase. This inhibition was correlated to the depletion of ATP caused by these substrates since dihydroxyacetone and D-glyceraldehyde did not affect the enzyme activity. When hepatocytes were washed with fresh medium after the incubation, but before the extraction of enzyme, fructose, dihydroxyacetone and D-glyceraldehyde all caused stimulation whereas glycerol was without effect. This stimulation correlated closely with the increase in concentrations of fructose-1, 6-bisphosphate and/or fructose-1-phosphate within the hepatocytes.

The accumulation of inhibitor(s) in the extrahepatocyte medium in response to fructose, prevented the stimulation of the enzyme by fructose-1, 6-bisphosphate which occurs despite the large dilution of the cellular contents during the extraction and assay procedure. It was found that the accumulation of two inhibitors of pyruvate kinase could explain the effect of fructose on hepatocyte pyruvate kinase activity. These are alanine, a known inhibitor of the enzyme, and allantoin.

The regulation of the intracellular pyruvate kinase was examined by estimating glycolytic flux from the accumulation of lactate and pyruvate. The glycolytic flux from fructose exceeded that from other substrates despite the inhibition of pyruvate kinase described above.

Glycolysis from dihydroxyacetone although initially rapid, was much slower than from fructose whereas glycerol and other reduced substrates caused an inhibition of glycolysis probably as a result of an increase in the $(NADH)/(NAD^+)$ ratio. The rapid glycolysis from fructose can be explained by the accumulation of fructose-1, 6-bisphosphate and fructose-1-phosphate, causing a stimulation of pyruvate kinase and a depletion of ATP, relieving the inhibition of the enzyme. Changes in hepatocyte phosphoenolpyruvate concentrations correlate well with the rates of glycolysis observed.

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ABREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3':5'-phosphate (cyclic)
DHAP	Dihydroxyacetone phosphate
EDTA	Ethylenediaminetetra-acetate
EGTA	Ethyleneglycolbis-(aminoethylether)tetra-acetate
FFA	Free fatty acid
Fru-1-P	Fructose-1-phosphate
Fru-6-P	Fructose-6-phosphate
$Fru_{16}BP$	Fructose-1, 6-bisphosphate
$Fru_{26}BP$	Fructose-2, 6-bisphosphate
$Fru_{16}BPase$	Fructose-1, 6-bisphosphatase
G3P	Glycerol-3-phosphate
Glu-6-P	Glucose-6-phosphate
Glu-6-Pase	Glucose-6-phosphatase
IMP	Inosine 5'-phosphate
KRB	Krebs-Ringer bicarbonate
LDH	Lactate dehydrogenase
NAD^+	Nicotinamide-adenine dinucleotide (oxidized)
NADH	Nicotinamide-adenine dinucleotide (reduced)
Pi	Orthophosphate (inorganic)
PEP	Phosphoenolpyruvate
PFK 1	Phosphofructokinase 1
PFK 2	Phosphofructokinase 2
1,3PGA	1,3-diphosphoglycerate
PK	Pyruvate Kinase
Tris	2-Amino-2-hydroxymethylpropane-1, 3-diol.

INTRODUCTION

INTRODUCTION

The first indication of the potential toxicity of fructose was considered when intravenous injection of a high fructose dose was found to cause hyperuricemia (Peheentupa & Raivo, 1967) and lactic acidosis (Bergström et al., 1968) in normal humans. The noxious influence of excess dietary consumption of fructose was also suspected when it was shown that in experimental animals and in human subjects, plasma triglyceride levels are raised by this ketose (Nikkilä, 1969).

The hepatic metabolism of fructose is different from that of glucose in several important respects. However, the glycolysis of both these substrates involve a common regulatory enzyme, pyruvate kinase. Indeed, pyruvate kinase is the only regulatory enzyme in the glycolysis of fructose to lactate. The activity of this enzyme in the liver is under hormonal and dietary regulation (Seubert & Schoner, 1971). In the present study the activity of pyruvate kinase in isolated hepatocytes is studied under various conditions which allow investigation of the regulation of the enzyme by fructose and other related substrates. This thesis reports on the metabolic effects of various substrates and investigates the metabolic regulation of pyruvate kinase in vivo.

I. CARBOHYDRATE METABOLISM

A MEDICAL INTEREST IN FRUCTOSE METABOLISM

Several reports in the literature indicate that the nature of dietary carbohydrate is a contributory factor in the causation of human diseases (Grande, 1967; Burke, 1971; Yudkin, 1971; Nikkilä, 1974). It has been repeatedly shown that when part or all of the starch in the diet of experimental animals or man is replaced with sucrose, plasma triglyceride and cholesterol levels increase (Bender & Damji, 1971; Macdonald, 1971; Naismith, 1971). Since starch and sucrose are composed of different carbohydrates, the suggestions put forward to explain these

observations were that either the fructose component of the sucrose molecule might be more readily converted to lipids than is glucose derived from starch, or that sucrose was more rapidly digested and absorbed and this would result in a higher concentration of monosaccharides entering the liver after a sucrose load, which in turn would lead to changes in the activity of the enzymes responsible for the conversion of monosaccharides to lipids.

The mechanisms by which fructose causes medical problems are manifold. For example, the role of this ketose in the causation of coronary disease may be due to an alteration in the circulating hormones and in the enzyme patterns that determine the metabolic rates (Yudkin, 1971).

Oral administration of fructose has also been shown to cause elevation of serum uric acid levels (Stripe et al., 1970; Emmerson, 1974) in normal human subjects. In patients with gout and in their healthy children, a more pronounced and prolonged effect of fructose on the serum level of uric acid has been reported (Stripe et al., 1970). This hyperuricemic effect of fructose is also observed in higher apes which like humans lack the capacity to oxidize uric acid (Simkin, 1972).

Thus, from this evidence it is clear that fructose, unlike glucose, may cause various diseases presumably because of the nature of its metabolism. However, it should be noted that dietary sucrose (and therefore fructose) is not the sole or the major cause of these diseases, since for example, cigarette smoking and physical inactivity are also involved in the causation of coronary diseases.

B. FATE OF DIETARY CARBOHYDRATES

Dietary carbohydrates are absorbed into the blood stream as monosaccharides which means that their initial hydrolysis plays a major role in their metabolism. Higher molecular weight starches are hydrolysed by α-amylase, an enzyme present in the saliva, while the

disaccharides and oligosaccharides are split into glucose, fructose and other related monosaccharides by enzymes located in the mucosal cells (Widdas, 1971).

The simple sugars are absorbed across the brush border of the mucosal cells. Glucose and galactose are actively absorbed by a carrier mechanism which utilizes the existing Na^+ gradient into the cell and which is therefore indirectly dependent on the cellular metabolism (Crane, 1968). In rat and man fructose is absorbed across the intestinal epithelial cells by a mechanism which may not involve active transport (Crane, 1960; Herman, 1974). However, this is not a simple passive diffusion of the ketose since its transport in rat small intestine is a saturable rate-limiting process (Guy & Deren, 1971; Gracey et al., 1972; Sigrist-Nelson & Hopfer, 1974). Evidence for a distinct transport system of D-fructose in humans came from studies on patients with malabsorption symptoms, where it was observed that patients with glucose-galactose malabsorption were able to absorb fructose at a normal rate (Dahlqvist (1974).

C. GLUCOSE METABOLISM

Glucose is a major metabolic fuel utilized by most tissues. The blood glucose level in the fasting human adult ranges from 3 to 6 mM (Bold & Wilding, 1975), and is under hormonal and dietary regulation. Excess glucose can be stored as glycogen in the liver or as triglycerides in the adipose tissue (Newsholme & Start, 1976). The relative constancy of blood glucose levels despite various disturbing factors is an example of homeostatic regulation.

The entry of blood glucose into the tissue Glu-6-P pool(s) may be controlled by many factors such as, the permeability of the tissue to glucose, the type(s) of hexokinase present in the tissue, the inhibition of hexokinase by Glu-6-P, the availability of ATP and the rate of supply of glucose to the tissue. Skeletal muscle and heart are not freely permeable to glucose and require the presence of insulin for

maximal glucose transport (Scrutton & Utter, 1968), whereas transport through the blood-brain barrier is insulin independent (Bachelard, 1970; Pardridge & Oldendorf, 1977; Sokoloff et al.,1977) and may be governed by the metabolic rate of glucose since brain hexokinase is inhibited by Glu-6-P (Wilson, 1980).

Liver provides a different aspect of glucose uptake because this organ has been reported to be freely permeable to the inward and outward flow of glucose (Cahill et al., 1958a). However, experiments using liver slices indicated that insulin is necessary for proper utilization of glucose by the liver (Spiro et al., 1958), and furthermore, Cahill et al.,(1958b) and Spiro (1959) reported that uptake of this hexose by liver slices is highly concentration dependent. The concentration dependency of glucose uptake by liver cells was also observed using perfused liver (Williams et al., 1968) and isolated hepatocytes (Baur & Heldt, 1976). The hexokinase present in hepatocytes is glucokinase (E.C. 2.7.1.2) (Crisp & Pogson, 1972), an enzyme which differs from brain and muscle hexokinase in several aspects (Purich et al., 1973). Hepatic glucokinase is under hormonal and dietary control (Salas et al., 1963; Sharma et al., 1963; Walker & Rao, 1964; Pilkis, 1970; Niemeyer et al., 1975; Weinhouse, 1976). The most important feature of glucokinase is possibly its high K_m for glucose (about 10 mM) which enables the liver to respond to changes in blood glucose level in the physiological range (Di Pietro & Weinhouse, 1960; Walker, 1963; Vinuela et al., 1963).

1. Glycolysis - Gluconeogenesis

The liver is thought to be the major gluconeogenic tissue in fasted animals (Scrutton & Utter, 1968). In fed animals gluconeogenesis is inhibited and glycolysis may predominate. The glycolytic enzymes occur exclusively in the cytoplasm whereas part of the gluconeogenic pathway involves the mitochondria. However, since flux through both of these pathways can occur within the same cell, a precise control of the regulatory enzymes is of great importance.

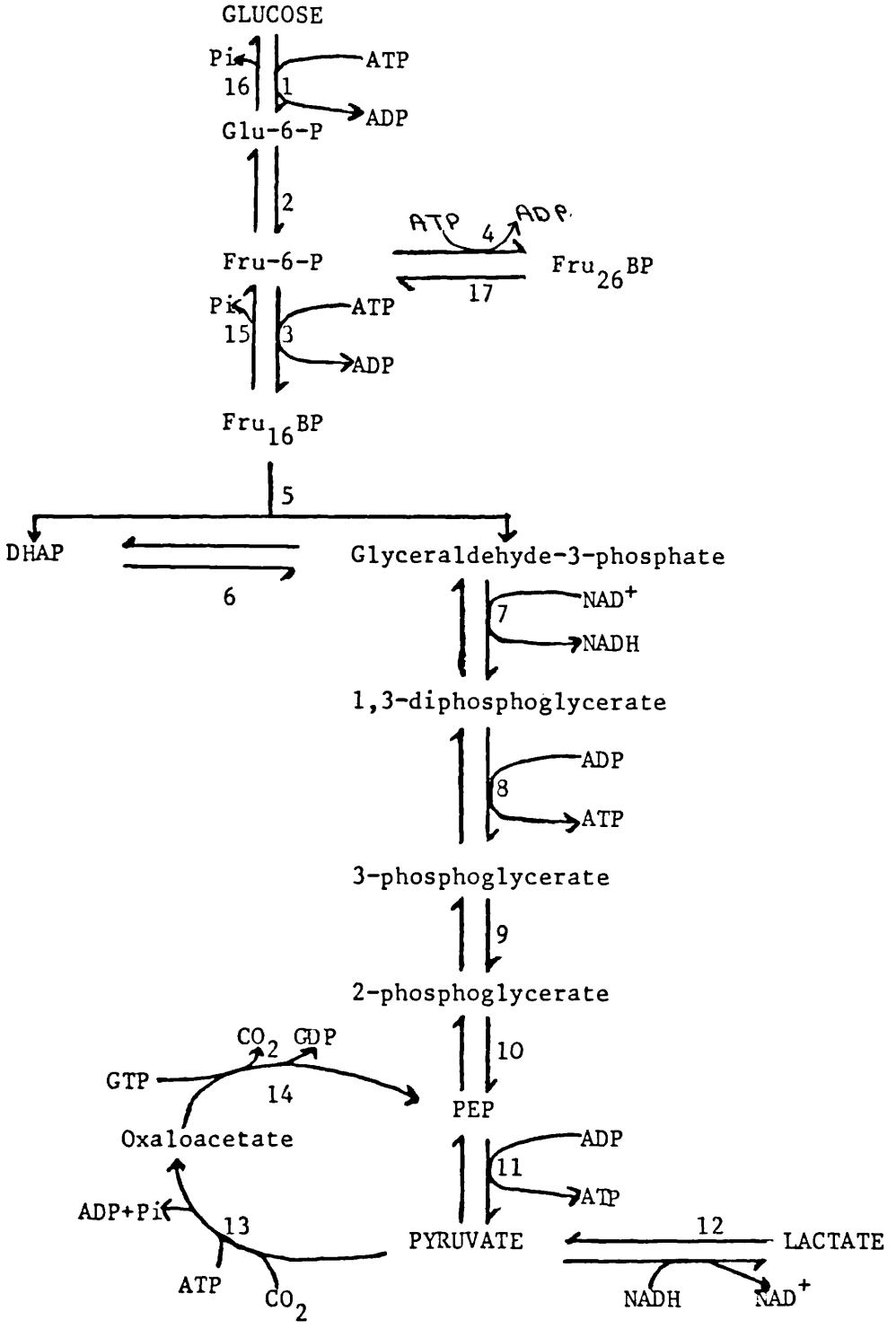


Figure 1. Hepatic glycolysis and gluconeogenesis

1. glucokinase
2. glucose phosphate isomerase
3. phosphofructokinase 1
4. phosphofructokinase 2
5. aldolase
6. triose phosphate isomerase
7. glyceraldehyde-3-phosphate dehydrogenase
8. phosphoglycerate kinase
9. phosphoglyceromutase
10. enolase
11. pyruvate kinase
12. lactate dehydrogenase
13. pyruvate carboxylase
14. phosphoenolpyruvate carboxy-kinase
15. fructose-1,6-bisphosphatase
16. glucose-6-phosphatase
17. fructose-2,6-bisphosphatase

The glycolytic pathway common to both glycogen and glucose starts at the Glu-6-P crossroads and ends as pyruvate and lactate. The reaction sequence between glucose and pyruvate is catalysed by a group of ten enzymes which are thought to have no physical dependence on each other, i.e. they appear not to be associated into a stable multienzyme complex. There are two major stages of glycolysis. The first stage serves as a preparatory or collection phase, in which a number of different hexoses after phosphorylation by ATP enter the glycolytic sequence and are converted into a common product glyceraldehyde-3-phosphate. In this stage, two ATP molecules are expended to phosphorylate the 1 and 6 positions of glucose. The second stage of glycolysis is the common pathway for all sugars. This involves the oxidoreduction steps and the energy conserving mechanisms by which ADP is phosphorylated to ATP. In this second stage four molecules of ATP are formed, so that the net yield is two molecules of ATP per molecule of glucose degraded to pyruvate.

The key glycolytic enzymes are phosphofructokinase 1 (E.C.2.7.1.11) which catalyses the phosphorylation of Fru-6-P by ATP to $Fru_{16}BP$, and pyruvate kinase (E.C. 2.7.1.40) which catalyses the formation of pyruvate from PEP with the production of one ATP molecule (figure 1). The reactions catalysed by these two enzymes are irreversible under physiological conditions and their precise control affects the flow of glycolysis. The other enzymes catalyse reversible steps and are also involved in gluconeogenesis.

Gluconeogenesis is the process whereby lactate, pyruvate and certain amino acids are converted to glucose and glycogen. The meaning of this term has been expanded to include synthesis of glucose from fructose (Exton & Park, 1967; Vernon et al., 1968; Assimacopoulos-Jeannet et al., 1973). The liver is the major site of gluconeogenesis, although the kidney becomes important during prolonged starvation. The most important function of gluconeogenesis is the maintenance of blood glucose levels when food intake is restricted and/or glycogen stores are depleted.

During gluconeogenesis lactate and most amino acids are converted to pyruvate in the cytoplasm. Pyruvate enters the mitochondria where it is converted to oxaloacetate by pyruvate carboxylase (E.C. 6.4.1.1) or to acetyl CoA by pyruvate dehydrogenase (E.C. 1.2.-4.1). The distribution of PEP carboxykinase (E.C.4.1.1.32) between the mitochondria and cytoplasm largely determines the fate of mitochondrial oxaloacetate (Tilghman, 1976). In chicken, pigeon and rabbit liver, this enzyme is in the mitochondria and thus oxaloacetate is directly converted to PEP which is then transported to the cytoplasm. In the rat liver PEP carboxykinase is located in the cytoplasm and thus oxaloacetate is first converted to malate and/or aspartate which are then transported to the cytoplasm where they can be reconverted to oxaloacetate. This complicated series of interconversions is apparently necessary because the mitochondrial membrane is presumed to be impermeable to oxaloacetate. However, Gimpel et al., (1973) reported that oxaloacetate may be transported across the mitochondrial membrane in exchange for dicarboxylate or phosphate ions. Cytosolic PEP is then converted to glucose by a series of enzymes, some of which are common to both glycolysis and gluconeogenesis (figure 1). The conversion of PEP to $Fru_{16}BP$ is catalysed by a series of reversible enzymes. The bisphosphate is converted to Fru-6-P by $Fru_{16}BPase$ (E.C.3.1.3.11) and the resulting Fru-6-P is converted to Glu-6-P which in turn is dephosphorylated to glucose by Glu-6-Pase (E.C.3.1.3.9).

Glycolysis and gluconeogenesis have been reported to be under hormonal control. The pathways have opposite functions so that factors that stimulate one process inactivate the other. In the early 1960's it was reported that glucagon stimulates gluconeogenesis in the rat liver (Schimassek & Mitzkat, 1963; Gracia et al., 1964; Struck et al., 1965; Exton & Park, 1966). Exton & Park (1966, 1969); postulated that the effect of glucagon in the perfused liver system was modulated by an increase in cAMP levels. The evidence for this was based on the fact that both glucagon and adrenaline caused a rapid elevation of cAMP levels, and also the concentration of hormone necessary to elicit the elevation of cAMP concentrations was similar to that needed to stimulate

gluconeogensis. In addition, exogenous cAMP mimicked the effect of these hormones (Garrison & Haynes, 1973). Regulation of cAMP levels has also been used to explain the inhibitory effect of insulin on gluconeogenesis. Exton et al.,(1971), reported that cAMP levels are lowered by insulin in perfused liver exposed to glucagon and adrenaline. Similar effects have also been reported to occur in isolated hepatocytes (Pilkis et al., 1975; Claus & Pilkis, 1976). However, insulin does not suppress the effects of high concentrations of glucagon which is probably because the hepatic cyclic nucleotide concentrations had become so great that a reduction in its level by insulin was not apparent.

These observations are consistent with the concept of cAMP as the second messenger (Robison, et al., 1971). According to this concept, the hormone is the first messenger which carries the relevant information to the cell where it binds to specific hormone receptors. This event stimulates the conversion of ATP to cAMP by adenylate cyclase (E.C. 4.6.1.1). cAMP acts as the second messenger which transfers the information to the cell's enzymatic mechanisms.

While it appears that glucagon effects in the liver are mediated by cAMP, recent evidence suggests that catecholamine effects may not involve cAMP. Adrenaline has been shown to stimulate gluconeogenesis by an α-adrenergic mechanism which is not associated with changes in the cAMP levels (Sherline et al.,1972; Tolbert et al., 1973; Tolbert & Fain, 1974) and that catecholamine effects can be blocked by α-adrenergic blocking agents such as phentolamine even though cAMP levels remain unchanged. These observations were confirmed by Exton & Harper, (1975) and Cherrington et al., (1976), who showed that the α-effect of catecholamines in rat hepatocytes was not associated with an activation of cAMP dependent protein kinase. However, it is also possible that catecholamines activate gluconeogenesis in some species by a predominantly β-adrenergic mechanism and in some by an α-adrenergic mechanism.

Calcium ions have also been shown to play an important role in the regulation of gluconeogenesis in isolated hepatocytes (Kneer et al., 1979). These authors reported that Ca^{2+} was required to enhance hormonal stimulation of gluconeogenesis from substrates that enter the gluconeogenic pathway prior to PEP and from reduced substrates that enter at the triose phosphate or at the Fru-6-P stage, whereas extracellular Ca^{2+} was not required to enhance the rate of glucose production from the oxidised substrates that enter at the triose phosphate stage. Ca^{2+} is also believed to mediate catecholamine effects, since activation of the α-adrenergic receptors has been associated with cytosolic Ca^{2+} mobilization (Stubbs et al., 1976; Assimacopoulos-Jeannet et al.,1977; Keppens et al., 1977; Van de Werve et al., 1977; Blackmore et al., 1978; Chen et al., 1978), though some of this Ca^{2+} may originate from the mitochondria (Blackmore et al., 1979).

The other possible regulatory mechanism involved in achieving an unidirectional flow of metabolites is the regulation of a few key reactions. Between glucose and pyruvate there are three irreversible steps which are; the interconversion of glucose and Glu-6-P which is catalysed by glucokinase and Glu-6-Pase; the interconversion between Fru-6-P and $Fru_{16}BP$ where the action of PFK 1 is opposed by $Fru_{16}BPase$; and at the level of pyruvate and PEP where PK is opposed to a multi-step conversion of pyruvate into PEP involving pyruvate carboxylase and PEP carboxykinase.

Therefore, if the antagonistic reactions operate simultaneously and at the same rate, there is no net flux of metabolites only a 'futile' recycling of substrates. Such a cycling of substrates either between glucose and Glu-6-P or between Fru-6-P and $Fru_{16}BP$ results in the apparently wasteful hydrolysis of ATP. However, the demonstration of the operation of such cycles makes it possible that these apparently 'futile' processes have a physiological role. For example, since these processes are characterised by energy dissipation, i.e. heat production, they were ascribed a possible role in the regulation

of thermogenesis (Clark, M.G., et al.,1973; Newsholme, 1978). Another useful function of cycling, particularly between Fru-6-P and $Fru_{16}BP$ is that it might provide an amplification mechanism for allosteric control of metabolic flow (Newsholme & Crabtree, 1976; Newsholme, 1978). Thus it seems logical not to dismiss these cycles as 'futile' and to use the term substrate cycles instead. Therefore the regulation of the three cycles occurring in the glycolytic/gluconeogenic pathways may change the direction of metabolic flow.

i) The glucose/glucose-6-phosphate substrate cycle

The operation of this cycle as well as its role in the regulation of the glucose flux is of interest since Glu-6-P is located at a major metabolic crossroads which involves synthesis and breakdown of glycogen, glycolysis, gluconeogenesis and the oxidative pentose phosphate pathway. Glucose phosphorylation is catalysed by glucokinase, an enzyme found exclusively in the parenchymal cells (Crisp & Pogson, 1972), and the rate of phosphorylation is proportional to the extracellular glucose concentrations (Clark,D.G., et al., 1973). The hydrolysis of Glu-6-P is catalysed by Glu-6-Pase which serves to produce glucose under hypoglycemic conditions. Glu-6-Pase is bound to microsomes (Hers et al., 1951) and has been purified for kinetic studies (Cori et al., 1973). This enzyme is half-saturable between 2.0 and 7.0 mM Glu-6-P (Beaufay & de Duve,1954; Arion et al., 1972). The concentration of Glu-6-P in the liver is far below these values (Faupel et al., 1972; Williamson & Brosnan, 1974; Hue & Hers, 1974) and therefore under most conditions the rate of Glu-6-P hydrolysis is a first order reaction regulated by the substrate concentrations.

Although the activities of glucokinase and Glu-6-Pase do not appear to be sensitive to short-term regulation by hormones, their simultaneous operation may be important for the regulation of carbohydrate metabolism, because this substrate cycle allows large changes in the net flux of carbon to be controlled by substrate concentration (Hue & Hers, 1974). Thus a small elevation in blood glucose level

leads to a decrease in Glu-6-P concentration. The decrease in the level of this intermediate is attributed to an activation by glucose of glycogen synthetase (E.C. 2.4.1.11) and increased incorporation into glycogen.

ii) The fructose-6-phosphate/fructose-1, 6-bisphosphate substrate cycle

The enzymes involved in this cycle are $Fru_{16}BPase$ and PFK 1 whose activities are tightly regulated by allosteric control and, perhaps, by covalent modification of the enzyme molecule. Since the hexose-6-phosphates are located at a metabolic crossroads, a change in the activity of PFK 1 and/or $Fru_{16}BPase$ determines whether the flux of carbon occurs in the direction of glycolysis or of gluconeogenesis.

A study of hepatic $Fru_{16}BPase$ has revealed that this enzyme displays a K_m for $Fru_{16}BP$ in the µmolar range (Taketa & Pogell, 1963; 1965; Underwood & Newsholme, 1965; Tejwani et al., 1976). The enzyme is inhibited by concentrations of substrate higher than 0.1 mM (Mendicino & Vasarhely, 1963; Taketa & Pogell, 1965; Underwood & Newsholme, 1965), but this probably has no physiological significance since the concentration of $Fru_{16}BP$ in the liver, under physiological conditions ranges between 20 and 40 µM (Faupel et al., 1972; Williamson & Brosnan, 1974; Lawson et al., 1976). There is also a good indication that the major part of $Fru_{16}BP$ is bound to proteins and that the concentration of free bisphosphate could be as low as 1 µM (Sols & Marco, 1970; Lawson et al., 1976). The products of the reaction, i.e. Fru-6-P and Pi, are competitive inhibitors of the enzyme with K_i values of 0.07 and 2.7 mM respectively (Dudman et al., 1978). These values are in the range of the concentrations usually found in the liver (Williamson & Brosnan, 1974). Therefore the decrease in Pi known to occur in the liver after a fructose load (Van den Berghe, 1978) could increase the activity of $Fru_{16}BPase$. The activity of $Fru_{16}BPase$ is also subject to a multiplicity of metabolic controls (Horecker et al., 1975) including allosteric inhibition by AMP (Mendicino & Vasarhely, 1963; Taketa &

Pogell, 1963; 1965; Underwood & Newsholme, 1965; Rosenberg et al., 1973; Data et al., 1974; Nimmo & Tipton, 1975; Tejwani et al., 1976; Riou et al., 1977), and activated by a number of substances such as histidine (Pogell et al., 1968), chelators and fatty acids (Carlson et al., 1973). Furthermore, $Fru_{16}BPase$ is also inhibited by $Fru_{26}BP$ (Van Schaftingen & Hers, 1981a) a metabolic product of PFK 2 (Van Schaftingen & Hers, 1981b; Furuya & Uyeda, 1981) (see also figure 1). The main characteristics of this inhibition are that it is much stronger at low than at high substrate concentrations, it changes the substrate saturation curve from almost hyperbolic to a sigmoidal curve and the inhibition is synergistic with the inhibition by AMP.

There is evidence to suggest that $Fru_{16}BPase$ is phosphorylated by ATP in vitro in the presence of relatively high concentrations of a purified catalytic subunit of the cAMP-dependent protein kinase (Riou et al., 1977; Mendicino et al., 1978). However, although in vivo studies have suggested that short-term treatment with hormones causes changes in the activity of $Fru_{16}BPase$ (Greene et al.,1974; Stifel et al., 1974; Taunton et al.,1972; 1974), these findings did not give conclusive evidence that the activity of this enzyme depends on its phosphorylation state. Indeed recent evidence provided by Van Schaftingen et al., (1981a) indicates that the effect of glucagon on $Fru_{16}BPase$ is mediated indirectly via a cAMP-dependent phosphorylation of PFK 2 leading to an elevation of $Fru_{26}BP$. $Fru_{16}BPase$ may also be subject to long-term adaptive changes in the amount of enzyme protein (Weber et al., 1965a).

The other enzyme in this cycle is PFK 1 which is regulated by various effectors, dietary status and hormonal conditions. The amount of enzyme has been shown to decrease during starvation (Dunaway & Weber, 1974; Söling & Kleineke, 1976), or when the rats are made diabetic with alloxan. Refeeding the starved rats or insulin treatment for 72 h increases enzyme levels between 6 and 8-fold. The activity of hepatic PFK 1 can also be affected by phosphorylation and dephosphorylation (Brand & Söling, 1975; Brand et al., 1976; Söling et al.,1981).

The rat enzyme can be phosphorylated by cAMP-independent kinase with a resulting increase in enzyme activity. Glucagon and catecholamines have been reported to affect the activity of PFK 1 within minutes of their administration (Taunton et al., 1974; Stifel et al., 1974; Castaño et al., 1979). The same hormones inhibit flux through the enzyme in intact hepatocytes (Clark et al., 1974; Kneer et al., 1974; Rognstad & Katz, 1976).

PFK 1 is also regulated by various effectors. ATP, citrate, PEP and creatine phosphate are negative effectors and increase the sigmoidal nature of the saturation curve for Fru-6-P, whereas positive effectors such as AMP, Pi and Fru-6-P, have the opposite effect (Ramaiah, 1974; Hofmann, 1976). Monovalent cations such as K^+ and NH_4^+ activate PFK 1 and decrease the allosteric inhibition by ATP (Otto et al., 1976). A newly recognized positive effector of PFK 1 is $Fru_{26}BP$ (Claus et al., 1980; Richards & Uyeda, 1980; Van Schaftingen & Hers, 1980; Van Schaftingen et al., 1980c, Pilkis et al., 1981; Uyeda et al., 1981a; 1981b). The hepatic concentration of this effector is greatly increased under conditions in which glycolysis occurs and greatly decreased by glucagon so that glycolysis is inhibited (Van Schaftingen et al., 1980b). Thus, although it has been suggested that the glucagon effects on PFK 1 might result from a phosphorylation of the enzyme (Kagimoto & Uyeda, 1979; 1980) it seems more likely that the effect of glucagon is mainly based on the decrease in the concentration of $Fru_{26}BP$.

$Fru_{26}BP$ and AMP have in common the properties of inhibiting $Fru_{16}BPase$ and stimulating PFK 1. Changes in the concentration of these effectors could therefore explain the effects of glucose and glucagon on this substrate cycle. The concentration of $Fru_{26}BP$ in the liver is greatly increased by glucose and decreased by glucagon (Van Schaftingen et al., 1980b), whereas in contrast, the concentration of AMP in the liver is increased by glucagon but

is not affected by glucose (Van Schaftingen et al., 1980a). Therefore it seems that $Fru_{26}BP$ rather than AMP acts as a second messenger in the regulation of glycolysis and gluconeogenesis at this substrate cycle. The importance of this effector on the Fru-6-P/$Fru_{16}BP$ substrate cycle is further emphasized by the observation that Pi and AMP at physiological concentrations increase the activity of PFK 2, whereas, both PEP and citrate are inhibitory in the presence and absence of Pi (Van Schaftingen & Hers, 1981b). Thus if PEP levels are elevated during glycolysis, PFK 2 activity is decreased. This in turn would result in a decrease in the $Fru_{26}BP$ levels, which may be the signal that activates $Fru_{16}BP$ase and inactivates PFK 1. From these reports it is evident that the most important factor in the regulation of the Fru-6-P/$Fru_{16}BP$ cycle is the $Fru_{26}BP$ concentration.

iii) The pyruvate/phosphoenolpyruvate substrate cycle

The most complicated substrate cycle in the regulation of glycolysis and gluconeogenesis is that between PEP and pyruvate; it consists of various steps involving two different compartments within the cell. In rat liver, the following steps are involved in this cycle; the transport of pyruvate to the mitochondria, the carboxylation of pyruvate yielding oxaloacetate, the reduction of oxaloacetate to malate or its transamination to aspartate, the transport of these metabolites to the cytosol, their reconversion to oxaloacetate, the formation of PEP catalysed by PEP carboxykinase and the conversion of PEP to pyruvate via the reaction catalysed by PK.

Pyruvate entry into the mitochondria has been shown to be under hormonal control (Adam & Haynes, 1969). Support for this hypothesis is provided by the observation that mitochondria from hepatocytes treated with glucagon, adrenaline or cAMP, as well as liver mitochondria from rats treated with glucagon or adrenaline take up more pyruvate than those from control rats (Adam & Haynes, 1969; Garrison & Haynes, 1975; Titheradge & Coore, 1976a; 1976b). Since there is some indirect evidence to suggest that pyruvate carboxylation may be

limited by the rate of pyruvate entry into the mitochondria (Haynes, 1972), the regulation of gluconeogenesis at the pyruvate uptake stage is a plausible mechanism.

The synthesis of oxaloacetate from pyruvate in the mitochondria is catalysed by pyruvate carboxylase (Utter & Keech, 1960; 1963; Keech & Utter 1963), an enzyme located exclusively in rat liver mitochondria (Walter, 1976). In rat liver the maximal activity of this enzyme is higher in starved than in fed animals (Söling & Kleineke, 1976). The K_m for pyruvate is 0.15 mM (McClure & Lardy, 1971) and this is in the physiological range (Williamson & Brosnan, 1974). The activity of pyruvate carboxylase is regulated by the concentrations of pyruvate, acetyl CoA,adenine nucleotides and certain amino acids (Pilkis et al., 1978a). The rat liver enzyme requires acetyl CoA for activity (Keech & Utter, 1963) with a K_a of the purified enzyme ranging from 15 to 70 µM (Barritt et al., 1976), but values as high as 170 µM have been found with intact mitochondria (Walter, 1976). The hepatic mitochondrial acetyl CoA concentration ranges between 0.24 and 1.25 mM (Sobell et al., 1976; Siess et al., 1977; Tischler et al., 1977). Part of this mitochondrial acetyl CoA may be bound to protein (Sols & Marco, 1970; Barritt et al., 1976), so that the actual concentration of free acetyl CoA might be smaller. Thus the importance of the regulation of pyruvate carboxylase by acetyl CoA is difficult to evaluate. The other possible regulatory mechanism of pyruvate carboxylase is hormonal regulation. Glucagon is known to decrease the concentration of lactate and pyruvate in the liver (Exton & Park, 1969), but there is evidence to suggest that the decrease in pyruvate concentrations is confined within the cytosolic compartment (Siess et al., 1977), while the mitochondrial pyruvate remains unaltered. However, glucagon has been reported to cause a 25% increase in the concentration of mitochondrial acetyl CoA, which acting together with the small increase in the mitochondrial ATP/ADP ratio could slightly stimulate pyruvate carboxylase activity (Siess et al., 1977). Nevertheless, the most important regulator of this enzyme seems to be the inhibitor glutamate (Scrutton & White, 1974; Siess et al., 1977), whose concentration is known to decrease

after glucagon treatment (Ui et al., 1973a; 1973b; Cook et al., 1977). Therefore,it appears that pyruvate carboxylase is regulated by glucagon,not by a phosphorylation- dephosphorylation mechanism (Leiter et al., 1978) but by an alteration of the mitochondrial concentrations of acetyl CoA, ATP and glutamate.

Since rat liver PEP carboxykinase is located mainly in the cytosol (Nordlie & Lardy, 1963), the oxaloacetate formed via the pyruvate carboxylase reaction has to be transported from the mitochondrial compartment to the cytosol. The efflux of oxaloacetate from the mitochondria is too slow a process to keep pace with the rate of gluconeogenesis, thus the transport of malate and aspartate formed from oxaloacetate is an obligatory step in gluconeogenesis from lactate and pyruvate (Lardy et al., 1965). In the cytosol, oxaloacetate is resynthesized by malate dehydrogenase (E.C.1.1.1.37) and aspartate aminotransferase (E.C.2.6.1.1).

Oxaloacetate is metabolised to PEP in a reaction catalysed by PEP carboxykinase. This enzyme is also responsive to dietary and hormonal changes. The level of the enzyme is raised by fasting, by diabetes and by administration of glucagon if the hormone is administered for periods of hours or days to the intact animal. The level is reduced by prolonged administration of insulin (Tilghman , 1976). The apparent K_m for oxaloacetate is reported to range from 1 to 10 µM (Ballard, 1970; Walsh & Chen, 1971; Jomain-Baum et al.,1976), but reaches about 60 µM in the presence of 0.15 mM Mn^{2+} with saturating concentrations of GTP (Jomain-Baum & Schramm, 1978). Since the concentration of cytosolic oxaloacetate ranges between 5 and 50 µM (Zuurendonk et al., 1976; Siess et al., 1977; Tischler et al., 1977) and is not influenced by glucagon (Siess et al., 1977),and the total concentration of GTP in the liver is about 0.5 mM (Van den Berghe et al., 1977a) which is sufficient to saturate the enzyme (K_m 0.02 mM) according to Jomain-Baum & Schramm,(1978); it therefore appears that the variations in the concentrations of oxaloacetate may affect the activity of PEP carboxykinase.

The remaining enzyme involved in this cycle is PK. The liver content of PK is greatly increased by a diet enriched in carbohydrate and decreased upon starving (Seubert & Schoner, 1971; Ibsen, 1977; Van Berkel et al., 1977a). This enzyme is allosterically inhibited by ATP and several amino acids such as alanine, cysteine, phenylalanine and serine and it is stimulated by $Fru_{16}BP$, Fru-1-P and by cations such as K^+, NH_4^+ and Mg^{2+} (Seubert & Schoner, 1971). The liver PK is also under hormonal regulation by a mechanism which involves phosphorylation-dephosphorylation of the enzyme (Engström, 1978). Phosphorylation of the enzyme increases the apparent $S_{0.5}$ for PEP 2 or 3-fold (Ekman et al., 1976; Ljungström et al., 1976) and the affinity for the allosteric inhibitors alanine and ATP increases whereas the affinity for the allosteric activator $Fru_{16}BP$ decreases. Gluconeogenesis stimulating hormones such as glucagon inactivate PK whereas insulin activates the enzyme. A detailed review of the properties of this enzyme is given in section II.

Thus, the regulation of the pyruvate/PEP cycle may occur at various points. The operation of this cycle is energetically wasteful because it consumes 1 mol. of GTP per mol. of pyruvate recycled. It must be noted that the occurrence of substrate cycling between pyruvate and PEP has been reported (Friedman et al., 1971;Rognstad & Katz, 1972). The rate of gluconeogenesis from pyruvate in perfused liver from starved animals is greater than its rate of recycling whereas the reverse situation is observed with fed animals (Friedmann et al., 1971).

Therefore, generally it seems that factors that stimulate glycolysis have the opposite effect on gluconeogenesis which implies that when one pathway is dominant the other is depressed but not completely blocked and hence substrate cycling occurs.

2. Glycogen Metabolism

Glycogen is stored in the liver as a reserve of glucose to meet the energetic need of extrahepatic tissues. When glucose is abundant (for example after feeding), glycogen accumulates and it is subsequently

mobilized when there is extrahepatic demand for glucose. Glucose itself is a potent regulator of the hepatic uptake or output of glucose, so that the hepatic blood glucose homeostasis is auto-regulated. The hepatic threshold of glucose is the concentration at which the liver stops producing glucose and starts to take up the hexose. The glucose is produced as a result of both gluconeogenesis and glycogenolysis.

The rate-limiting steps of glycogen synthesis and breakdown in the liver are catalysed by glycogen synthetase (E.C. 2.4.1.11) and glycogen phosphorylase (E.C.2.4.1.1). Each of these enzymes exists in two forms, 'a'(active) and 'b'(inactive), which are inter-convertible through phosphorylation by kinases and dephosphorylation by phosphatases (Hers, 1976). Thus stimulation of glycogen degradation by hormones will therefore be achieved by the inactivation of glycogen synthetase and activation of phosphorylase (see figure 2).

The activation of phosphorylase can be brought about by the cAMP-dependent activation of protein kinase (E.C.2.7.1.37), which in turn leads to the activation of a phosphorylase kinase (E.C.2.7.1.38) and the consequent activation of phosphorylase. On the other hand, cAMP-dependent protein kinase can phosphorylate glycogen synthetase and hence cause its inactivation. Thus, hormones which raise cAMP levels (glucagon and adrenaline) can stimulate glycogenolysis via this mechanism while insulin is thought to have the reverse effects (Friedman et al., 1970; Hue & Feliú, 1978; Nyfeler et al., 1981). Stimulation of glycogenolysis in rabbit liver by adrenaline, noradrenaline and phenylephrine has been shown to involve β-adrenergic mediated mechanisms (Rufo et al., 1981). Changes in physiological concentrations of glucose can also regulate glycogen metabolism (Hers et al., 1973). When glucose binds to phosphorylase'a', the enzyme is inhibited and thus the conversion of phosphorylase 'a' to phosphorylase 'b' is increased. A secondary effect is that the inhibition of synthetase phosphatase by phosphorylase 'a' is reversed and thus glycogen synthetase is activated (Stalman, 1976).

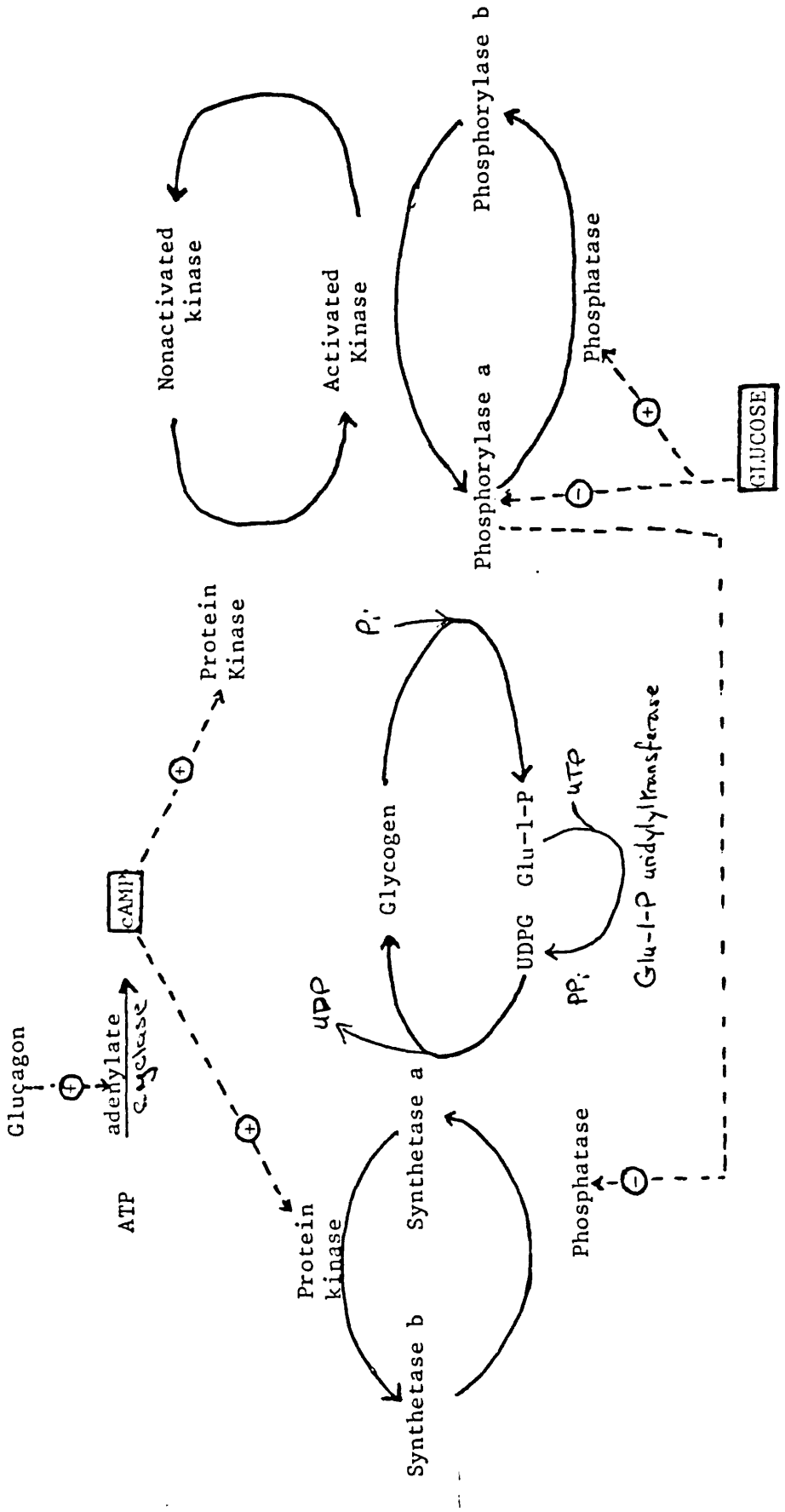


Figure 2. The control of glycogen metabolism in the liver

D. FRUCTOSE METABOLISM

In humans, Medeloff & Weichselbaum,(1953) and Wolfe et al., (1975) demonstrated that after prolonged infusion with fructose, the liver accounts for 75% of its removal. Similar results were obtained by Topping & Mayes,(1971) who administered high fructose concentrations to rats by gastric intubation. Utilization of fructose by kidney and intestine has also been documented but the quantitative participation of these organs is unclear and seems to be species dependent (Bollman & Mann, 1931; Reinecke, 1944). In humans, the use of splanchnic vein catheterization during infusion indicates that the small intestine accounts for less than 10% of the total metabolism of fructose and that a considerable proportion of the hexose is absorbed unchanged (Medeloff & Weichselbaum,1953).

Fructose is transported to the liver from the small intestine via the portal blood vessel. Liver is the major site of fructose metabolism. A steep gradient exists between the extra- and intracellular concentrations of the sugar (Sestoft, 1974) and thus elimination of fructose from the blood is a function of its concentration and follows Michaelis-Menten kinetics (Sestoft et al., 1972). In rat liver the carrier - mediated membrane transport of fructose has a high K_m and V_{max} (67 mM and 30 µmole $min^{-1}g^{-1}$ respectively); in comparison to the intracellular phosphorylation constants calculated for fructokinase (E.C.2.7.1.1) (K_m of about 1.0 mM and V_{max} of 10.3 µmole $min^{-1}g^{-1}$ with fructose) (Sestoft et al., 1972; Sestoft, 1974; Sestoft & Fleron, 1974). The transport values for fructose suggest that at physiological fructose concentrations, membrane transport limits the rate of uptake, thus protecting the liver from severe depletion of adenine nucleotide (see section I.D.4).

The first step of fructose metabolism is its phosphorylation by hepatic fructokinase to yield Fru-1-P and ADP (Cori et al., 1951; Hers, 1952a). The enzyme utilizes ATP in the form of the Mg^{2+} complex as the phosphate donor (Hers, 1952b; Parks et al., 1957). Hepatic fructokinase has a high affinity for fructose with K_m values between

0.46 and 1.0 mM reported and K_m values between 0.2 and 2.0 mM reported for Mg^{2+} ATP (Adelman et al., 1967; Sanchez et al., 1971; Sestoft, 1974). Fructokinase, however, is not specific for fructose because it also catalyses the phosphorylation of L-sorbose, D-tagatose, D-xylulose and L-galactoheptulose (Adelman et al., 1967; Sanchez et al., 1971) and therefore can be regarded as a ketohexokinase.

The next step is the cleavage of Fru-1-P by hepatic aldolase (E.C.4.1.2.13) to yield DHAP and D-glyceraldehyde. This same enzyme also catalyses the splitting of $Fru_{1,6}BP$ to DHAP and D-glyceraldehyde-3-phosphate in addition to condensation of the triose phosphates (Hers & Kusaka, 1953). This differentiates the liver enzyme from muscle or brain aldolase, because unlike hepatic aldolase, the latter types are more specific for $Fru_{1,6}BP$ (Penhoet et al., 1966). The metabolism of D-glyceraldehyde is discussed in section I.E.

Metabolic effects of fructose in the liver

As already discussed, liver is the major site of fructose metabolism. The effects of increased fructose concentrations on various metabolites and metabolic pathways in the liver have been studied.

1. Accumulation of fructose-1-phosphate

The accumulation of Fru-1-P in the liver after a fructose load has been demonstrated using intact animals (Burch et al., 1969; Bode et al., 1973; Van den Berghe et al., 1973; 1977a) as well as perfused organ (Woods et al., 1970; Sestoft et al., 1972). This accumulation of the monophosphate in these conditions indicates that the breakdown of Fru-1-P is much slower than its formation. However, from an examination of the in vitro data available, it seems clear that both the K_m and the V_{max} of fructokinase and aldolase are in the same range (Van den Berghe, 1978). Thus an explanation for this accumulation is not immediately apparent. One possible explanation offered by Woods et al.,(1970) is that the accumulation of

an inhibitor of aldolase (IMP), which is apparent after a fructose load, may play a role in the regulation of Fru-1-P accumulation. However, the finding that the accumulation of the fructose ester preceeds the increase of IMP *in vivo* (Van den Berghe *et al.*, 1977a) contradicts this interpretation. Furthermore, measurement of the initial rate of hepatic Fru-1-P formation *in vivo* (Van den Berghe *et al.*, 1977a) reveals that it is several times faster than that expected from the consideration of the enzyme activities determined *in vitro*. The reason for this discrepancy is not clear. One possibility is that since fructokinase has been shown to require high concentrationsof K^+ (Hers, 1952a; 1952b), the possibility exists that all ionic and metabolite requirements have not been met for optimal activity in the *in vitro* assay.

From these observations, it has been proposed that the accumulation of Fru-1-P is due to the fact that both fructokinase and aldolase can act much more rapidly than the metabolic pathways that convert triose phosphates into glucose and pyruvate (Exton and Park, 1967).

2. Glycolysis

Fructose enters the glycolytic pathway at the triose phosphate stage (figure 3) and can produce lactate and pyruvate as its end products. In liver slices (Thieden & Lundquist, 1967), perfused organ (Exton & Park, 1967) as well as in the isolated liver cells (Seglen, 1974), lactate formation from fructose is several fold faster than from glucose. The explanations offered for this difference in the rate of utilization are;(i) hepatic fructokinase has a much higher activity than the glucose phosphorylating capacity of glucokinase,(ii) fructose metabolism bypasses the PFK regulatory step of glycolysis, (iii) pyruvate kinase is stimulated by Fru-1-P (Eggleston & Woods, 1970). Seglen (1974) showed that fructose was utilized very effectively by isolated liver cells from both fed and fasted rats, the glycolytic rate being 10-30 times higher than with glucose under aerobic as well

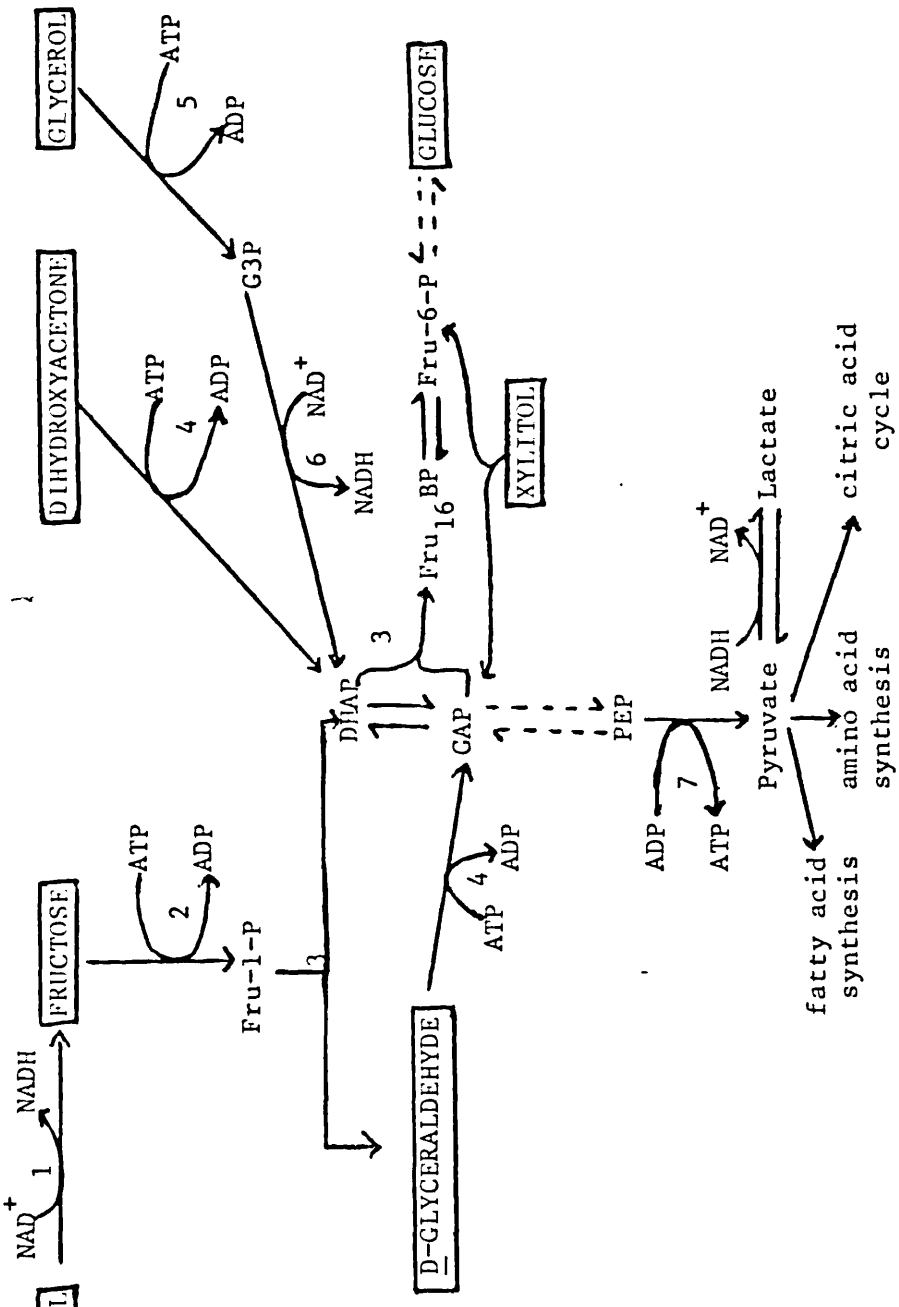


Figure 3. The metabolism of various carbohydrate substrates in the liver. (GAP - glyceraldehyde-3-phosphate).

1 = sorbitol dehydrogenase; 3 = aldolase; 5 = glycerokinase; 7 = pyruvate kinase.

2 = ketohexokinase; 4 = triokinase; 6 = glycerol-3-phosphate dehydrogenase;

as anaerobic conditions. In the presence of ethanol, lactate production from fructose was considerably lowered (Scholz & Nohl, 1976). Similar results were obtained by Lundquist et al.,(1974) and Sestoft (1974) and were interpreted to be due to an inhibition of the glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12) reaction as a consequence of NAD^+ redox changes induced by ethanol (Furfine & Velick, 1965).

3. Gluconeogenesis

Glucose is the major end product of hepatic fructose metabolism. Exton & Park,(1967) used perfused liversfrom fasted rats and showed that after 1 h perfusion with 20 mM fructose, 52% of the ketose was recovered as glucose, 18% as lactate and pyruvate, 8% as glycogen and the remaining 22% is assumed to be metabolised to glycerol, sorbitol, triglycerides, carbon dioxide and ketone bodies. Fructose is a very good gluconeogenic substrate in perfused liver, the maximum rate of glucose produced being about twice that from lactate (Ross et al., 1967a). Using isolated hepatocytes,Veneziale & Lohmar,(1973); Seglen, (1974) and Arinze & Rowley (1975) reported similar findings.

The fructose carbon enters the glycolytic/gluconeogenic pathways as DHAP and D-glyceraldehyde-3-phosphate (figure 3), thus bypassing pyruvate carboxylase and PEP carboxykinase, the regulatory enzymes for gluconeogenesis from lactate and pyruvate. Fructokinase, aldolase and triokinase (E.C.2.7.3.10) are required for the conversion of fructose to triose phosphates. Thus the segment of gluconeogenesis between DHAP or D-glyceraldehyde-3-phosphate and glucose serves as the common pathway for gluconeogenesis whether the carbon source for the newly formed glucose is pyruvate or fructose.

Such pathway differences between pyruvate and fructose provide a useful model, in gluconeogenic studies, to evaluate which metabolic segment(s) is actually influenced by glucagon or its mediator, cAMP. An action of glucagon on the initial segment of fructose metabolism

should have no influence of gluconeogenesis from pyruvate, while an action of glucagon in the early segment of pyruvate metabolism should have no influence on the rate of gluconeogenesis from fructose. Failure to observe a glucagon induced stimulation of gluconeogenesis from fructose by Ross et al.,(1967b) led to the suggestion that the stage between pyruvate and oxaloacetate is the site of action of glucagon, and that ....'a physiologically meaningful stimulation of reactions (by glucagon) between the triose phosphate and glucose seems unlikely'(Exton et al., 1970). In contrast, Veneziale, (1971) perfused isolated fasted rat liver with glucagon and fructose in the presence of quinolinate and observed a stimulation of fructose conversion to glucose. Quinolinate was added to minimize, if not prevent, the glucagon stimulation of gluconeogenesis from endogenous substrates via PEP carboxykinase. However,Garrison & Haynes,(1973) also found a lack of stimulation of gluconeogenesis from fructose by glucagon and dibutryl cAMP in isolated hepatocytes.

This discrepancy between these reports may be related to the use of different fructose concentrations. It seems that gluconeogenesis from fructose at low concentrations is stimulated by glucagon but not when high levels of the ketose were used. This may be related to the observation that low fructose concentrations raise $Fru_{26}BP$ levels in isolated hepatocytes whereas high fructose lowers the level of this effector (Hue, 1981).

Furthermore, a study of the rate of uptake of D-fructose by isolated rat liver in the presence and absence of glucagon indicates that approximately 40% of the added fructose is cleared from the perfusate, both in the presence and absence of glucagon (Veneziale, 1971). Therefore, the action of glucagon must be on an intracellular aspect of the process.

Other factors such as diabetes (Renold et al., 1954; Friedmann et al., 1970; Exton et al., 1973) and fasting (Seglen, 1974) also increase glucose production from fructose. Ethanol administration has

been shown to increase gluconeogenesis from fructose (Madison et al., 1967; Exton & Park, 1969; Lundquist et al., 1974; Scholz & Nohl, 1976; Cederbaum & Dicker, 1979), but contradictory to these reports, Krebs (1968), Krebs et al., (1969), Papenberg et al., (1970) and Ylikahri et al., (1972) reported that hepatic gluconeogenesis from fructose is inhibited by ethanol. Studies carried out on the rate of total fructose metabolism indicate that ethanol does not affect fructose entry into hepatocytes nor its phosphorylation nor its aldolytic cleavage (Scholz & Nohl, 1976). This suggests that at the level of triose phosphates, fructose metabolism is switched over from fructolysis to glucose production. Since ethanol administration induces an increase in the (NADH)/(NAD^+) ratios, which in turn would cause the inhibition of glyceraldehyde-3-phosphate dehydrogenase (Furfine & Velick, 1965), it is more plausible that ethanol would inhibit glycolysis from fructose and increase the gluconeogenic rates from this ketose.

4. Adenine nucleotide metabolism

When large doses of fructose are injected intravenously to experimental animals the concentration of Fru-1-P in the liver increases within a few min (Burch et al., 1969; Van den Berghe et al., 1977a) and the concentration accumulated depends on the dose injected (Burch et al., 1970). This effect was also observed by Mäenpää et al., (1968) who reported that as a consequence of this rapid accumulation of Fru-1-P, the intracellular concentrations of Pi and ATP decrease. However, the decrease in ATP levels is not followed by an equivalent increment in the sum of ADP plus AMP levels, but preceeds an increase in plasma concentrations of uric acid and allantoin, providing evidence for the degradation of the adenine nucleotide pool (Burch et al., 1969; Burch et al., 1970; Bode et al., 1973). Woods et al.,(1970) also showed the degradation of adenine nucleotides and accumulation of IMP in the perfused liver treated with fructose.

When ATP levels are depleted, two ADP molecules combine in the presence of adenylate kinase (E.C.2.7.4.3) to yield ATP and AMP. The

resulting AMP can be catalysed by either of two pathways (figure 4) which lead to the formation of uric acid and allantoin. These end products thus come from the nucleotide pool as a whole since adenylate kinase maintains an equilibrium between the nucleotides. The amino group of the adenine nucleotide can be removed either directly by AMP deaminase (E.C. 3.5.4.2) or following hydrolysis of the phosphate group by 5' nucleotidase (E.C. 3.1.3.5). In the latter case the enzyme responsible for the deamination is adenosine deaminase (E.C. 2.5.4.4). The common product of both pathways is inosine, which is further metabolised to hypoxanthine and ribose-1-phosphate by purine nucleoside phosphorylase (E.C. 2.4.2.1). Hypoxanthine is converted to xanthine and uric acid by xanthine oxidase (E.C.1.2.3.2) and further metabolised by uricase (E.C.1.7.3.3) to allantoin in rats.

Adenine nucleotide catabolism *in vivo* is kept to a minimum by inhibition of the enzymes AMP deaminase by Pi (Nikiforuk & Colowick, 1956), and 5'-nucleotidase by ATP (Baer *et al.*, 1966). The precise role of these enzymes was studied by Van den Berghe *et al.*, (1977a), (1977b). An investigation of the kinetic properties of the 5'-nucleotidase present in the cytosol of rat liver showed that when IMP is used as substrate the enzyme displays hyperbolic kinetics; similar kinetics are observed with GMP whereas AMP displays sigmoidal kinetics. At pH 7.4 the enzyme activity with 0.2 mM AMP is undetectable under physiological conditions. This led to the conclusion that *in vivo*, AMP is not hydrolysed by 5'-nucleotidase and that normal as well as fructose-induced catabolism of adenine nucleotides requires prior deamination to IMP (Van den Berghe, 1973).

A study of the kinetic properties of AMP deaminase at physiological substrate concentrations (0.2 mM AMP) reveals that a low activity can be measured which is inhibited by Pi (Van den Berghe *et al.*, 1977a, 1977b). ATP (3mM) increases the enzyme activity 200-fold. Pi alone does not influence the ATP activated enzyme, but GTP (0.5 mM) causes a 60% inhibition. The combined effects of the inhibitors at their

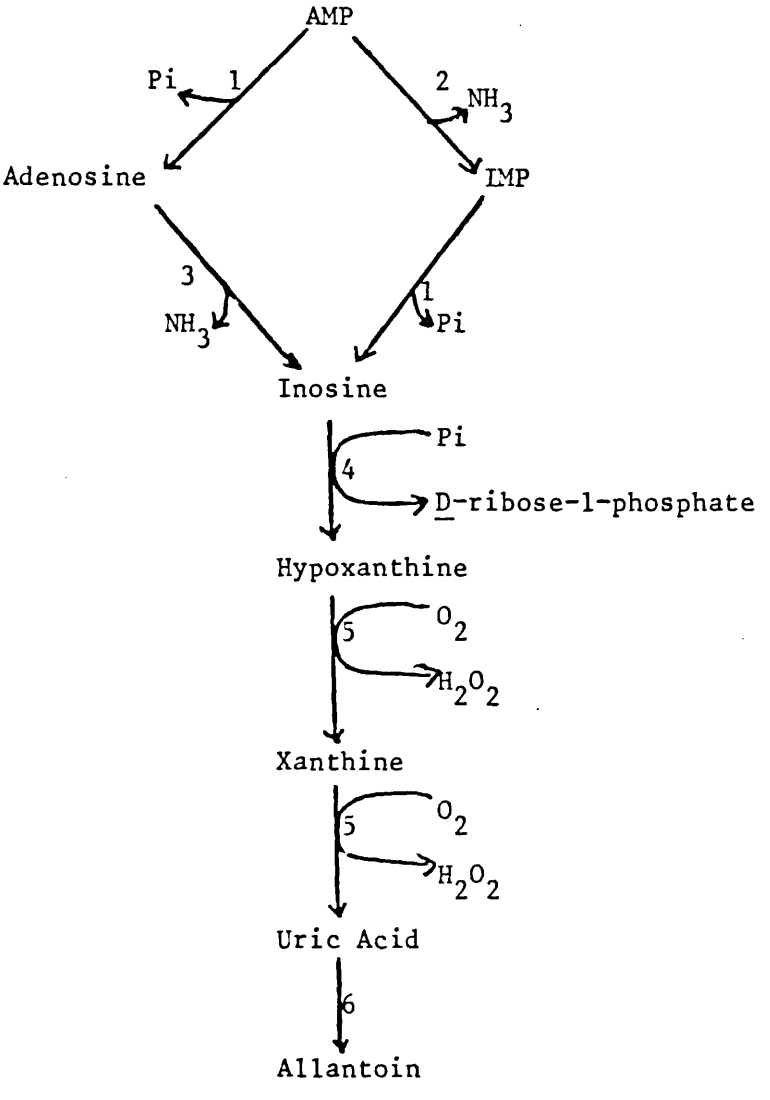


Figure 4. Pathway of AMP degradation in the liver

1. 5'-nucleotidase
2. AMP deaminase
3. adenosine deaminase
4. purine nucleoside phosphorylase
5. xanthine oxidase
6. uricase

physiological concentrations cause a 95% inhibition of the enzyme. Thus Van den Berghe and co-workers proposed that the rapid degradation of adenine nucleotides that occurs after a fructose load is caused by a decrease in the concentration of both AMP deaminase inhibitors, Pi and GTP. This activation is then counteracted by the decrease in the concentration of AMP deaminase stimulator, ATP. The increase in IMP concentrations, initially described by Woods et al., (1970) provides further evidence that the pathway of AMP deamination occurs through AMP deaminase in vivo. Accumulation of adenosine cannot be detected in isolated hepatocytes treated with fructose (Smith et al., 1977a, 1977b).

Uric acid is formed from hypoxanthine by two consecutive reactions catalysed by xanthine oxidase. This enzyme loses its ability to catalyse the oxidation reaction when it is incubated with allopurinol (4-hydroxypyraxolo(3,4-d)pyrimidine). Allopurinol is an analogue of hypoxanthine which is converted to oxipurinol (4,6-dihydroxyparazolo(3,4-d)pyrimidine) by this enzyme (Spector & Johns, 1970). Both allopurinol and oxipurinol inhibit xanthine oxidase (Elion et al.,1963).

Uric acid constitutes the end product of the catabolism of adenine nucleotides in humans and higher apes which lack uricase (Simkin, 1972). In species lacking this enzyme, the catabolism of AMP is normally kept to a minimum because the low solubility of uric acid, coupled with limited renal excretion constitutes a potential danger of precipitation of crystals with the damaging consequences found in gout. In experimental animals (e.g.rats) that possess uricase, the administration of a fructose load causes an increase in plasma allantoin concentration in addition to increased uric acid levels (Menpa et al., 1968; Kekomaki et al., 1972). Smith et al., (1977b) also reported that incubating isolated hepatocytes with fructose causes an accumulation of allantoin.

5. Glycogen Metabolism

Fructose has been shown to activate the enzyme cascade responsible for glycogen breakdown, whereas glucose promotes glycogen

synthesis (Miller, 1978). These findings are in contrast to the suggestion put forward by Seglen (1974), that fructose is a good glycogen precursor. In the perfused liver, the activation of phosphorylase by fructose appears to be transient (Walli et al.,1975; Jakob, 1976) and to be followed by an inactivation of the enzyme; whereas in vivo a late inactivation of phosphorylase by fructose in mouse liver has been reported (Van den Berghe et al., 1973; Thurston et al., 1974). The activation of phosphorylase by fructose in isolated hepatocytes was explained by Miller (1978), to be the result of increased cAMP levels. This mechanism was found to be applicable only at high fructose concentrations (about 30 mM) (Van de Werve & Hers, 1979). Another factor that can be considered is the depletion of hepatic Pi and ATP which occurs after a fructose load. Liver and muscle phosphorylase kinase are optimally active at an ATP/Mg ratio of about 0.5 (Van de Werve & Hers, 1979). In the liver the ATP is present mainly as an ATP/Mg (1:1) complex with only slight magnesium excess (Veloso et al., 1973), such that the depletion of ATP could result in an increase of free Mg^{2+} thus shifting the ratio towards 0.5, which results in the activation of phosphorylase. This was confirmed by Wood et al., (1981) who observed that low concentrations of fructose stimulate glycogen synthesis whereas high concentrations (above 5 mM) are inhibitory.

6. Other fructose effects

Studies with humans and animals have indicated that serum lipid levels are affected by the type of carbohydrate in the diet. A high fructose diet (Zakim et al., 1967),as well as addition of the ketose to the perfused liver (Söling et al., 1970) increase the hepatic concentration of acetyl CoA by 40-60% compared to controls perfused with glucose. This effect can be attributed to the high rate of fructolysis as compared to glycolysis. Harris, (1975) concluded that the rate of de novo fatty acid synthesis by isolated hepatocytes is dependent on the accumulation of pyruvate and lactate. This was also confirmed by Walli (1978), using isolated perfused liver.

The rate of disappearance of blood ethanol has been shown to be stimulated by fructose. Intravenous injection of the ketose increases the ethanol elimination rate by 25-80% (Lowenstein et al., 1970; Brown et al., 1972), whereas oral ingestion is less efficient (Pawan, 1968; Soterakis & Iber, 1975). Thieden et al., (1972) using isolated perfused liver demonstrated that this effect was due to a stimulation of ethanol oxidation by fructose. The rate-limiting step in the metabolism of ethanol is the formation of acetaldehyde, catalysed by NAD^+- dependent alcohol dehydrogenase (E.C.1.1.1.1). The maximum activity of this enzyme in liver slices (Videla & Israel, 1970) and perfused liver (Lindros et al., 1972) is approximately twice the rate of ethanol metabolism and it is widely accepted that the capacity to reoxidize NADH is rate limiting (Lieber & De Carti, 1968). Addition of fructose lowers the cytosolic $(NADH)/(NAD^+)$ ratio (Thieden & Lundquist 1967) which is raised 5 to 10 fold as a consequence of ethanol metabolism (Forsander et al., 1965; Krebs et al., 1969).

Essentially two mechanisms have been proposed to explain the influence of fructose on ethanol metabolism, depending upon whether the effect is thought to be exerted on the cytoplasmic reoxidation of NADH or on the respiratory chain. Extramitochondrial reoxidation of NADH can be accomplished by the reduction of fructose and its metabolites, DHAP and D-glyceraldehyde, to sorbitol, G3P and glycerol. However, measurements of the production of glycerol and sorbitol from fructose by the perfused liver (Scholz & Nohl, 1976) and calculations based on the kinetic parameters of the enzymes involved (Thieden et al., 1972) indicates that the formation of sorbitol and glycerol accounts for the utilization of only 30% of the reducing equivalents provided by the supplementary oxidation of ethanol. The other possible mechanism for the reoxidation of cytoplasmic NADH is involved with the coupling of cytoplasmic NADH reoxidation to the reduction of oxaloacetate to malate by malate dehydrogenase (see figure 5B). The second postulated mechanism is the reoxidation of NADH by the respiratory chain. Fructose increases the hepatic consumption of oxygen by up to 100% in vivo (Tygstrup et al., 1965), in slices (Thieden & Lundquist,

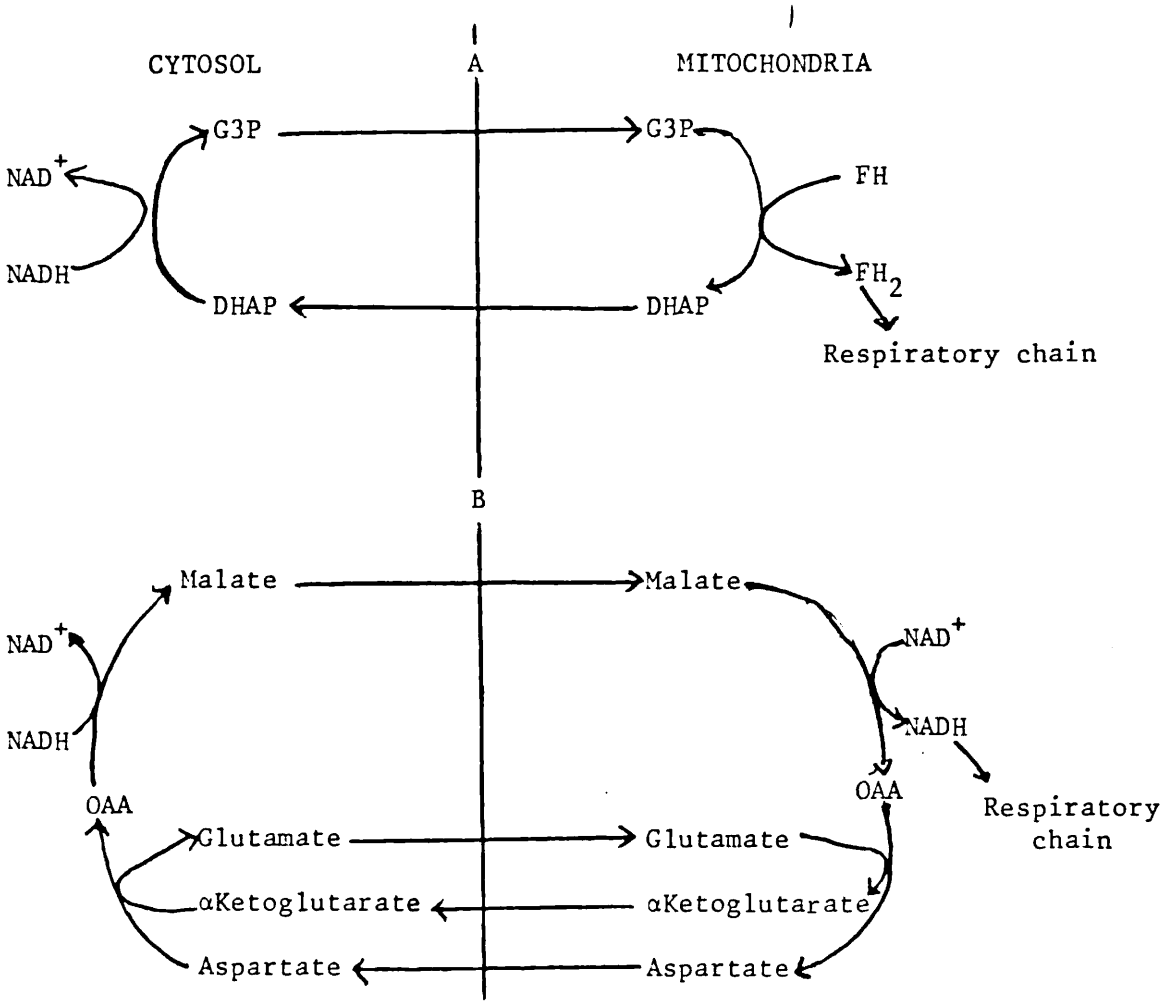


Figure 5. Shuttle mechanisms proposed for the transport of reducing equivalents into the mitochondria. (OAA - oxaloacetate; FH - flavine nucleotide (half-reduced form); FH_2 - reduced flavine nucleotide).

A - glycerol-3-phosphate shuttle;

B - malate aspartate shuttle.

1967), in the perfused liver (Sestoft et al., 1972; Zehner et al., 1973; Sestoft, 1974) and in isolated cells (Seglen, 1974). Ethanol by itself has no effect on this parameter (Forsander et al.,1965) but if fructose is added while ethanol is being metabolised the increase in oxygen consumption is approximately doubled as compared with the fructose effect alone (Thieden & Lundquist, 1967). Scholz & Nohl, (1976) proposed that fructose stimulates the oxidation of ethanol by enhancing the hepatic energy utilization, which is reflected by increased oxygen consumption.

E. D-GLYCERALDEHYDE, DIHYDROXYACETONE AND GLYCEROL METABOLISM

In contrast to fructose, D-glyceraldehyde is not a normal dietary constituent. However, it is important as a natural product mainly because it is formed enzymatically from fructose (figure 3). This product appears to be metabolised by three different routes which are; (i) direct conversion into D-glyceraldehyde-3-phosphate by triokinase; (ii) reduction to glycerol by NADH- or NADPH- linked alcohol dehydrogenase (E.C.1.1.1.1 or E.C.1.1.1.2) followed by phosphorylation via glycerokinase (E.C.2.7.1.30); (iii) oxidation to D-glycerate by aldehyde dehydrogenase (E.C.1.2.1.2), followed by phosphorylation to 2-phosphoglycerate by glycerate kinase (E.C.2.7.1.31). The first route is believed to be favoured (Burch et al., 1970) because fructose is utilized more rapidly than glycerol. The demonstration of a D-glyceraldehyde K_m (0.01 mM) for triokinase, in contrast to the higher K_m values for aldehyde dehydrogenase (0.3 mM) NAD^+-linked alcohol dehydrogenase (11 mM) and $NADP^+$ -linked alcohol dehydrogenase (0.6 mM) also points to preferential transformation of D-glyceraldehyde into D-glyceraldehyde-3-phosphate (Veneziale, 1976).

Dihydroxyacetone is phosphorylated by triokinase in the presence of ATP as the phosphate donor to yield DHAP. A triokinase preparation from rat liver exhibits a V_{max} for dihydroxyacetone, which is 2-5 times greater than the V_{max} for D-glyceraldehyde

(Frandsen & Grunnet, 1971). The effect of a dihydroxyacetone load on hepatic adenine nucleotides was studied by Woods & Krebs, (1973) who concluded that in the perfused liver there is no significant decrease of adenine nucleotides.

The enzyme which catalyses the phosphorylation of glycerol is glycerokinase. This enzyme has been found to be inhibited by high levels of glycerol when the concentration of ATP is low (Robinson & Newsholme, 1969). The physiological significance of this inhibition may be to prevent phosphorylation of glycerol when ATP concentration is low (e.g. in ischemia, or after a fructose or glycerol load). Glycerol effects on hepatic metabolite levels have been examined by Burch et al. (1970) who concluded that following a glycerol load, large increases in G3P are observed which are not followed by a rise in the DHAP levels. These findings have been confirmed by Woods & Krebs, (1973).

The limiting step in hepatic glycerol utilization is clearly G3P oxidation. This involves the enzyme glycerol-3-phosphate dehydrogenase (E.C. 1.1.1.8) which exists as two distinct forms. One form is associated with the outer part of the inner membrane of the mitochondria (Green, 1936), whilst the other form is found in the cytoplasm (Ho & Pace, 1958). The NADH produced during glycerol metabolism by the cytoplasmic enzyme has to be reoxidized if G3P oxidation is to be maintained. Thus the limiting factor in glycerol metabolism is the rate of NADH reoxidation. Since pyridine nucleotides cannot cross the mitochondrial membrane (Lehninger, 1951), cytoplasmic NADH produced cannot be directly oxidized by the mitochondria. This is partially overcome by the G3P shuttle (figure 5A). Glycerol-3-phosphate dehydrogenase in the cytoplasm can reduce DHAP to G3P and oxidize NADH to NAD^+ (cytoplasmic glycerol-3-phosphate dehydrogenase at physiological pH favours the production of G3P from DHAP). This G3P can then move into the mitochondria (Klingenberg & Bucher, 1960). Inside the mitochondria G3P is oxidised by a flavin - containing glycerol-3-phosphate dehydrogenase. The reduced flavin can then be oxidized via the respiratory chain. The net result of this shuttle is to transfer excess reducing equivalents from the cytosol to the mitochondria.

The other possible mechanism for the import of excess NADH from the cytosol into the mitochondria is via the malate-aspartate shuttle (figure 5B) (Cederbaum et al., 1973; Rognstad & Clark, 1974). In this shuttle malate enters the mitochondria where oxaloacetate can be synthesized in a reaction catalysed by malate dehydrogenase and coupled to NAD^+ reduction. Since the mitochondrial membrane is not freely permeable to oxaloacetate (Haslam & Krebs, 1968), it is first transaminated to aspartate in the mitochondria and the products of the transamination (i.e. aspartate and α-ketoglutarate) are exported to the cytosol where they re-form oxaloacetate via cytosolic glutamate-oxaloacetate transaminase (E.C. 2.6.1.1). The net result of this shuttle is the transfer of excess cytosolic NADH to the mitochondria and its oxidation via the respiratory chain.

F. XYLITOL AND SORBITOL METABOLISM

The liver cell is permeable to both these reduced substrates which enter the glycolytic-gluconeogenic sequence after an oxidation and a phosphorylation step (figure 3 and figure 6).

In the liver xylitol is first oxidised to D-xylulose by the NAD^+ linked xylitol dehydrogenase causing the cytoplasmic (NADH)/(NAD^+) ratio to increase (Williamson et al , 1971). The next step is the phosphorylation of xylulose to xylulose-5-phosphate by xylulose kinase (E.C.2.7.1.17). The phosphorylated product is further metabolised by means of the transaldolase and transketolase reactions of the pentose phosphate cycle to D-glyceraldehyde-3-phosphate and Fru-6-P. Xylulose has also been shown to be a good substrate for fructokinase in vitro (Adelman et al., 1967; Raushel & Cleland, 1977). The K_m for the pentose is 0.45 mM (cf to a K_m of 1.0 mM for fructose) and the V_{max} is 65% of the V_{max} with fructose as substrate (Barngrover et al., 1981). The product from this reaction is xylulose-1-Phosphate which can undergo aldolytic leavage yielding DHAP and glycolaldehyde. The

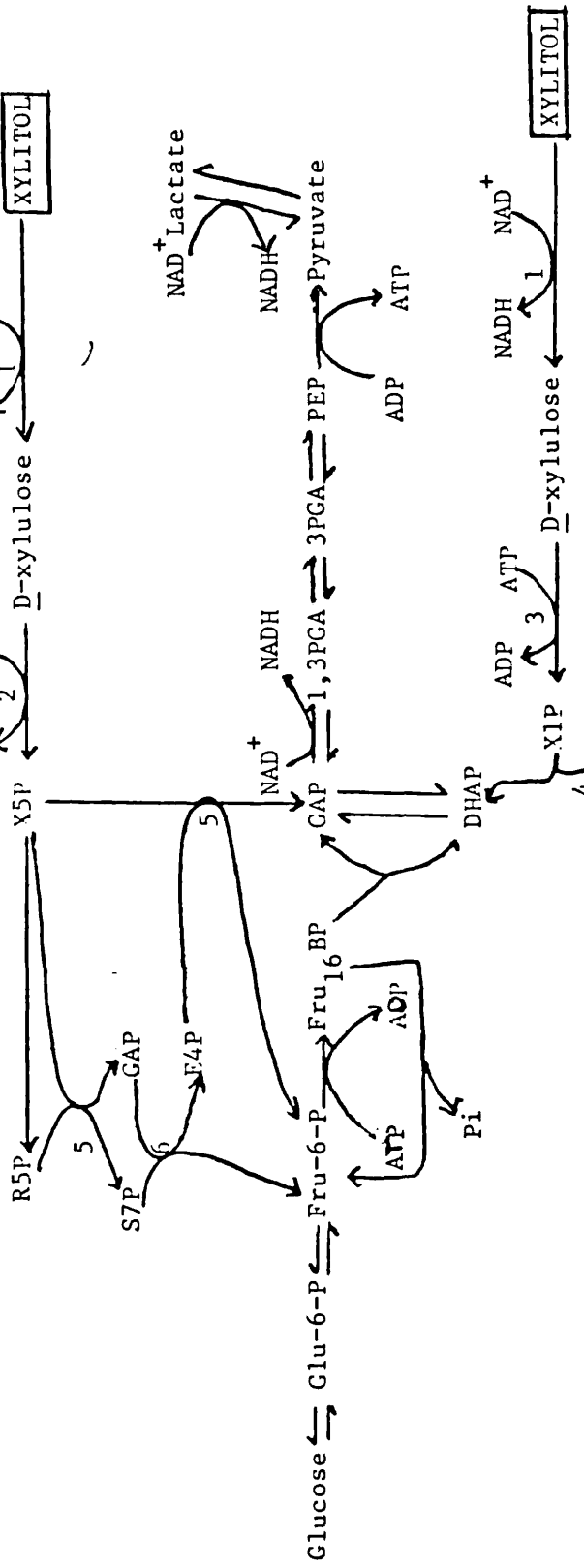


Figure 6. The metabolism of xylitol in the liver(E4P-erythrose-4-phosphate;GAP -glyceraldehyde-3-phosphate; R5P - ribose-5-phosphate; S7P - sedoheptulose-7-phosphate; X1P - xylulose-1-phosphate; X5P - xylulose-5-phosphate; 1,3PGA - 1,3 diphosphoglycerate; 3GPA 3-phosphoglycerate).

1 = xylitol dehydrogenase
2 = xylulose kinase
3 = fructokinase
4 = aldolase
5 = transketolase
6 = transaldolase
7 = pyruvate kinase.

DHAP produced can thus enter the glycolytic/gluconeogenic sequence while glycolaldehyde is further metabolised to oxalate (Gessner et al., 1961; Hodgkinson, 1977; Barngrover et al., 1981) (see also figure 6).

Sorbitol is oxidized to fructose in the liver by NAD^+ linked sorbitol dehydrogenase (E.C.1.1.1.14). The fructose produced is then phosphorylated by fructokinase and follows the remainder of the fructolysis sequence already discussed (figure 3).

II. PYRUVATE KINASE

Pyruvate kinase (ATP: pyruvate 2-O-phosphotransferase) catalyses an undirectional step in the glycolytic sequence. The reaction involves the removal of a phosphate group from PEP and the phosphorylation of ADP yielding pyruvate and ATP. This enzyme has a central role in the regulation of both glycolysis and gluconeogenesis and is regulated by a number of allosteric and covalent modifications (Seubert & Schoner, 1971; Engström, 1978; Pilkis et al., 1978a).

A. MULTIMOLECULAR FORMS OF PYRUVATE KINASE

Kinetic studies of PK from rat and human tissues have led to the identification of three classes of isoenzymes with different electrophoretic, immunological and kinetic properties (Tanaka et al., 1965; Susor & Rutter, 1968; Imamura & Tanaka et al., 1972; Imamura et al., 1972). These isoenzymes have been designated M-, A- and L- type PK.

Type M is present in muscle, brain and a small proportion has also been found in the liver. Studies on the kinetic properties of M-type PK from rat liver have shown that it is similar to the muscle enzyme; plots of initial velocities versus PEP or ADP concentrations give hyperbolic curves (Jiménez De Asúa et al., 1971; Muirhead et al., 1981). Muscle enzyme has been shown to be inhibited by phenylalanine; this inhibition is pH dependent (Gregory & Ainsworth,1981)

and can be reversed by cysteine, serine and alanine (Rozengurt et al., 1970; Kwan & Davies, 1981). In contrast to this the liver M-type enzyme is inhibited not only by phenylalanine but also by alanine, valine, tyrosine, proline, threonine and tryptophan. The inhibition by phenylalanine and alanine of the liver M-type PK is only slightly pH dependent (Jiménez De Asúa et al., 1971). The PK from cardiac tissue was shown to have physical properties similar to those of the muscle enzyme but it displays different kinetic properties with regard to activation by Fru_{16} BP and inhibition by phenylalanine and alanine (Flanders et al., 1971).

Type A PK is present as a major component in adipose tissue and kidney and is a minor component in the liver. It shows slightly sigmoidal substrate saturation curves, is allosterically inhibited by alanine and activated by $Fru_{16}BP$ (Carbonell et al., 1973); Van Veelen et al., 1981).

Type L is the major component of liver PK and a minor component in the case of the kidney and intestine. A modified type L PK is also present in the erythrocytes (Nakashima, 1974; Lincoln et al., 1975; Marie et al., 1976; Kahn et al., 1978). Type L PK shows markedly sigmoidal kinetics with respect to the concentration of PEP; allosteric inhibition by ATP and alanine; and allosteric activation by $Fru_{16}BP$ (Tanaka et al., 1967a; Seubert & Schoner, 1971). Thus in contrast to the type M enzyme, type L is an allosteric enzyme which is under hormonal and dietary control.

In order to explain the existence of two different species of PK with different regulatory properties in one organ, different compartmentation either within the cells or between the various hepatic cell types is postulated. Type L PK was shown to be found exclusively in the parenchymal cells while type M was restricted to the non-parenchymal cells (Crisp & Pogson, 1972; Van Berkel et al., 1972; Bonney et al., 1973). Furthermore, the capacity for gluconeogenesis and

glucokinase have also been shown to be confined to the parenchymal cells of the rat liver by these workers. Thus, there may be two kinds of glycolytic pathways in the liver; one which utilizes hexokinase and type M PK and has been termed the basal pathway; the other involves glucokinase and type L PK and is probably the regulatory pathway (Tanaka et al., 1967b; Van Berkel et al., 1972). The localization of these two pathways is further emphasized by the evidence that gluconeogenesis, glucokinase, and L-type PK are confined to the parenchymal cells and these cells respond to metabolic changes in the organ.

B. MOLECULAR PROPERTIES OF L-TYPE PYRUVATE KINASE

A highly purified PK preparation was first obtained by Tanaka et al., (1967b) who crystallized the rat liver enzyme. Later, bovine (Cardenas & Dyson, 1972); pig (Kutzbach et al., 1973); chicken (Eigenbrodt & Schoner, 1975) and human (Marie et al., 1976) PK's were also purified. The molecular weight of the L-type enzyme has been reported to be 200,000-228,000 (Tanaka et al., 1967b; Bischofberger et al., 1970; Ibsen & Trippet, 1972; Cardenas & Dyson, 1973; Riou et al., 1978). The enzyme has a quaternary structure with identical subunits. The molecular weight of the subunits has been estimated to range from 54,000 to 62,000 using either an SDS-polyacrylamide gel technique or sedimentation equilibrium in guanine hydrochloride (Kutzback & Hess, 1970; Cardenas & Dyson, 1973; Ljungström et al., 1974; Riou et al., 1978).

C. REGULATION OF L-TYPE PYRUVATE KINASE

Recent studies have provided evidence that the pyruvate/PEP substrate cycle is very important in the regulation of gluconeogenesis (see section I.C.1). PK is also involved in the formation of various metabolic products from carbohydrates, thus making its regulation an important concept in liver metabolism.

1. Allosteric Activation

Stimulation of L-type PK by $Fru_{16}BP$ was first observed by Hess et al., (1966); Tanaka et al., (1967a) and Taylor & Bailey, (1967). The stimulatory effects of $Fru_{16}BP$ depend not only on the concentration of the effector but also on PEP concentrations and pH (Seubert & Schoner, 1971). The interrelationship between PEP and $Fru_{16}BP$ is best illustrated by plotting reaction velocity and substrate concentrations. In the absence of $Fru_{16}BP$, the curve for the initial velocity against PEP concentrations is sigmoidal and in the presence of $Fru_{16}BP$, the response to PEP concentrations is transformed to give a Michaelis-Menten type curve (Rozengurt et al., 1969; Taylor & Bailey, 1967). This effect of $Fru_{16}BP$ is apparent with both phosphorylated and unphosphorylated enzyme (Ekman et al., 1976). Changes in the pH strikingly alter the response to $Fru_{16}BP$. Taylor & Bailey (1967) using a crude preparation of PK showed that at pH 6.0 only a small increase in activity was observed in response to 0.5 mM $Fru_{16}BP$, whereas at pH 8.0 a 20-fold increase was observed. The pH optimum for PK activity in the presence of $Fru_{16}BP$ lies between pH 7.0 - 7.5 and in its absence is about pH 6.5.

Fru-1-P at concentrations which occur in the rat liver during perfusion with 10 mM fructose is also an activator of PK (Eggleston & Woods, 1970). This activation of PK by Fru-1-P is greatest at physiological PEP concentrations (i.e. PEP concentrations which are present in fresh freeze-clamped or perfused liver). At a higher PEP concentration (0.8 mM), Eggleston & Woods, (1970) found that activation by Fru-1-P is abolished. Koster et al., (1972) observed that $Glu_{16}BP$ also stimulates L-type PK. Maximum stimulation of the enzyme was obtained with 1 mM $Glu_{16}BP$. However, it is unlikely to have any physiological significance because of low levels of this intermediate in vivo.

An effect of Pi on L-type PK has been observed by Koster & Hulsman (1970), who found that depending on the substrate concentrations Pi can either stimulate or inhibit the enzyme. When PEP

concentrations are kept constant and ADP concentrations varied, Pi can activate PK at high ADP concentrations, whereas at low ADP concentrations an inhibitory effect is apparent. The physiological relevance of such effects is doubtful since a relatively high concentration of Pi was required to produce these effects.

Only the feed-forward activation of the enzyme by $Fru_{16}BP$ (and possibly Fru-1-P) have been considered to have any physiological significance. However, 6-phosphogluconate, an intermediate of the pentose phosphate pathway, has recently been shown to activate L-type PK (Smith & Freedland, 1979; 1981). This activation is considered to be a means of communication between the two pathways central to fatty acid synthesis. The physiological significance to this effect is confirmed by the observations that the stimulatory effect of 6-phosphogluconate is apparent using concentrations of the effector which occur *in vivo* and that in the physiological range of PEP concentrations, 6-phosphogluconate has the greatest effect on PK from sucrose-fed rats and the least effect on the enzyme from 48 h starved rats (Smith & Freedland,1979). Furthermore, incubation of isolated hepatocytes with 6-aminonicotinamide, an inhibitor of 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) causes the accumulation of 6-phosphogluconate, and also increases the flux of PEP through PK (Smith & Freedland, 1981).

L-type PK has also been shown to have an absolute requirement for mono- and divalent ions (Seubert & Schoner, 1971). K^+ is known to stimulate the enzyme (Irving & Williams, 1973); the optimum activation is obtained with 50 mM K^+. Ekman *et al.*, (1976) and Ljungström *et al.*, (1976) observed that both the phosphorylated and unphosphorylated PK were dependent on K^+. The stimulatory effect of K^+ is increased by $Fru_{16}BP$ (Jiménez De Asúa *et al.*, 1970). The requirement of PK for monovalent ions can also be fulfilled by NH_4^+ (Jiménez De Asúa *et al.*, 1970). Mg^{2+} has also been shown to be necessary for PK activity (Carminatti *et al.*, 1968); the optimum concentration which gives maximum activation of PK is about 7 mM

(Ljungström et al., 1976). Higher concentrations of Mg^{2+} were inhibitory and the inhibition is almost complete at concentrations 10-fold higher than the optimum. The activating effect of Ca^{2+} (1 mM) is absolutely dependent on Mg^{2+} concentration (Gabrielli & Baldi, 1972). In the absence of Mg^{2+}, PK has no catalytic activity even in the presence of Ca^{2+}. The activation by Ca^{2+} is only observed at low PEP concentrations. In the presence of $Fru_{16}BP$ (0.1 mM), Ca^{2+} ions are inhibitory at all PEP concentrations.

2. Allosteric Inhibition

ATP is thought to be an important inhibitor of L-type PK in vivo, since the enzyme is inhibited by physiological concentrations of the nucleotide (Llorente et al., 1970; Seubert & Schoner, 1971). The sigmoidal kinetic properties in response to increasing concentrations of PEP is enhanced in the presence of ATP, an effect which can be reversed by $Fru_{16}BP$ (Llorente et al., 1970). GTP has also been reported to be an inhibitor of chicken liver PK (Williams et al., 1969).

A number of amino acids such as alanine, cysteine, proline, serine, tyrosine, valine and phenylalanine have been reported to inhibit L-type PK. However, of these amino acids, only alanine inhibited the enzyme in the concentration range found under physiological conditions (Seubert et al., 1968; Weber et al., 1968b; Schoner et al., 1970). The inhibitory action of alanine is stereospecific for the L-isomer; D-alanine and β-alanine are without effect (Schoner et al., 1970). Alanine only inhibits PK at subsaturating PEP concentrations; the inhibition is competitive with respect to the substrate. The inhibitory action of alanine can be counteracted by physiological concentrations of $Fru_{16}BP$ (Llorente et al., 1970; Carbonell et al., 1973).

Llorente et al., (1970), found that a 50% inhibition of the enzyme was observed with concentrations of 0.1 mM alanine and 0.5 mM ATP, which are both below their physiological concentration range

(0.5 to 1.5 mM for alanine; Williamson et al., 1967b; Brosnan et al., 1970; Schoner et al., 1970;and 2 to 3 mM for ATP; Bücher et al., 1964; Schoner et al., 1970). ATP and alanine have also been shown to inhibit both the phosphorylated and unphosphorylated PK (Ekman et al., 1976; Ljungström et al., 1976).

Therefore it is clear that L-type PK is allosterically inhibited by ATP and alanine and activated by $Fru_{16}BP$. The presence of physiological concentrations of these effectors can have profound effects on the degree of co-operativity displayed by the enzyme as well as on the $S_{0.5}$ for PEP. With physiological concentrations of ATP, alanine and PEP, it can be predicted that the enzyme would be completely inhibited in the absence of $Fru_{16}BP$. Thus the activity of PK in vivo probably depends on the concentration of $Fru_{16}BP$ available to bind to PK. However, the concentration of $Fru_{16}BP$ in the cell is such that it would completely activate the enzyme even in the presence of physiological concentrations of ATP and alanine. $Fru_{16}BP$ reverses the effects of ATP and alanine on both the phosphorylated and unphosphorylated enzyme (Ekman et al., 1976; Ljungström et al., 1976). Therefore the regulation of L-type PK in vivo is mainly dependent on the concentrations of ATP, alanine and $Fru_{16}BP$ as well as the phosphorylation state of the enzyme.

Other metabolites which have been reported to inhibit PK are the free fatty acids (FFA), which also stimulate glucose production by the perfused liver (Williamson et al., 1968 ; Friedman et al., 1965). In addition to stimulation of gluconeogenesis, the effect of FFA may also be due to a direct inhibition of PK (Lea & Weber, 1968; Weber et al., 1968a). Quantitative studies by Weber et al., (1968b) revealed that long-chain FFA were more effective inhibitors than medium-chain FFA. The inactivation is time-dependent and irreversible. FFA also inhibit the other key glycolytic enzymes (glucokinase and PFK 1), but their selectivity was questioned when they were found to inhibit a gluconeogenic enzyme (Pande & Mead, 1968). These authors concluded that FFA are unspecific inhibitors and suggested detergent properties of the FFA to be the cause of their inhibitory action.

Acetyl-CoA is an end product of FFA metabolism and its levels increase in gluconeogenic conditions (Williamson et al., 1966); it seemed possible that it could exert an inhibitory action on some key glycolytic enzymes. Weber et al., (1968b) studied the inhibition of PK by acetyl-CoA and showed that it was time dependent, thus it is obvious that a chemical modification of the enzyme rather than an allosteric effect is responsible for the alteration of the catalytic properties of PK. As with FFA the interaction of acetyl-CoA with PK is irreversible.

Oxalate exerts a dual effect on PK.In the absence of $Fru_{16}BP$ and at low PEP concentrations, oxalate behaves as an allosteric activator. In the presence of $Fru_{16}BP$ and at low PEP concentrations it is a powerful competitive inhibitor with respect to PEP (Buc et al., 1978; 1981).

PK has been shown to be sensitive to Cu^{2+} (Passeron et al., 1967; Bailey et al., 1968a; Carminatti et al., 1968). At low PEP concentrations type L is strongly inhibited by Cu^{2+}. The inhibition produced by 6.6×10^{-3} M Cu^{2+} can be reversed by chelating agents such as EDTA and EGTA. $Fru_{16}BP$ also reverses the inhibition and it is by far the most effective, since more than 60% reactivation can be obtained at a $Fru_{16}BP$ concentration five times lower than that of Cu^{2+}. This indicates that $Fru_{16}BP$ is not acting as a simple chelating agent, but rather has a direct effect on the enzyme. The sensitivity of the enzyme towards Cu^{2+} is greatly increased at high Cu^{2+} concentrations (Carminatti et al., 1968).

3. Effect of pH

The allosteric properties of L-type PK are strongly affected by changes in pH (Rozengurt et al., 1969). The enzyme obeys Michaelis-Menten kinetics at pH 5.9 with respect to PEP and there is little activation by $Fru_{16}BP$. As the pH is raised, the kinetic response to increasing PEP concentrations becomes increasingly sigmoidal and $Fru_{16}BP$ activation is more pronounced. This altered kinetic profile

suggests an activation of an ionizing group (possibly imidazole) having a pK value around 7.0 (Irving & Williams, 1973). The inhibitory action of alanine and ATP is also modified by changes in pH; the inhibition is reduced at low pH values (Schoner et al., 1970).

4. Effect of Temperature

The kinetic behaviour of PK is affected by temperature. If crude liver extracts are obtained at room temperature, instead of the usual 2-4°C, the extracts show marked sigmoidal kinetics. Storage of the enzyme extracts in the cold diminishes the sensitivity of the PK to the inhibitory action of alanine and ATP. Resensitization to almost 90% of the original activity is observed upon incubation of the extracts at 37°C (Llorente et al., 1970). Van Berkel et al., (1975) observed that ATP and alanine both stabilize PK when assayed at 53°C. The sensitivity of PK to $Fru_{16}BP$ in warmed extracts is lost on cooling and can be recovered upon rewarming (Llorente et al.,1970).

5. Hormonal Regulation

The activity of key glycolytic and gluconeogenic enzymes can be changed rapidly by administration of hormones (Taunton et al., 1972; 1974; Stifel et al., 1974).

Administration of glucagon reduces flux through PK, thus diminishing recycling and increasing the conversion of PEP to glucose. This effect is consistent with the observations made by Exton & Park (1969), who found a fall in pyruvate and a rise in PEP steady-state concentrations in livers perfused with the hormone or with cAMP. These findings indicated that one site of action of glucagon involves the regulation of PK. Further investigations performed by independent workers demonstrated conclusively that addition of glucagon to the perfused rat liver (Blair et al., 1976) or to isolated hepatocytes (Felíu et al., 1976; Foster & Blair, 1976; Friedrichs, 1976; Pilkis et al., 1976a; 1976b; Riou et al., 1976; Van Berkel et al., 1976; 1977a; 1977b) results in the inhibition of PK assayed at subsaturating PEP concentrations. When the PK activity was determined at saturating PEP concentrations,

the hormonal influence was not detected. The regulation of PK by glucagon, therefore, does not alter the total enzyme content in the liver, but rather appears to influence the dependence of the enzyme activity on PEP concentrations. PK inactivated by low glucagon concentrations can be rapidly reactivated by the addition of insulin (Felíu et al., 1976). In the presence of maximally effective concentrations of $Fru_{16}BP$ (20 μM), 10 nM glucagon has no effect on PK activity (Riou et al., 1976). These workers also reported that the maximum stimulation of the enzyme by $Fru_{16}BP$ in the control was only 1.4-fold but was 6-fold in the extracts of glucagon-treated hepatocytes. PK from glucagon-treated hepatocytes is further inhibited by ATP and alanine (Riou et al., 1976).

Cyclic nucleotides, such as, cAMP and dibutyryl cAMP have been shown to have the same effect as glucagon (Felíu et al., 1976; Van Berkel et al., 1976; Pilkis et al., 1978b). This is consistent with the suggestion that cAMP is a second messenger (Robison et al., 1971) and any changes observed after glucagon administration are due to an increase in cAMP levels. There is strong evidence that the effect of glucagon is mediated via a cAMP-dependent protein kinase (see section II.E.1).

Catecholamines have also been reported to inhibit hepatocyte PK. The inhibition is quite small compared to that observed with glucagon (Felíu et al., 1976). Inhibition of L-type PK is consistent with stimulation of gluconeogenesis by adrenaline reported by Claus & Pilkis, (1976). Insulin can suppress, at least in part, the inhibition caused by maximally effective concentrations of adrenaline (Felíu et al., 1976). In this respect, the adrenaline effect is qualitatively different to that of glucagon; in the latter case only the effect of submaximal concentrations of the hormone can be antagonized by insulin. The inhibition is associated with an increase in the $S_{0.5}$ for PEP (Felíu et al., 1976) which suggests phosphorylation of PK. The mechanism is possibly mediated via both α- and β-receptors (see section II.E.2).

Insulin in its role of homeostatic regulator of the blood glucose levels exerts its effects on liver metabolism where several enzyme changes are well documented (Pitot & Yatvin, 1973). In general, insulin promotes the synthesis of enzymes associated with carbohydrate utilization and storage and represses the synthesis of enzymes associated with glucose synthesis. The role of insulin in induction of PK was studied by Weber et al., (1965b). They observed that PK activity was low in alloxan-diabetic rats, but was returned to normal following insulin injection. Further investigation showed that insulin induction of PK was blocked by ethionine (an inhibitor of protein synthesis) and actinomycin D (an inhibitor of RNA synthesis), suggesting that the insulin-induced rise in this enzyme is due to the de novo synthesis of enzyme which is dependent on the synthesis of new RNA.

The short term effects of insulin on PK were demonstrated by Taunton et al., (1974), who injected insulin into the portal vein of experimental animals and detected a rapid effect involving the activation of hepatic PFK 1 and PK. The effect was maximal after 10 min and gradually declined over 30 min. Pre-treatment of the rat with actinomycin D and puromycin did not alter the response of the enzymes to this hormone, indicating that de novo protein synthesis was not responsible for the changes in enzyme activities. These rapid effects of insulin on PK activity have also been demonstrated in experiments using isolated perfused liver (Blair et al., 1976) and isolated hepatocytes (Felíu et al., 1976).

Insulin alone, slightly increases the activity of PK in the perfused liver compared to the activity found in homogenate from control livers. More pronounced effects are observed in livers which have been treated with low concentrations of glucagon (Blair et al., 1976). This effect of insulin could be explained under some conditions by a fall in the level of cAMP. Another explanation of the insulin effect is that it may increase $Fru_{16}BP$ levels. However, Claus et al.,(1979) reported that even though insulin suppresses the inactivation of PK by glucagon and phenylepherine, it did not change $Fru_{16}BP$ levels.

6. Dietary Regulation

During starvation, hepatic gluconeogenesis is predominant. The glucose produced is used as a source of energy by other non-gluconeogenic tissues (e.g. muscle). Thus, there is an obvious need for hepatic PK activity to be low during starvation (Krebs & Eggleston, 1965; Tanaka et al., 1965). Meal-trained rats fed on a high carbohydrate diet were used to show maximum induction of hepatic PK after 8 days (Hopkirk & Bloxham, 1979). PK is also subject to a diurnal variation. A rapid increase in enzyme activity (V_{max}) also occurs as a response to feeding; this is accompanied by an apparent increase in the affinity of the enzyme for PEP which is consistent with the decreased phosphorylation of the enzyme.

Kohl & Cottam (1976), made similar observations when they starved and re-fed rats on a high carbohydrate and low protein diet. Using a precipitating antiserum directed specifically against hepatic PK, they found that rats maintained on a high carbohydrate diet 4 days prior to sacrifice have at least 20 mg of precipitable hepatic PK protein per liver. Starving the animal results in a marked reduction in hepatic PK, so that after 3 days starvation less than 7 mg of hepatic PK protein per liver remains.Re-feeding the animals results in a return to the normal situation. Furthermore, the specific activity of PK purified from liver fasted for 72 to 84 h is approximately one third of the specific activity of the enzyme isolated from rats fed on a high carbohydrate diet (Kohl & Cottam, 1977). The lower specific activity of the enzyme from fasted rats must reflect a modification of the enzyme (Van Berkel et al., 1978; Hall et al., 1979). It is suggested that phosphorylated PK is more susceptible to proteolytic modification than the dephosphoenzyme and that this factor plays a role in the irreversible deactivation of PK under starvation conditions (Bergström et al., 1978).

D. MECHANISM OF ALLOSTERIC CONTROL

The co-operative kinetics of allosteric enzyme can be explained using the model of Monod et al., (1965). This postulates that two conformational states (R and T) of the enzyme occur which are in equilibrium.These can exhibit different affinities for the substrates and effectors (K system) or the same affinities but a different catalytic efficiency (V system). According to this model, positive effectors, interacting preferentially with the R state, would shift the equilibrium in favour of the state with a higher affinity for the substrate (K system); or the more active form of the enzyme (V system). Negative effectors, interacting preferentially with the T state would shift the equilibrium in favour of the state with a lower affinity (K system) or a lowered catalytic activity (V system).

In the case of PK, these predictions are fulfilled with respect to the interaction of PEP with various effectors(Schoner et al., 1970). In the presence of a positive effector, e.g. $Fru_{16}BP$, the homotropic interaction of PEP is lowered as indicated by a change in the Hill coefficient in the direction of unity (Jiménez De Asúa et al., 1970). On the contrary, the negative effectors, e.g. ATP and alanine, cause a strengthening of the homotropic interaction of PEP as indicated by the change of the Hill coefficients to a greater value (Schoner et al., 1970). These findings are compatible with the model of Monod et al., (1965), suggesting that PK exists as an equilibrium between two conformational states, with various effectors controlling its activity.

$$\text{pyruvate kinase}_{(R)} \underset{Fru_{16}BP, H^+}{\overset{ATP,\ alanine,\ OH^-}{\rightleftharpoons}} \text{pyruvate kinase}_{(T)}$$

Further investigation of the allosteric model of L-type PK revealed that ATP and alanine both stabilize the enzyme, the degree of maximum stabilization for alanine being greater than for ATP (Van Berkel et al., 1975). These workers also observed that further

stabilization occurs when ATP is added to the enzyme maximally stabilized with alanine or vice versa, indicating additive effects of alanine and ATP on the stabilization. These observations reinforce the suggestion made earlier, that alanine and ATP introduce different conformational states, probably by binding to different sites (Van Berkel et al., 1974). Rozengurt et al., (1973) reported that the allosteric activators K^+ and $Fru_{16}BP$ each introduce a different R conformation. These findings on allosteric activation and inhibition are difficult to reconcile with a two-state model of Monod et al., (1965), and suggests that sequential conformational changes are involved in the allosteric transition (Koshland & Neet, 1968; Koshland, 1970).

E. MECHANISM OF HORMONAL REGULATION

The intracellular physiological response to glucagon seems to be mediated by cAMP (Sutherland, 1972), whose main action at the molecular level is to stimulate specific protein phosphorylation (Kuo & Greengard, 1969). Such a phosphorylation of enzymes results in a change in their kinetic properties, as has been clearly demonstrated for phosphorylase kinase and glycogen synthetase (Hers, 1976). The cAMP-stimulated phosphorylation of enzymes and other proteins is reversible because of the presence of phosphoprotein phosphatases. Thus metabolic regulation via phosphorylation-dephosphorylation of PK can occur in vivo.

1. Phosphorylation-dephosphorylation

Highly purified L-type PK from rat liver has been shown to be phosphorylated by ATP in a reaction catalysed by cAMP-dependent protein kinase (Ljungström et al., 1974). Phosphorylation appears to modify the ease with which the enzyme undergoes conformational changes in response to its effectors. The end result is to shift the equilibrium between the active and inactive forms. This hypothesis is illustrated diagramatically in figure 7a.

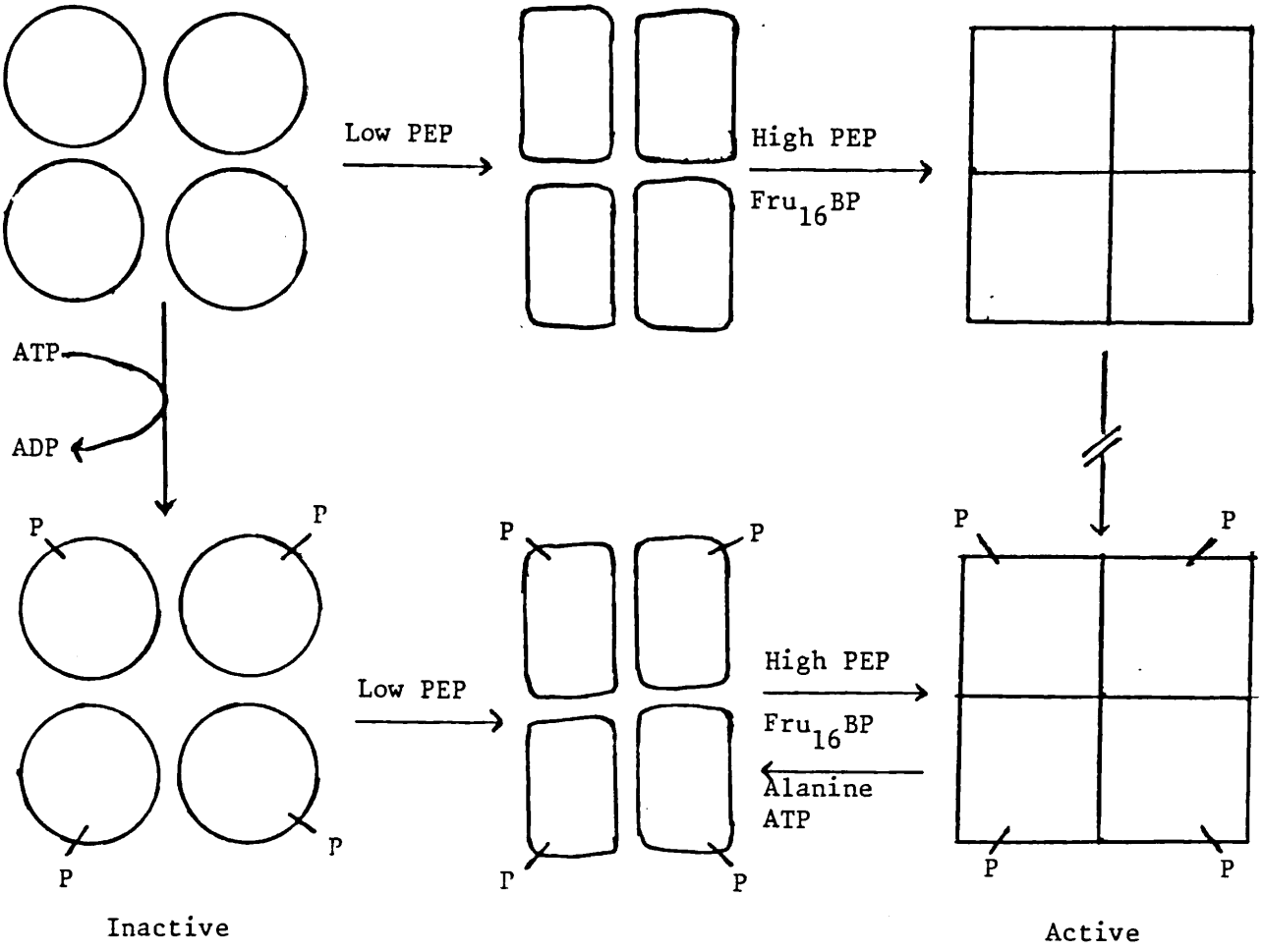


Figure 7a. Schematic representation of the effect of phosphorylation on the equilibrium between active and inactive forms of PK (circles - inactive form of PK; squares - active form of PK; other symbols - transitional forms; P represents covalently bound phosphate groups) (after Pilkis et al., 1978a).

It is postulated that increasing concentrations of PEP and $Fru_{16}BP$ cause a conformational change in the enzyme leading to a more closely associated quaternary structure and to increased activity, whereas ATP and alanine have the opposite effect. Phosphorylation of the enzyme subunits tends to impede this conformational change, resulting in a 'looser' quaternary structure at low PEP concentrations

and to decreased activity. High PEP and $Fru_{16}BP$ concentrations can overcome the effect of phosphorylation. It is also postulated that the active tetrameric form does not undergo phosphorylation (Eigenbrodt & Schoner, 1977a; 1977b).

i) Site of Phosphorylation

One major $[^{32}P]$ phosphopeptide containing $[^{32}P]$ phosphoryl-serine has been isolated from $[^{32}P]$ PK formed by the incubation of the purified enzyme from rat liver (Edlund et al., 1975) or from pig liver (Hjelmquist et al., 1974) with cAMP-dependent protein kinase and $[^{32}P]$ ATP. This established that a specific seryl residue in the PK is phosphorylated. Humble et al., (1975) showed that alkali-inactivated pig liver PK and a peptide obtained by cyanogen bromide treatment could be phosphorylated by $[^{32}P]$ ATP, and in both cases the rate of phosphorylation was higher than that with the native enzyme. The amino acid sequences of the peptides have been determined and are shown to have similarities to peptides from other phosphorylated enzymes (Engström, 1978). The general sequence is that there are two basic residues and either one or two neutral amino acids preceeding the serine which is followed by another neutral amino acid. Hjelmquist et al., (1974) showed that the minimum structural requirements for the phosphorylation of L-type PK were met by the pentapeptide Arg-Arg-Ala-Ser-Val. This was confirmed by Humble (1980) who used chymotrypsin to remove the phosphorylated site of the enzyme. The rate of phosphorylation of these peptides is related to the amino acid sequence surrounding the phosphorylation site (Pilkis et al., 1980). These workers compared the rate of phosphorylation of $Fru_{16}BP$-ase (which contains only one basic residue preceeding serine) and PK (which contains two basic residues preceeding serine). PK is 10 times better as a substrate for phosphorylation than $Fru_{16}BP$ase.

ii) Phosphorylation of pyruvate kinase in vitro

Studies to date have revealed that the rat and pig liver enzymes appear to be phosphorylated by a cAMP-dependent protein kinase (Ljungström

et al., 1976; Riou et al., 1976; Titanji et al., 1976; Berglund et al., 1977). Phosphorylation has been studied using a homogeneous preparation of bovine liver or heart catalytic subunits of cAMP-dependent protein kinase and homogeneous rat liver PK (Pilkis et al., 1978c). In some cases however, crude preparations of protein kinase were used, and it is possible that they contained cAMP-independent as well as cAMP-dependent protein kinase. The possible involvement of an independent kinase in the phosphorylation of PK was studied by Pilkis et al., (1978b), using hepatocyte homogenates that had been gel filtered on Sephadex G-25 in order to remove all low-molecular-weight compounds. The addition of cAMP and ATP-Mg to these extracts produced a time dependent inactivation of L-type PK, whereas the addition of ATP-Mg alone had no effect. The cAMP-induced inactivation could be completely blocked by the addition of a protein kinase inhibitor, a compound specific for the catalytic subunit of cAMP-dependent protein kinase (Walsh et al., 1971). These results demonstrated that cAMP-dependent phosphorylation is linked to enzyme inactivation.

The rate of phosphorylation of PK by a partially purified cAMP-dependent protein kinase is increased by alanine (Berglund et al., (1977). This stimulation is observed at alkaline pH, but it is not clear whether the phosphate was incorporated at the same site or if there was any associated alteration in enzyme activity. Evaluation of these results is clouded by the fact that the protein kinase used was not purified. The effect of allosteric effectors on the phosphorylation of PK was further studied by El-Maghrabi et al., (1980) who used a purified protein kinase. They reported that physiological concentrations of the allosteric effectors PEP and $Fru_{16}BP$ inhibit the rate of phosphorylation catalysed by protein kinase. The negative allosteric effector, alanine, only had a small stimulatiory effect by itself, but it relieved the inhibition of phosphorylation by PEP and $Fru_{16}BP$. The ability of allosteric effectors to modify the phosphorylation state of PK extends considerably the flexibility and sensitivity of its regulation. Thus in addition to the regulation

of PK directly by allosteric effectors, they also act indirectly by modulating the phosphorylation state of the enzyme.

The kinetics of the phosphorylated and dephosphorylated rat liver enzyme have been studied. The major kinetic effect of phosphorylation is to reduce the affinity for the substrate PEP, the $S_{0.5}$ for this substrate increases from 0.3 mM for the dephosphoenzyme to 0.8 mM for the phosphorylated form (Ekman et al., 1976). Thus the enzyme activity versus PEP concentration curve is more sigmoidal for the phosphorylated enzyme. The affinity for the second substrate ADP is unchanged (Ljungström et al., 1976). $Fru_{16}BP$ increases the apparent affinity of both enzyme forms for PEP (Ekman et al., 1976; Ljungström et al., 1976). At saturating concentrations of this activator, the kinetics of both enzyme forms are transformed to approximately identical hyperbolic curves. The negative effectors, ATP and alanine, increase even further the $S_{0.5}$ for PEP of the phosphorylated enzyme. A higher $Fru_{16}BP$ concentration is necessary for the maximal activation of the phosphoenzyme than for the dephosphoenzyme assayed in the presence of physiological concentrations of ATP and alanine (Ekman et al., 1976). Ljungström et al., (1976) reported that hydrogen ions are effective in activating the phosphoenzyme. Thus when the pH is lowered from 8 to 6.5, the inhibition due to phosphorylation is abolished. The requirement for K^+ is unaffected by the phosphorylation state, whereas the phosphoenzyme requires a higher concentration of Mg^{2+} for maximal activity, compared with the control enzyme.

iii) Phosphorylation of L-type pyruvate kinase in vivo

Experiments using whole animals, perfused livers, liver slices and isolated hepatocytes have been performed in various laboratories to study the phosphorylation of L-type PK in vivo. Ljungström & Ekman, (1977) used liver slices and observed a small but significant phosphorylation of the enzyme (about 0.2 mol of phosphate/mol of enzyme). This value was low, but can be explained by the existence in the cell sap of a phosphatase activity (Titanji et al., 1976), which can cause

rapid dephosphorylation of PK. They observed a decrease in enzyme activity parallel to an increase in the phosphorylation of the enzyme. A similar decrease in enzyme activity to that observed when PK is phosphorylated *in vitro* is also apparent in experiments with perfused livers (Blair *et al.*, 1976).and isolated hepatocytes (Ishibashi & Cottam, 1978).

A comparison of the kinetic properties of the enzyme in extracts of glucagon-treated hepatocytes with those of the purified enzyme that has been phosphorylated *in vitro* shows several similarities (Riou *et al.*, 1976). Firstly, there is an apparent decrease in the affinity of the enzyme for PEP. Secondly, both preparations are more sensitive to inhibition by ATP and alanine. Thirdly, both enzymes are less sensitive to activation by $Fru_{16}BP$. Therefore, from the data available there is strong evidence that glucagon stimulates phosphorylation of PK (Garrison & Borland, 1978) which would result in a decrease in the enzyme activity.

Furthermore, Riou *et al.*, (1978) used $[^{32}P]$ phosphoric acid to demonstrate that glucagon stimulates the incorporation of ^{32}Pi into the enzyme *in vivo*. They injected the rats with ^{32}Pi, somatostatin (given to prevent the secretion of insulin in response to glucagon administration) and either saline or glucagon. The incorporation of ^{32}Pi into PK was stimulated 2- to 3-fold by glucagon. This increase in enzyme-bound phosphate was associated with an inhibition of PK and an increase in hepatic cAMP accumulation. These results demonstrate *in vivo* phosphorylation of the enzyme and support the hypothesis that glucagon regulates L-type PK activity, at least in part, by a phosphorylation mechanism.

iv) Dephosphorylation of L-type pyruvate kinase

The enzyme(s) responsible for the dephosphorylation of PK are protein phosphatases. A phosphoprotein phosphatase (E.C.3.1.3.16) which is capable of dephosphorylating $[^{32}P]$-PK has been purified

from rat liver (Titanji et al., 1976; Titanji, 1977). They observed that maximally phosphorylated PK (i.e. the enzyme activity is completely inhibited when assayed at subsaturating PEP concentrations) can be almost completely reactivated by incubation with the phosphatase. The PEP saturation curve is nearly the same after dephosphorylation of the enzyme as before phosphorylation (Titanji et al., 1976). Van Berkel et al., (1977a) reported that inactive PK in glucagon-treated hepatocyte homogenates could be reactivated by incubation with divalent ions. This reactivation is completely blocked in the presence of 20 mM KF, a well known inhibitor of phosphorylase 'b' phosphatase (Stalmans & Hers, 1975) and other protein phosphatases (Kato & Bishop, 1972; Siess & Wieland, 1972). These observations strongly suggest that dephosphorylation of PK is the result of the activation of protein phosphatase.

2. Other Possible Mechanisms of Hormonal Regulation

Another mechanism by which hormones affect PK activity involves changes in the intracellular levels of various allosteric effectors, particularly $Fru_{16}BP$. The addition of glucagon to intact hepatocytes lowers the level of $Fru_{16}BP$ (Blair et al., 1973; Pilkis et al.,1976a; 1976b). The fall in the concentration of $Fru_{16}BP$ reduces PK activity by inducing a conformational change. This change also converts the protein to a more favourable substrate for inactivation by protein kinase and/or to a less favourable substrate for activation by protein phosphatase. The effect of insulin appears to be unrelated to changes in $Fru_{16}BP$ concentrations (Claus et al., 1979) since the levels of this metabolite decline even further in the presence of insulin. This effect of insulin may be the result of the activation of glycogen synthetase (Craig et al., 1969).

Catecholamine effects are mediated via an α-adrenergic or a β-adrenergic mechanism. Adrenaline decreases the PK activity when assayed at subsaturating PEP concentrations, and this effect is partly blocked by α-adrenergic antagonists (e.g. phenoxybenzamine) and by

the β-adrenergic antagonists (e.g. propranolol) (Chan & Exton, 1978; Foster & Blair, 1978; Kemp & Clark 1978). Phenylephrine, an α-adrenergic agonist, causes small changes in PK activity and the effects are partly inhibited by α-blocking agents and to a smaller extent by β-blocking agents (Chan & Exton, 1978; Kemp & Clark, 1978). In the presence of 1-methyl-3-isobutylxanthine, an inhibitor of cyclic nucleotide phosphodiesterase (E.C. 3.1.4.17) the effect of both these agonists on PK activity is decreased by the α-adrenergic antagonists to a greater extent than by β-adrenergic antagonists, indicating that the cyclic nucleotide phosphodiesterase inhibitor potentiated mainly the α-adrenergic effects of these agonists. From these observations Chan & Exton, (1978) concluded that adrenaline inhibits PK by an α- receptor mediated, cAMP-independent mechanism. In contrast to these observations, Kemp & Clark (1978) obtained data which suggests that the regulation of PK by adrenaline is via a cAMP-dependent protein kinase. Furthermore, reports by other workers (Blair et al., 1979a; Yorek et al., 1981) suggest that both α- and β-adrenergic control of the enzyme may occur, and the relative contribution of these two pathways depends on the age of the rat used. Thus, β-adrenergic, cAMP-dependent regulation of PK activity and carbohydrate metabolism is consistent with the several observations that PK may be phosphorylated by a cAMP-dependent protein kinase (Berglund, et al., 1977; Ljungström & Ekman, 1977, Riou et al., 1978). The mechanism by which cAMP-independent α-adrenergic regulation occurs is not fully understood. However, the α-adrenergic regulation of glycogen metabolism seems to bypass cAMP and cAMP -dependent protein kinase, and intersect the β-mechanism at phosphorylase kinase via the stimulation of this enzyme as a result of an increase in cytosolic Ca^{2+} concentration (Exton, 1979).

F. THE PHYSIOLOGICAL SIGNIFICANCE OF THE REGULATION OF L-TYPE PYRUVATE KINASE

The specific activity of PK in the liver is much higher than that of PEP carboxykinase, which suggests that, in order to avoid

substrate cycling, PK must be substantially inhibited under gluconeogenic conditions (Pilkis et al., 1978a). Thus the regulation of L-type PK in vivo is of physiological importance.

As seen from the discussions earlier in this section, PEP, $Fru_{1,6}BP$, ATP, alanine and the phosphorylation state of the enzyme affect PK activity under physiological conditions, which seems to suggest that these are the major regulators of PK. PEP has marked effects on PK which result in the sigmoidal kinetics of the enzyme towards its substrate. At the PEP, ATP and alanine concentrations found in vivo, PK activity would be negligible unless activated by $Fru_{1,6}BP$. Therefore, although there exist in vivo concentrations of ATP and alanine which would completely inhibit PK, the $Fru_{1,6}BP$ available counteracts this effect. However, it is also clear that the concentration of $Fru_{1,6}BP$ in vivo is sufficient to maximally stimulate the phosphorylated PK assayed at subsaturating PEP concentrations in the presence of ATP and alanine (Ljungström et al., 1976). The usual explanation for this apparent anomaly is that the free concentrations of $Fru_{1,6}BP$ in the cytosol is much lower than the total concentration which is normally assayed (Sols & Marco, 1970; Lawson et al., 1976; Felíu et al., 1977). If these effects have any physiological significance, then the balance of the inhibitory and stimulatory effects and the phosphorylation state of the enzyme must determine the flux of carbon through PK.

Under physiological conditions, ATP levels are not altered to any great extent which suggests that the interaction of alanine and $Fru_{1,6}BP$ may be of great physiological importance in the regulation of PK. Alternatively, the phosphorylation state of the enzyme may be a determining factor or it may be that both types of regulation are important. The physiological significance of these effectors is apparent when dietary effects are examined. During starvation, PK activity is decreased and gluconeogenesis is dominant. Under these conditions, the in vivo concentrations of $Fru_{1,6}BP$ decrease (Claus

et al., 1979), which would ensure inactivation of the enzyme by ATP and alanine. At the same time there is a hormonally induced conversion of the enzyme to a less active phosphorylated form. The combined effects result in a deactivation of PK. Re-feeding the animal on a high carbohydrate diet, on the other hand, leads to a change in the liver metabolism from gluconeogenesis to net glycolysis. Under these conditions hexose phosphate levels are elevated and this leads to an increased synthesis of $Fru_{16}BP$ which would stimulate PK activity. At the same time the enzyme is dephosphorylated with a resulting increase in the affinity for PEP.

From these studies it is clear that these alterations in PK activity can account for the physiological changes which occur in animals in response to dietary and hormonal factors. A summary of the factors which affect PK activity in vivo is shown in figure 7b.

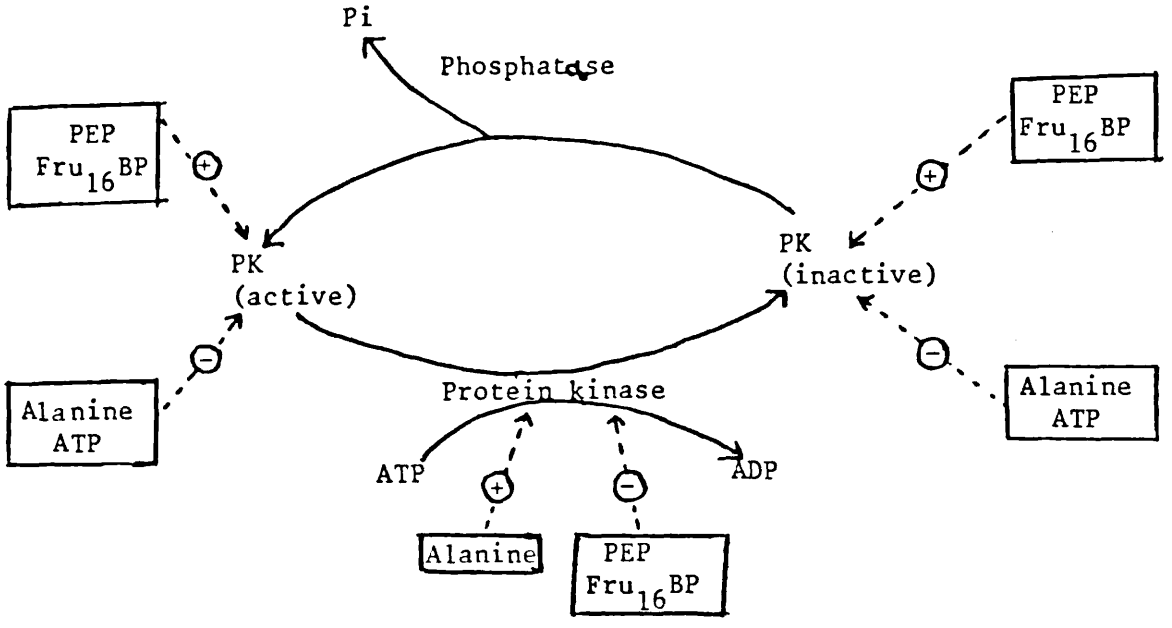


Figure 7b. Factors affecting L-type pyruvate kinase activity in liver parenchymal cells.

RESULTS AND DISCUSSION

III. RESULTS AND DISCUSSION

The L-type pyruvate kinase from rat liver is an allosteric enzyme which is inhibited by ATP and alanine and activated by $Fru_{16}BP$ (Seubert & Schoner, 1971). The enzyme can also be regulated by a cAMP dependent protein kinase (Engström, 1978).

The glycolysis of various carbohydrate substrates passes a common regulatory step, which is catalysed by PK (see figure 3). It has been reported that fructose causes the accumulation of hepatic Fru-1-P (Woods et al., 1970; Van den Berghe, 1978) a known activator of PK (Eggleston & Woods, 1970). Dihydroxyacetone has also been reported to increase $Fru_{16}BP$ concentrations in the perfused liver (Woods & Krebs, 1973) and isolated hepatocytes (Claus et al., 1979). The latter authors also reported that dihydroxyacetone stimulated PK in isolated hepatocytes from fasted rats, and this was correlated to the elevated $Fru_{16}BP$ levels.

It was of interest, therefore, to test the hypothesis that the activation of PK by accumulated Fru-1-P occurs when isolated hepatocytes are incubated in the presence of fructose, and to examine the PK activity from isolated hepatocytes incubated with other related substrates. The physiological importance of these effects was also investigated.

A. REGULATION OF PYRUVATE KINASE ACTIVITY BY CARBOHYDRATES

In this study isolated hepatocytes were incubated with various concentrations of carbohydrate substrates, and the PK activity was measured at 0.2 mM and 4.0 mM PEP in crude extracts. These values were compared to the control activities of PK measured in hepatocytes which were untreated.

1. Effect of glucose and fructose on pyruvate kinase activity

The metabolism of fructose by the liver differs from that of glucose in several important aspects (Section I.C. and I.D.) The conversion of fructose to the triose phosphates does not involve the

regulatory enzymes glucokinase and PFK 1 (figure 3), thus the only regulatory enzyme which is common to the glycolysis of glucose and fructose is PK. It is also well known that fructose loading causes the accumulation of Fru-1-P in the liver. This accumulation has been demonstrated in the livers of several species, using intact organisms, including laboratory animals (Kjerulf-Jensen, 1942; Günther et al., 1967; Burch et al., 1969; Heinz & Junghänel, 1969; Van den Berghe et al., 1973, 1977b), and normal human beings (Bode et al., 1973) as well as in the perfused organ (Woods et al., 1970; Sestoft et al., 1972). In addition, it has been reported that hepatic PK is activated by Fru-1-P at low substrate (PEP) concentrations, but no such activation occurs under saturating conditions (Eggleston & Woods, 1970).

In view of the reports described above, and the observations that there is a different rate of conversion of uniformly labelled glucose and fructose into various intermediates of carbohydrate and lipid metabolism (Bailey et al., 1968b; Bar-On & Stein, 1968; Gale & Crawford, 1969; Pereira & Jangaard, 1971), further studies of the differential effects of glucose and fructose on PK were carried out.

Freshly prepared hepatocytes from fed animals were incubated with 5 mM glucose and fructose and PK activity examined. A control experiment with no added hexose was run in parallel (Methods, section IV.B.2).

Incubation of the hepatocytes in the absence of hexose resulted in a gradual decrease in enzyme activity when measured at subsaturating PEP concentrations but no change in V_{max} (figure 8). Addition of glucose (5 mM) had no detectable effect on the rate of the decrease, but fructose (5 mM) caused a rapid fall in enzyme activity during the first 10 min of the incubation, followed by a further gradual decrease in activity. The effect of fructose was unexpected since it was expected that the accumulation of Fru-1-P would cause a stimulation of PK activity.

The effect of incubating isolated hepatocytes for 15 min with varying glucose and fructose concentrations (up to 50 mM) was studied. It was clear that glucose had little or no effect on PK activity at

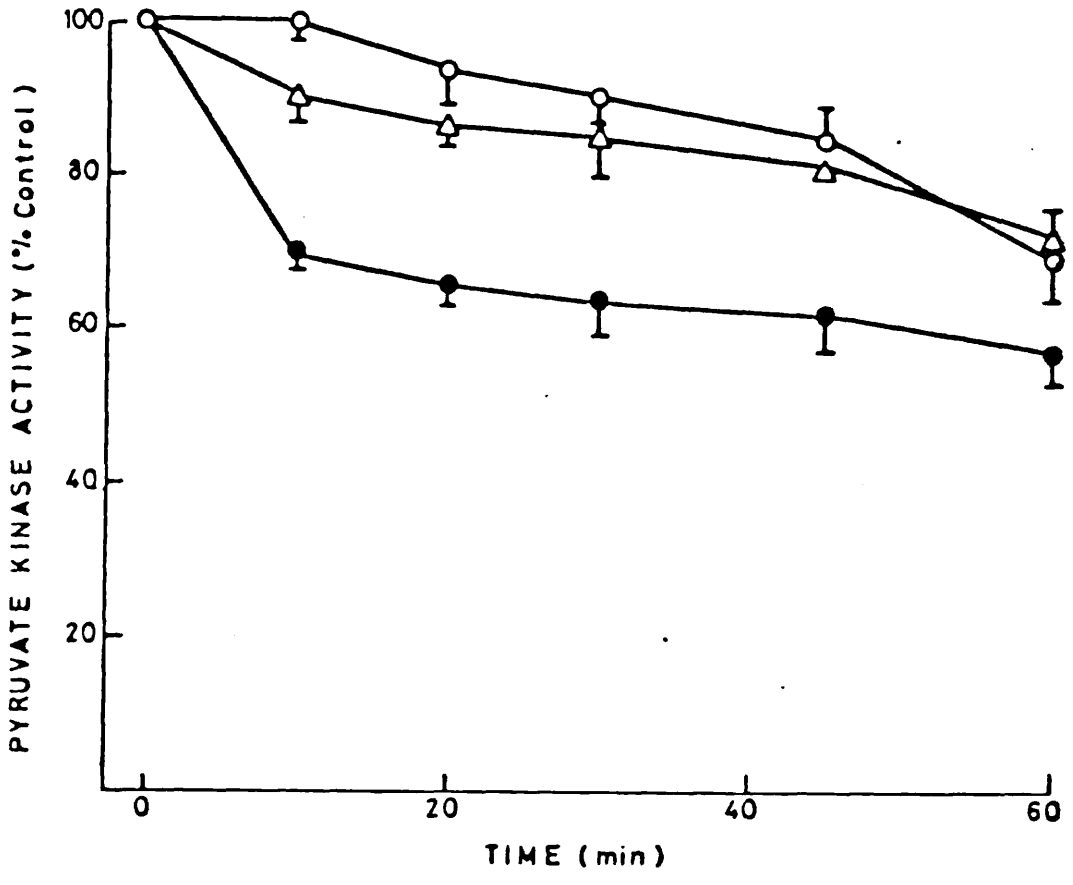


Figure 8. Time course of the effects of 5 mM fructose (●), 5 mM glucose (△) or no added hexose (○) on pyruvate kinase activity assayed with 0.2 mM PEP. The hexoses were added to the hepatocytes at zero time and the reaction stopped by freezing aliquots of the cell suspension at the appropriate time intervals as described in methods (section IV. B.3).

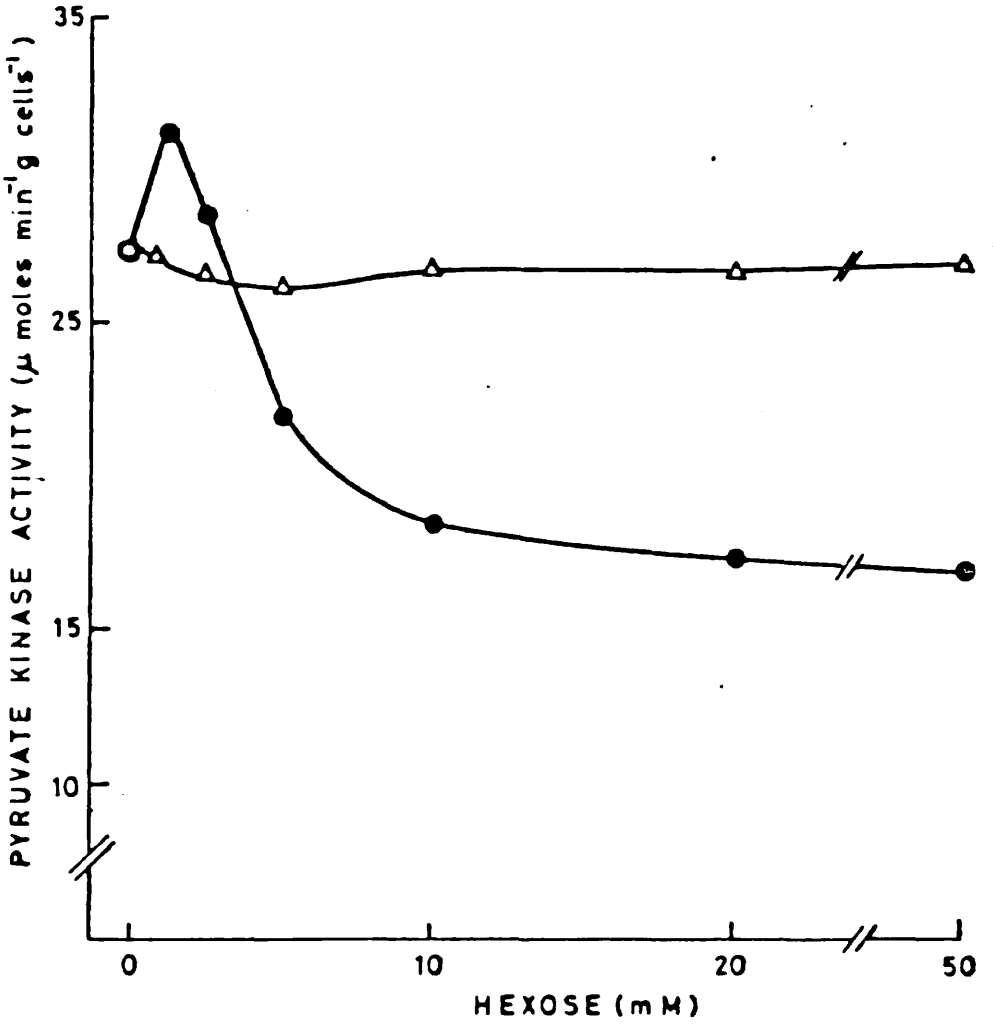


Figure 9. The concentration dependence of the effect of incubating hepatocytes (n=2) with fructose (●) or glucose (△) on pyruvate kinase activity. The incubation period was 15 min.

either low PEP concentrations (figure 9) or at saturating levels of the substrate (results not shown). Incubating the hepatocytes with low fructose concentrations caused a small but consistent activation of PK assayed at subsaturating PEP concentrations (figure 9) but the V_{max} was unchanged. Since the assay conditions represent a 120-fold dilution of the intracellular contents, the stimulation in situ is probably greater than that observed under these conditions. However, upon incubation of the hepatocytes with high fructose concentrations (5 mM or greater) the enzyme was inhibited at subsaturating PEP concentrations whereas V_{max} was unchanged.

These concentration dependent changes in the effect of fructose can be explained by either rapid changes in the levels of effectors of PK or by changes in the phosphorylation state of the enzyme. This was tested by examining the effects of salting out the enzyme with $(NH_4)_2SO_4$ (Table 1).

Table 1. The effect of $(NH_4)_2SO_4$ precipitation on the inhibition of pyruvate kinase in hepatocytes following fructose treatment.

	Pyruvate Kinase Activity (v/V_{max})	
	Untreated Enzyme	$(NH_4)_2SO_4$ Treated Enzyme
Control	0.42±0.02	0.34±0.01
Glucose (10 mM)	0.40±0.03	0.35±0.01
Fructose (10 mM)	0.33±0.03**	0.34±0.02

** $p < .01$ fructose versus control.

Hepatocytes were incubated for 15 min with or without substrate and aliquots removed as described in methods (Section IV.B.3).An aliquot was assayed for enzyme activity and the remainder was treated with saturated $(NH_4)_2SO_4$ to a final concentration of 50% $(NH_4)_2SO_4$. The precipitated protein was centrifuged at 17,000 g for 20 min, the pellet resuspended in 50% $(NH_4)_2SO_4$ and then recentrifuged. The resulting pellet was then resuspended in suspending medium (see methods IV.B.4) and used for enzyme assay.

The effect of this treatment on the activity from control and glucose treated cells is to decrease the v/V_{max}. This is probably related to the removal of $Fru_{16}BP$ from the crude extract. There was no such effect when the enzyme from the fructose treated hepatecytes was precipitated by $(NH_4)_2SO_4$. Thus it is not possible to conclude whether the inhibition is due to a stable change of the enzyme or a change in the concentration of effectors. However, when perchloric acid extracts of fructose (10 mM) treated heptocytes were incubated with a PK preparation which had been desalted by $(NH_4)_2SO_4$, the inhibition was observed (figure 10). This inhibition was much less marked when extracts from control and glucose treated hepatocytes were examined.

The evidence presented above appears to show that the inhibition of PK by fructose is the result of the accumulation of an inhibitor or the removal of a stimulator rather than a stable modification of the enzyme.

It was checked that fructose had no effect on $(NH_4)_2SO_4$ precipitated PK under these conditions (results not shown). It was also observed that the stimulation of PK which occurs when isolated hepatocytes are incubated with low fructose concentrations is abolished by the $(NH_4)_2SO_4$ treatment (results not shown), suggesting the removal of an activator.

While the initial rate of fructose uptake by hepatocytes appears to be slower than that of glucose (Baur & Heldt, 1976), the rate of phosphorylation of the ketose is considerably greater (Renold et al., 1954; Pereira & Jangaard, 1971; Bode et al., 1974; Sestoft & Fleron, 1974; Vessal et al., 1980). The consequence of the rapid phosphorylation of fructose is the accumulation of Fru-1-P and the depletion of ATP and Pi (Mienpaa et al., 1968; Burch et al., 1969; Woods et al.,1970; Sestoft et al., 1972; Van den Berghe et al., 1977a). Since ATP is present mainly as the ATP-Mg complex in the hepatocyte (Veloso et al., 1973), incubation of the cells with fructose also leads to an increase in the free Mg^{2+} in the extracts (Van de Werve & Hers, 1979).

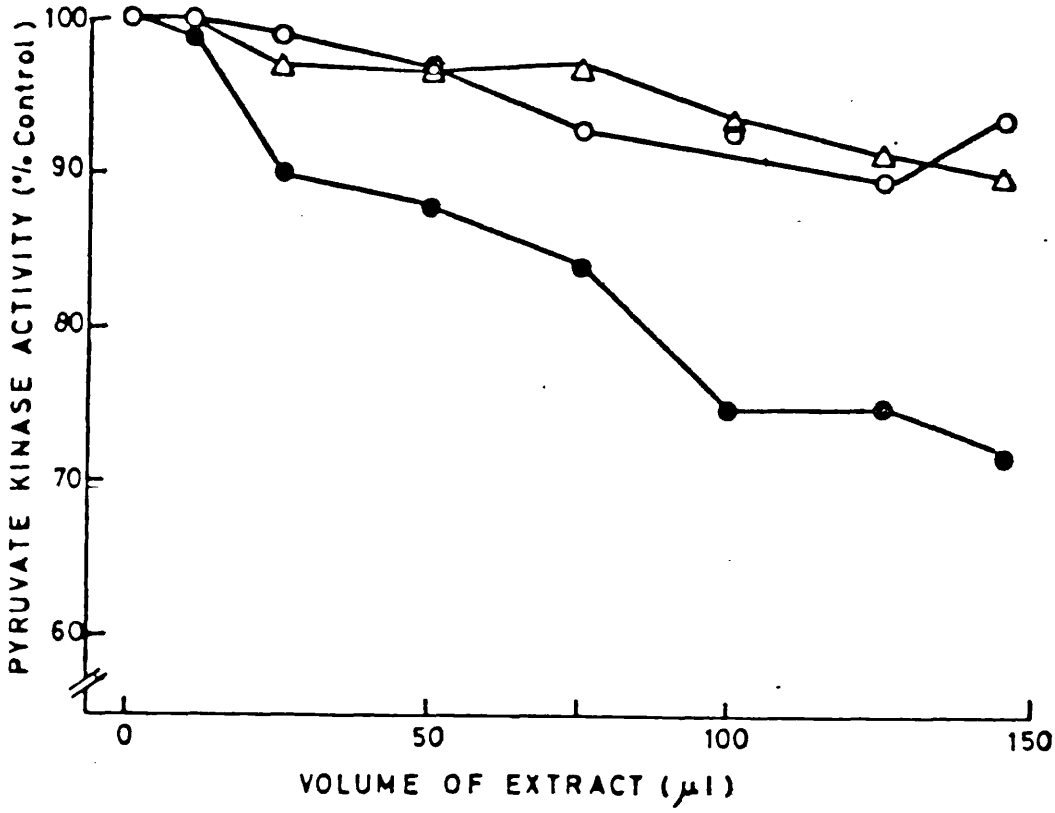


Figure 10. Inhibition of partially purified pyruvate kinase by a neutralized perchloric acid extract of hepatocytes treated with either 10 mM fructose (●) or 10 mM glucose (Δ) for 15 min. A control, with no added hexose (O) was also tested. The extracts were preincubated for 10 min with pyruvate kinase and PEP (0.2 mM) was added to start the enzyme assay (100 µl of extract is equivalent to 5.8 mg of hepatocytes).

Increased concentrations of glyceraldehyde, glyceraldehyde-3-phosphate, G3P and DHAP have been reported in perfused liver (Burch et al., 1969, 1970; Heinz & Junghänel, 1969; Woods et al., 1970). Fructose has also been reported to increase allantoin production by isolated hepatocytes (Smith et al., 1977b; Van den Berghe et al., 1980). Therefore there are a number of possible candidates responsible for the changes in PK activity observed in the present study.

It was of interest,therefore, to attempt to dissociate the stimulatory effect of Fru-1-P from the inhibitory effect by introducing a washing step (methods,section IV.B.3), following the incubation of hepatocytes with high fructose concentrations. This should have the effect of removing any metabolite which can permeate the cell membrane, whereas Fru-1-P should remain associated with the hepatocyte.

2. Pyruvate Kinase activity measured after removing the extrahepatocyte medium

i) Glucose and fructose effects

A comparison of the change in PK activity as a function of time after the addition of hexose revealed that the inhibition observed in the presence of fructose (10 mM) was abolished by the washing treatment, and a stimulation was now observed (figure 11 A & B). No significant change in PK activity in the glucose treated hepatocytes was observed following the washing step. These observations were confirmed when the concentration dependence of fructose effects were examined. Figure 12A shows that stimulation of the enzyme at low fructose levels and the inhibition at higher fructose concentrations are consistently reproducible. Upon subjecting the hepatocytes to a washing treatment (figure 12B), the inhibition was abolished and a stimulation of PK activity occurred at all concentrations examined. This data is consistent with the hypothesis that the stimulation of PK by fructose is due to the accumulation of a phosphorylated intermediate, possibly Fru-1-P, and that the cell membrane is freely permeable to the inhibitor which can be removed by washing the hepatocytes.

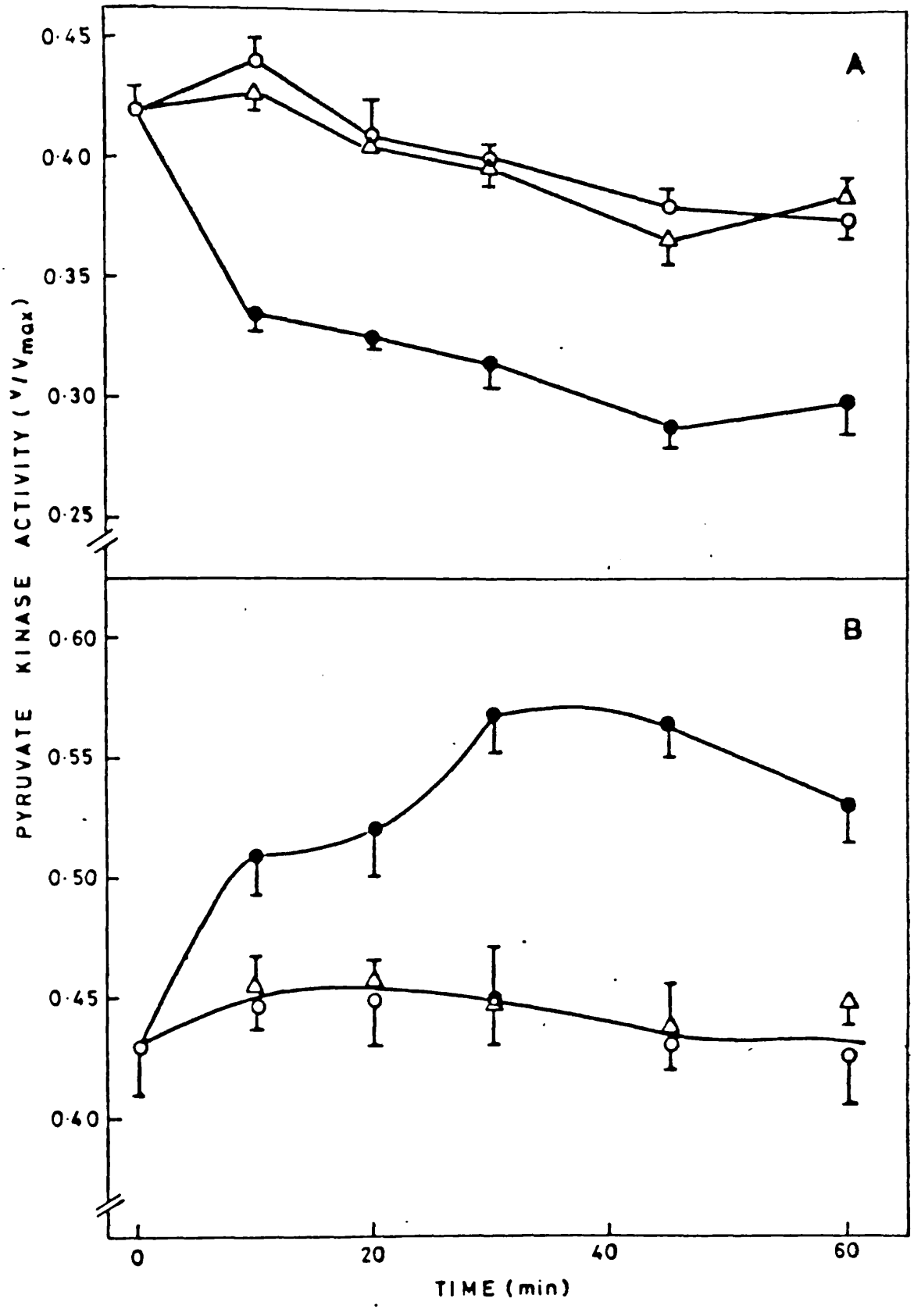


Figure 11. Time course showing the effect of 10 mM fructose (●), 10 mM glucose (△) or no added hexose (○) on pyruvate kinase activity. A, Hepatocytes were extracted with the incubation medium. B, Hepatocytes were washed with fresh medium before extraction; the cells were rapidly sedimented through ice cold KRB ($-Ca^{2+}$) containing 1.5 g % BSA. The supernatant fraction was aspirated and the pellet frozen quickly in liquid N_2. The cells from A and B were then homogenized in 5 volumes of ice cold suspending medium (see methods, section IV.B.3).

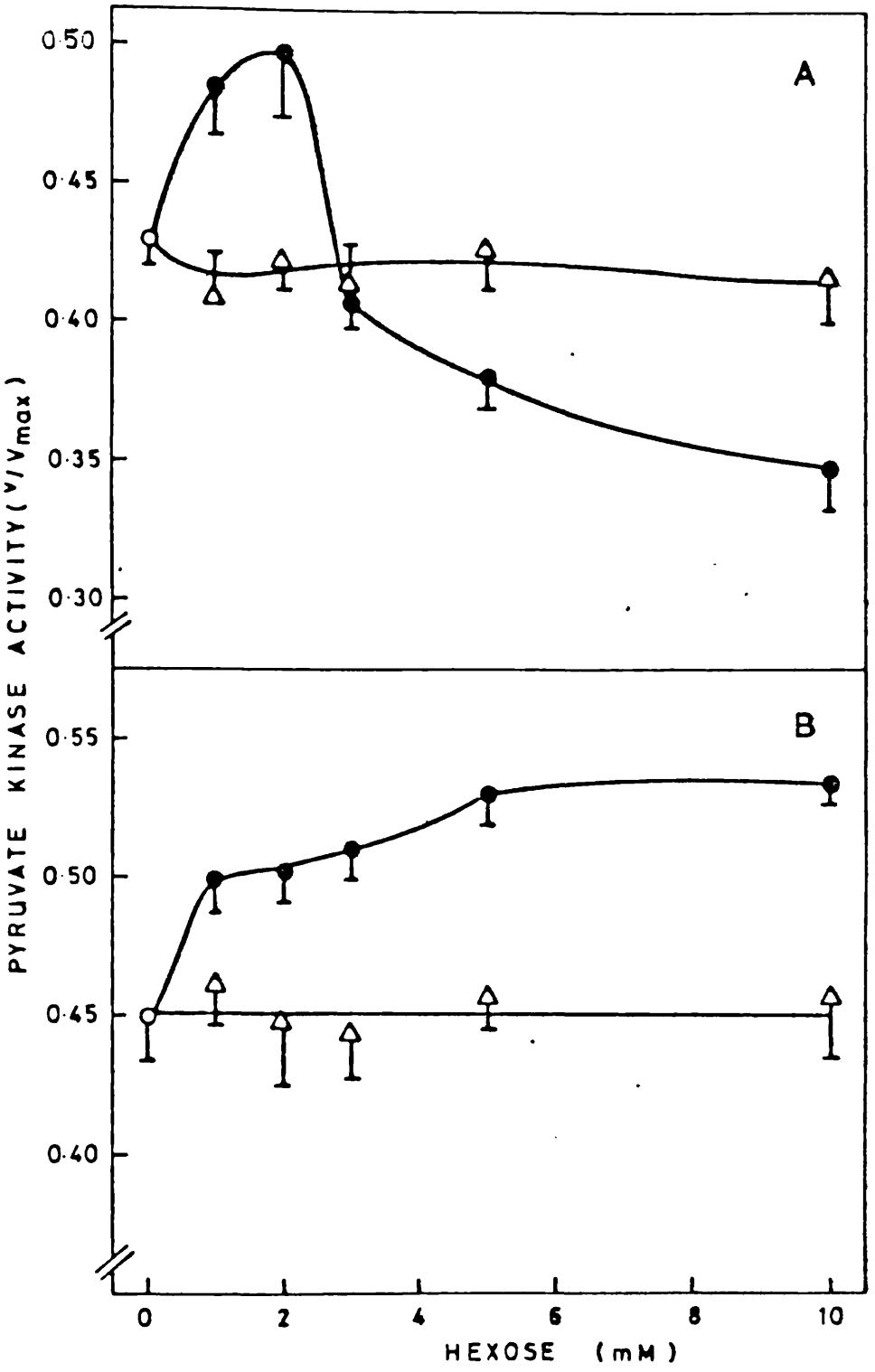


Figure 12. The concentration dependence of the effect of incubating hepatocytes with either fructose (●) or glucose (△) for 20 min on pyruvate kinase activity from; A, unwashed and B, washed hepatocytes.

A further examination of the time course of the fructose (10 mM) effects on PK activity show that both the inhibitory and stimulatory effects, in the unwashed and washed cells respectively, were very rapid, reaching a maximum level in the case of the stimulatory effect within 1 min (figure 13B). This latter change is consistent with the data in figure 23 which shows a rapid increase of Fru-1-P in the same time period. In the unwashed hepatocytes there was an immediate fall in the PK activity, followed by a more gradual decline (figure 13A). This suggests that there is a rapid burst in the formation of inhibitor, which overcomes the activating effect of Fru-1-P, followed by a more gradual accumulation of inhibitor (see also figure 8).

The primary events following a fructose load both in the intact animal (Burch et al., 1970; Van den Berghe et al., 1977a) and the perfused organ (Woods et al., 1970) are the rapid accumulation of Fru-1-P and a concomitant depletion of hepatic ATP. These events would be expected to lead to an activation of PK when hepatocytes are incubated with fructose. However, stimulation of the enzyme is observed only when the cells are either incubated with low fructose concentration (Figure 9 and 12A) or when cells incubated with a high concentrations of fructose are washed before assaying for the enzyme (see figure 11B & 12B). Incubation of hepatocytes with glucose has no significant effect on PK under these conditions, presumably because the hexose does not deplete ATP. One of the most important metabolic changes occurring as a result of fructose administration is the depletion of ATP. As a consequence of the depletion of this nucleotide, the levels of various intermediates increase (see above). It was thus interesting to study the hypothesis that, the difference in PK activity observed after glucose or fructose administration is due to a fall in hepatic ATP concentrations, or due to the accumulation of a metabolite of Fru-1-P.

ii) Effects of glycerol and dihydroxyacetone on pyruvate kinase activity in hepatocytes

Glycerol and dihydroxyacetone enter the glycolytic sequence at the triose phosphate stage (figure 3). This makes PK the common

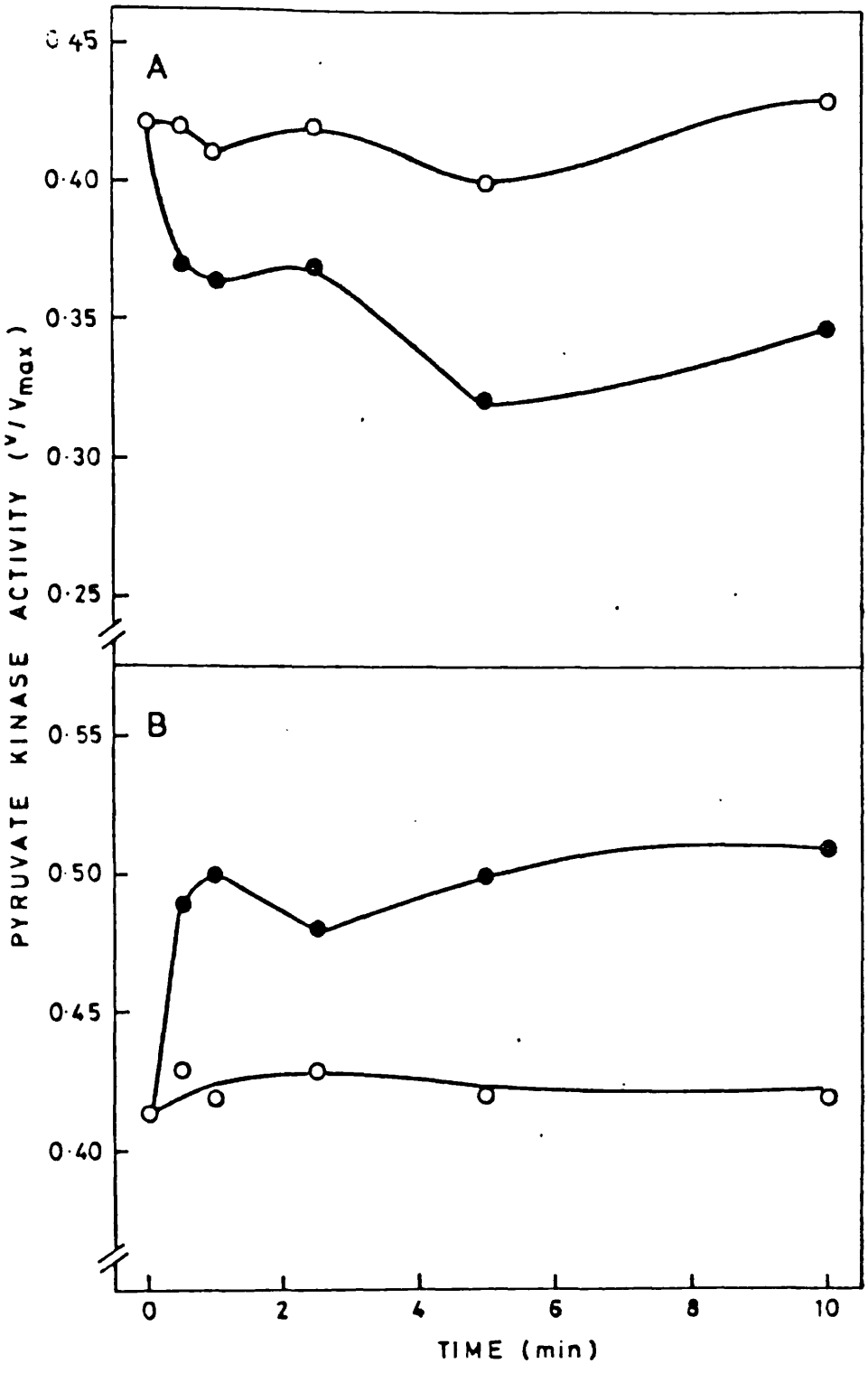


Figure 13. Rapid changes in pyruvate kinase activity from hepatocytes (n=2) incubated with (●) or without (O) 10 mM fructose. Comparison of, A, washed and B, unwashed hepatocytes.

regulatory enzyme for the glycolysis of these two substrates, and thus they share a common pathway to the metabolism of fructose. Glycerol is known to deplete ATP levels in the perfused liver (Burch et al., 1970; Woods & Krebs, 1973) whereas dihydroxyacetone does not cause extensive depletion of the nucleotide (Woods & Krebs, 1973). Thus the possibility that the effects of fructose are related to the ATP depletion could be examined by comparing the effects on PK using dihydroxyacetone and glycerol as substrates.

Dihydroxyacetone (10 mM) caused no significant change in PK activity (v/V_{max}) from unwashed cells whereas glycerol (10 mM) caused a rapid inhibition of the enzyme (figure 14A). Washing the cells relieved the inhibition caused by 10 mM glycerol (figure 14B), and also revealed a rapid activation of the enzyme when cells were incubated with 10 mM dihydroxyacetone. The maximum activation caused by dihydroxyacetone (40%) was reached after 20 min incubation and this was followed by a steady decline.

These observations indicate that glycerol causes the accumulation of an inhibitor which probably leaves the cell. This observation is similar to that made with 10 mM fructose. However, unlike fructose there is no activation observed when cells incubated with glycerol are washed, indicating that glycerol has little or no effect on the concentration of PK activator(s) within the cell . In contrast, dihydroxyacetone causes a stimulation of PK in washed cells (figure 14B); probably due to the accumulation of $Fru_{1,6}BP$ (Claus et al., 1979; see also figure 25B). The activation is not observed with unwashed cells (figure 14A) suggesting that incubation of hepatocytes with dihydroxyacetone also results in the production of inhibitor(s). However, the concentration of the inhibitor in the latter case is not sufficient to inhibit the enzyme because of the presence of the accumulated activator.

A study of the effect of varying concentrations of dihydroxyacetone and glycerol is shown in figure 15. The data confirms the observations made with 10 mM substrate and shows that significant changes were observed following incubation with 1 mM glycerol. It is of interest to note that there is a significant difference between

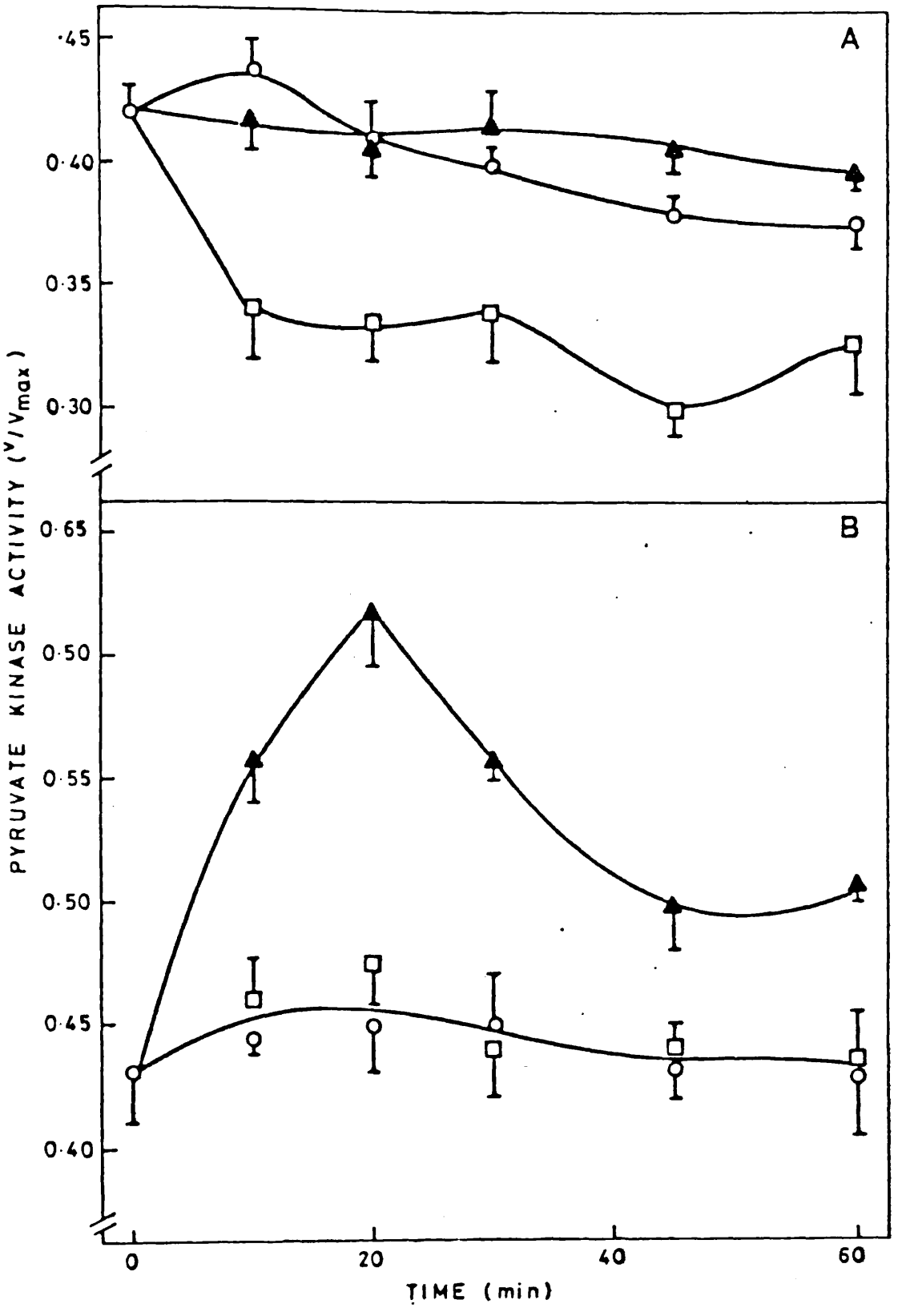


Figure 14. Time course of the effect of 10 mM dihydroxyacetone (▲), 10 mM glycerol (□) or no added substrate (O) on hepatocyte pyruvate kinase activity. A, Hepatocytes extracted with incubation medium. B, Hepatocytes washed before extraction.

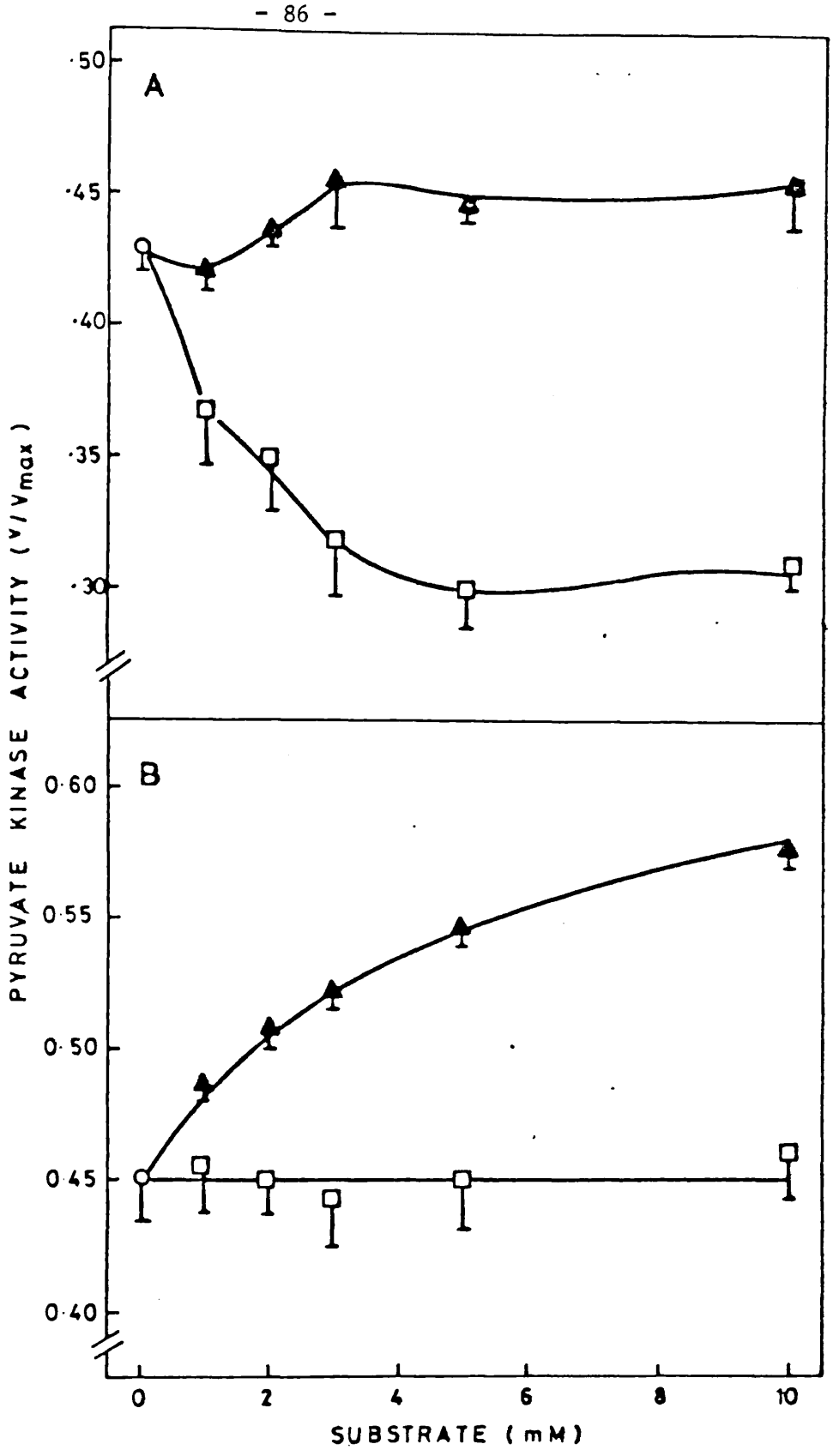


Figure 15. The concentration dependence of the effect of dihydroxyacetone (▲) and glycerol (□) on hepatocyte pyruvate kinase activity. A, Hepatocytes extracted without washing. B, Hepatocytes washed after incubation. The hepatocytes were incubated with substrate for 20 min.

washed and unwashed cells at all concentrations examined. However, when the depletion of ATP was examined it was found that 10 mM dihydroxyacetone induced a relatively small change in ATP level (Control versus 10 mM dihydroxyacetone: 2.49± .11 and 2.0 ± .09 μmoles g cells^{-1} respectively), whereas glycerol caused a more profound decrease in ATP concentration (to 0.8 ± 0.6 μmoles g cells^{-1}) a lower concentration of dihydroxyacetone (4 mM) had no effect on hepatic ATP concentration(2.45 μmoles g cells^{-1}) but as shown in figure 15A & B, there was some stimulation revealed after the washing step, again suggesting that an inhibitor had accumulated despite the fact that ATP was not depleted.

From these observations it is clear that both glycerol and dihydroxyacetone cause the accumulation of an inhibitor(s), presumably identical, or closely related to the inhibitor(s) produced by fructose. On the other hand, in contrast to dihydroxyacetone and fructose, incubating hepatocytes with glycerol does not result in the production of an activator of PK.

iii) Effect of D-glyceraldehyde on hepatic pyruvate kinase activity

D-glyceraldehyde is a product of fructose metabolism resulting from the aldolytic cleavage of Fru-1-P. There are three possible routes for the metabolism of this triose, but the major route under physiological conditions is probably its phosphorylation to give glyceraldehyde-3-phosphate (Burch et al., 1970). It was therefore of interest to examine the effects of D-glyceraldehyde on PK activity.

Again, at all concentrations of D-glyceraldehyde there was a stimulation of PK observed following the washing of the cells which was not apparent before washing (figure 16). As with dihydroxyacetone, it therefore appears that both the inhibitor and activator are produced in response to a D-glyceraldehyde load. However, maximum stimulation was observed at a relatively lower concentration in the case of the latter substrate (cf figure 15 & 16). The explanation for this is not immediately clear since the K_m and V_{max} of triokinase for the trioses

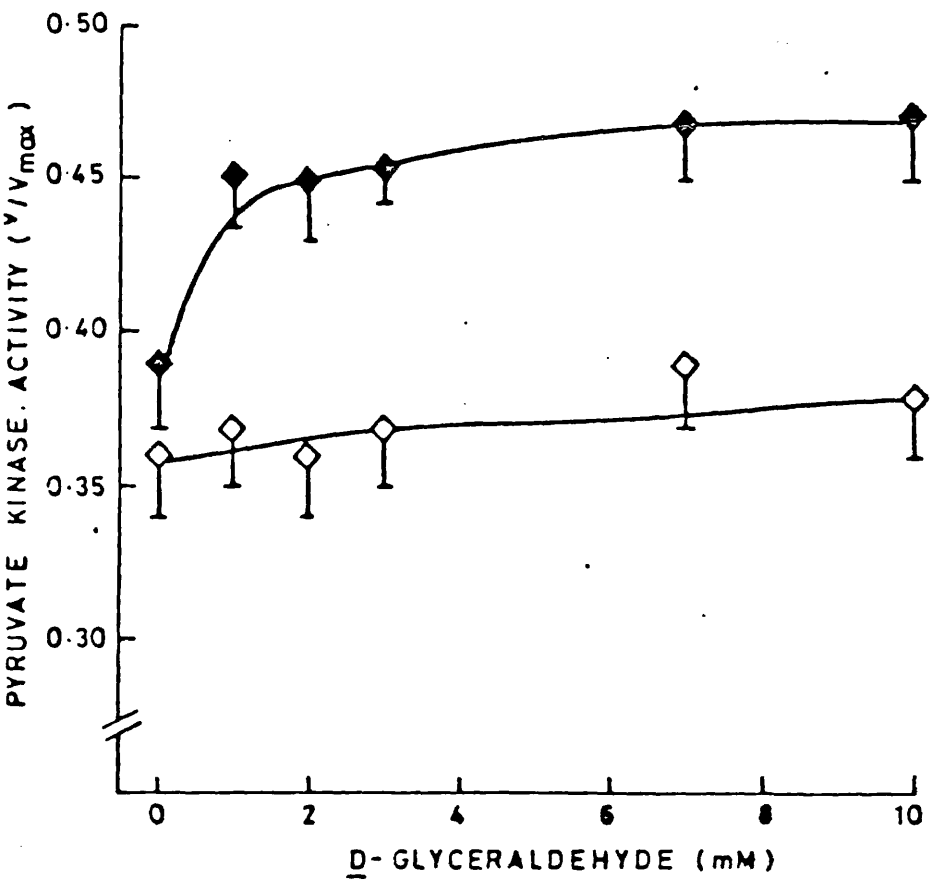


Figure 16. The concentration dependence of the effect of D-glyceraldehyde on hepatocyte pyruvate kinase activity in unwashed cells (◇) and washed cells (◆). The incubation period was 20 min.

would favour the metabolism of the ketose derivative. The most probable explanation is that a reversal of the aldolase reaction is occurring resulting in the formation of Fru-1-P from glyceraldehyde and DHAP (see section III.B.2). It is also likely that $Fru_{16}BP$ concentration is raised.

3. Pyruvate Kinase activity in hepatocytes from fasted rats

A study of the dietary regulation of L-type PK has revealed that the concentration of the enzyme obtained from fasted animals is decreased and that there is an increased proportion of PK in the less active form (Felíu et al., 1977; Kohl & Cottam, 1977; Van Berkel et al., 1977a; 1978). The changes in the kinetic properties brought about by fasting are similar to those observed in vitro when the purified enzyme is phosphorylated by a cAMP-dependent protein kinase (Ekman et al., 1976; Kohl & Cottam, 1977; Van Berkel et al., 1977a).

In the fasting animal the stimulation of gluconeogenesis is accompanied by a decrease in PK activity (Felíu et al., 1976 ; Foster & Blair, 1978; Blair et al., 1979b; Yorek et al., 1981), which results in reduced substrate cycling at the PEP-pyruvate stage. Studies of gluconeogenesis from various precursors have shown that fructose and dihydroxyacetone are very good precursors of glucose (Assimocopoulos-Jeannet et al., 1973; Garrison & Haynes, 1973, Veneziale & Lohmar, 1973; Brocks et al., 1980; Yorek et al., 1981). It was therefore of interest to study the effect of these precursors on PK activity in hepatocytes from fasted animals.

The v/V_{max} for the PK obtained from untreated hepatocytes of fed rats was 0.43 $\pm$ .03 compared to 0.33 $\pm$ .03 for cells from 24 h fasted animals. This difference ($p<.02$) is probably related to the cAMP-dependent phosphorylation of the enzyme in fasted animals and to a lowering of hepatic $Fru_{16}BP$ in the fasted state together with a decrease in the sensitivity of the enzyme to the effector (Claus et al., 1979) (see section II.).

Incubating hepatocytes from fasted animals with 10 mM glucose (figure 17 A & B) had no significant effect on PK activity in either washed or unwashed cells. PK in unwashed cells was activated when isolated hepatocytes were incubated with fructose (10 mM). A 30% activation compared to the control ($p<.02$) was observed after 20 min incubation (figure 17A). This result contrasts with that obtained with cells from fed animals (see figure 11A), where inhibition was observed. However, washing of the cells incubated with fructose resulted in a further stimulation of PK activity (figure 17B), suggesting that an inhibitor is produced in the presence of fructose, but it is not as effective as in the hepatocytes from fed animals. There are various possible explanations for this difference, for example, it may be that less inhibitor is produced in the fasted state or that the enzyme is less susceptible to the inhibitor.

Figure 18 shows a time course for the effect of 10 mM dihydroxyacetone on PK in washed and unwashed hepatocytes. Dihydroxyacetone activated PK in both preparations. As found with 10 mM fructose, the percentage of enzyme activation caused by this substrate in unwashed cells (39%) from fasted rats is greater than that in unwashed cells from fed animals. Again a greater stimulation was observed in washed cells (67%) than in the unwashed hepatocytes (39%). It is also clear that the activation of PK caused by this triose in washed hepatocytes obtained from fasted animals (67%) is greater than that from fed animals (40%). This is presumably due to the lower v/V_{max} in the fasted state so that $Fru_{16}BP$ would have a greater percentage effect. There is no evidence that less of the effector is produced in the hepatocytes from fed rats than those from fasted animals. The observations made using fasted cells make it clear that PK from fed rats is affected somewhat differently to that from fasted rats.

Thus it is clear that incubation of hepatocytes with fructose leads to two distinct effects, depending on the fructose concentration used. Low fructose concentrations in the physiological range cause the accumulation of an activator of PK. However, a high fructose concentration in addition to causing elevation of the stimulator also results in the production of an inhibitor which overcomes the effect of the

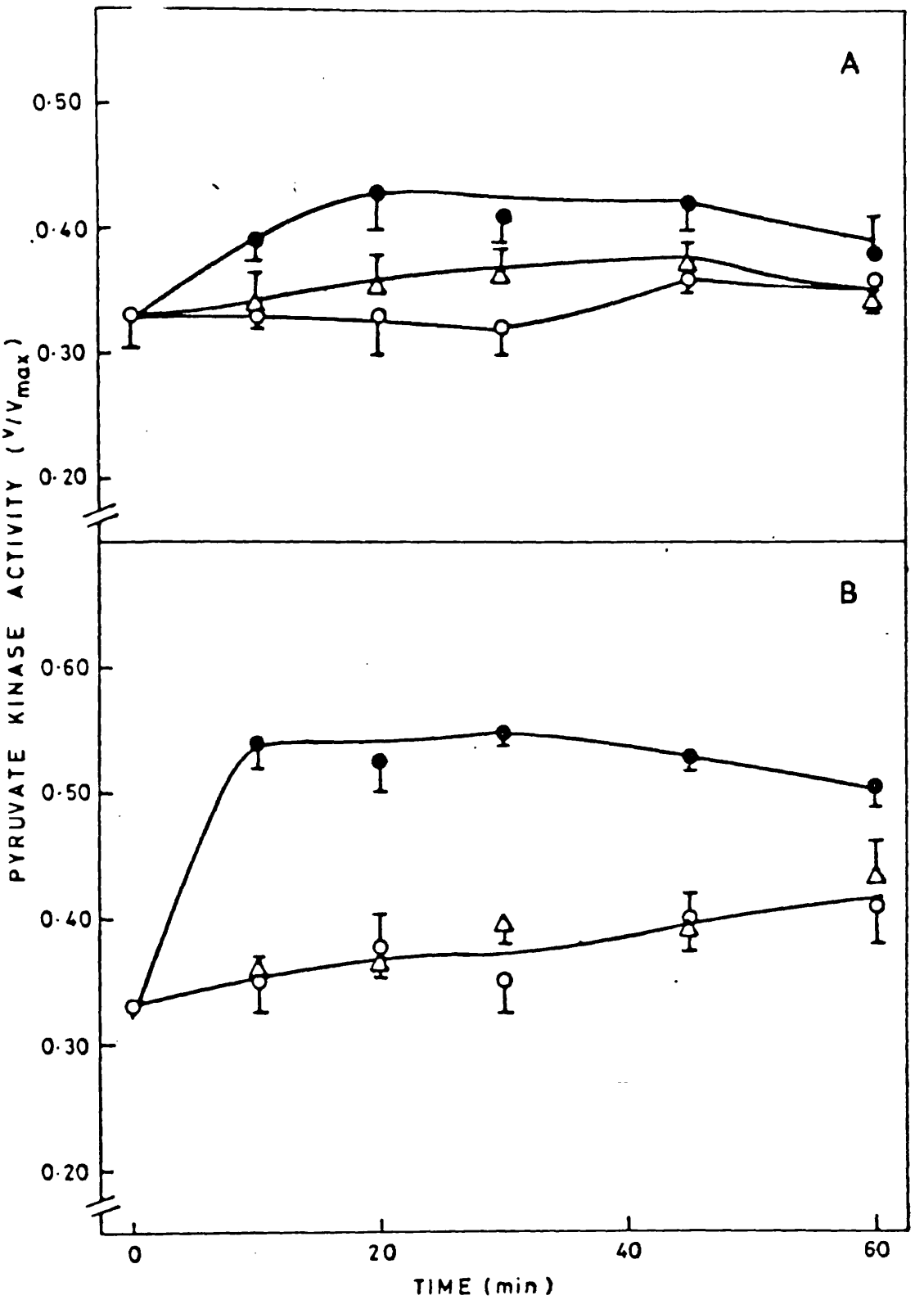


Figure 17. Time course showing the effect of 10 mM fructose (●), 10 mM glucose (Δ) or no added substrate (O) on pyruvate kinase activity in hepatocytes isolated from 24 h fasted rats. Comparison of A, unwashed and B, washed hepatocytes.

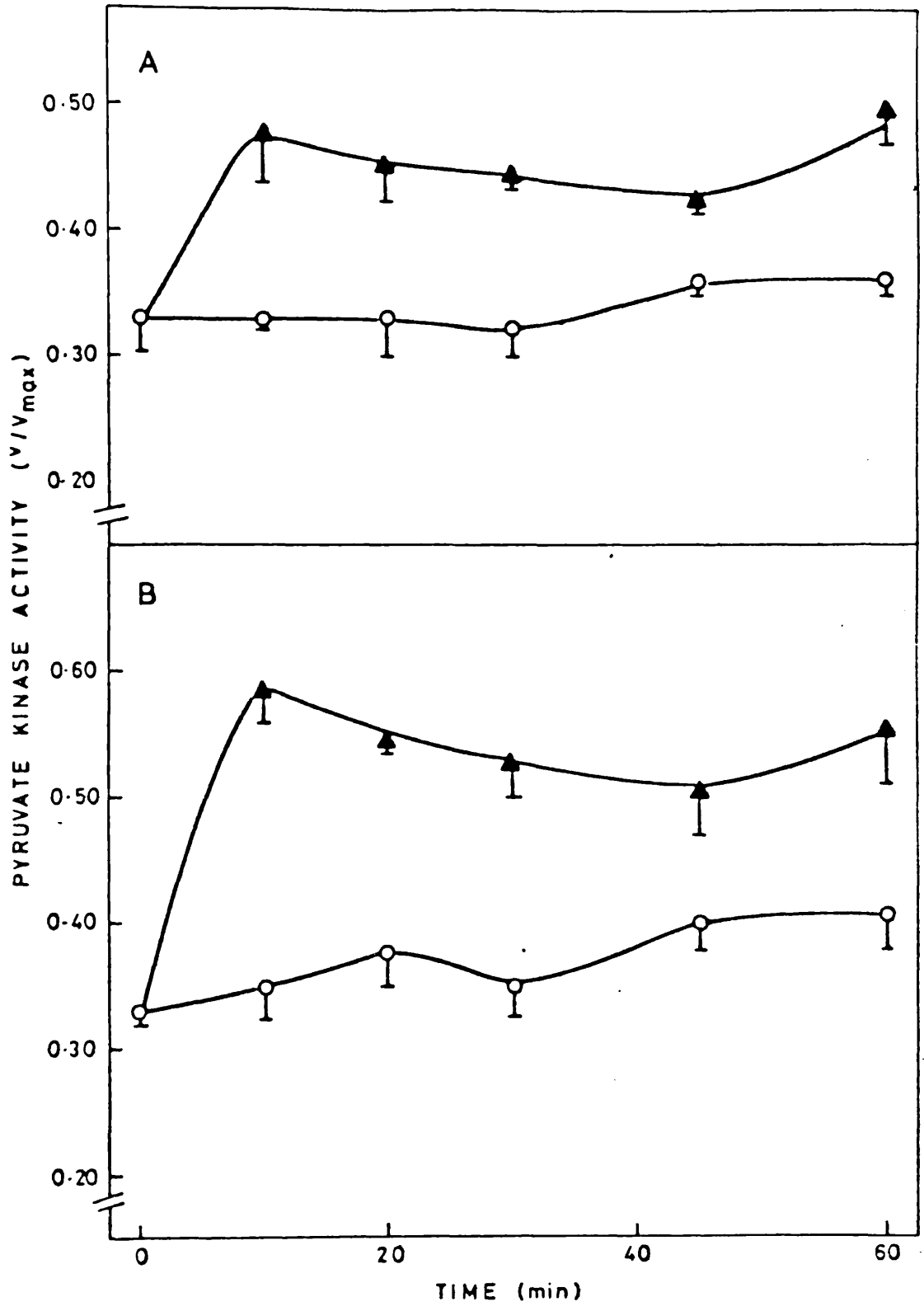


Figure 18. Time course showing the effect of 10 mM dihydroxyacetone (▲) and no added substrate (○) on pyruvate kinase activity in hepatocytes isolated from 24 h fasted rats; A, unwashed hepatocytes and B, washed hepatocytes.

stimulator. The cell membrane appears to be freely permeable to the inhibitor. The inhibitor is either produced to a lesser extent in hepatocytes from fasted animals or the enzyme from these hepatocytes is less susceptible to the inhibitor. The formation of inhibitor in cells from fed rats appeared to be related to the depletion of ATP since glycerol also causes an inhibition of the enzyme. On the other hand dihydroxyacetone and D-glyceraldehyde appear to cause some inhibition of the enzyme since the enzyme from washed cells is more active than that from unwashed cells.

B. METABOLITE CONCENTRATIONS IN ISOLATED HEPATOCYTES INCUBATED WITH VARIOUS CARBOHYDRATES

The evidence presented in section III.A shows conclusively that PK is activated by dihydroxyacetone and fructose when its activity is measured after discarding the extrahepatocyte medium, and that an inhibitor accumulates especially in the medium of cells incubated with high concentrations of fructose. Studies carried out on the nature of inhibition caused by various substrates, show that it can be reversed either by salting out the enzyme with $(NH_4)_2SO_4$ or removing the extrahepatocyte medium. The activation by fructose can be abolished by $(NH_4)_2SO_4$ treatment but not by washing the cells. It was therefore decided to examine the changes in the level of various metabolites which occur in response to different carbohydrate substrates. This was in order to define the nature of the inhibitor and activator of PK and to yield some data which may elucidate the nature of the metabolic regulation of glycolysis and gluconeogenesis from substrates which enter the glycolytic-gluconeogenic pathways at the triose phosphate level.

1. ATP

ATP levels in perfused organ have been reported to be decreased by glycerol and fructose but not by dihydroxyacetone (10 mM substrates) (Woods et al., 1970; Woods & Krebs, 1973). It is possible that the changes in PK observed in section III.A are related to the depletion

of ATP. Therefore, the changes in the hepatocyte concentration of ATP in response to glucose, glycerol, fructose and dihydroxyacetone were measured.

There was no change in ATP levels (over a 60 min incubation period) in hepatocytes from fed rats whether or not the cells were incubated with glucose (figure 19). ATP levels are considered a good indicator of hepatocyte viability and the levels found are comparable with the data obtained by other workers (Mdenpdd et al., 1968; Woods et al., 1970; Bode et al., 1973; Van den Berghe et al., 1977a). In addition it was found that when the ATP was measured in hepatocytes that had been washed, the level of ATP was identical to that found in unwashed cells (results not shown), suggesting that the impermeability of the hepatocyte membrane to the phosphorylated intermediates was maintained during the incubation period.

The most likely explanation for the fact that incubating isolated hepatocytes with 10 mM glucose does not affect hepatic ATP, is the comparatively low rate of glucose phosphorylation in the liver at this concentration of the hexose, one factor being the limited uptake of glucose by the liver cell (Scrutton & Utter, 1968). In addition, the activity of glucokinase is considerably lower than that of ketohexokinase. (Heinz & Lamprecht, 1967; Zakim et al., 1967).

Upon incubating isolated hepatocytes with fructose (figure 19) depletion of ATP was detected. The extent to which the hepatocyte ATP levels were lowered was concentration dependent; the highest concentration of fructose causing the most severe depletion of ATP. The minor depletion observed with a physiological concentration of fructose (2 mM) was reversed by further incubation. The more profound depletion measured after incubating with 5 and 10 mM fructose was partially reversed by incubation of hepatocytes for up to 60 min, but complete restoration of ATP to the pre-fructose value was not achieved.

The depletion of ATP is in agreement with the work of Clark et al.,(1979) and Van den Berghe et al.,(1980), but these workers failed

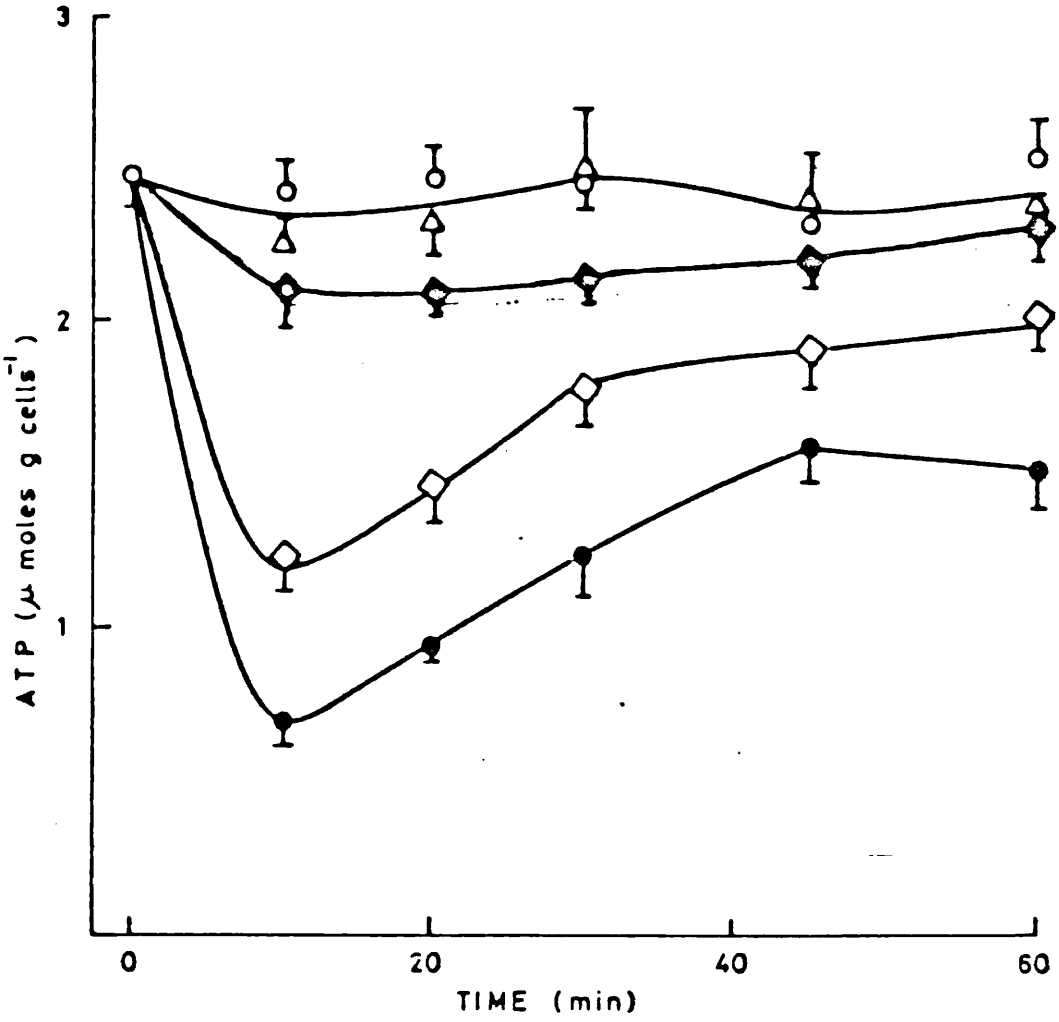


Figure 19. ATP content of isolated hepatocytes following treatment of cells with no added hexose (○), 2 mM fructose (◆), 5 mM fructose (◇), 10 mM fructose (●) or 10 mM glucose (△). Hepatocytes were extracted with perchloric acid and the ATP determined by an enzymic method (see methods, section IV.B.6a).

to show any recovery in ATP levels during the incubation period. The reason for this discrepancy is not clear. However, the former authors used hepatocytes from fasted rats and a high level of fructose (25 mM) whereas the latter authors used cells from fasted animals but incubated for 30 min only. The recovery in ATP levels observed in the present study, however, are a further indication of the viability of the hepatocyte preparation used.

The hepatic changes observed following fructose loading can be satisfactorily explained on the basis of the properties of ketohexokinase and of the enzymes of adenine nucleotide catabolism. The concentration of ATP in isolated hepatocytes incubated with fructose (5 and 10 mM) is not completely recovered over the 60 min incubation period because of the irreversible catabolism of AMP, which is formed by the adenylate kinase reaction with ADP. Fructose administration causes a decrease in the concentration of ATP, GTP and Pi (Van den Berghe et al., 1977a, 1980), of which the latter two are inhibitors of AMP deaminase, whereas ATP is a stimulator (Van den Berghe et al., 1977a; see also section I.D.). Thus the total hepatic adenine nucleotide concentrations drop after fructose administration, but ADP and AMP levels remain relatively constant (Mäenpää et al., 1968; Topping and Mayes, 1977; Van den Berghe et al., 1977a; Yip & Lardy, 1981).

Figure 20 shows that depletion of hepatocyte ATP concentrations caused by fructose (10 mM) is rapid and complete after 2.5 min incubation. This data is in agreement with the effect of fructose loading in vivo (Van den Berghe et al., 1977a) and in isolated hepatocytes (Van de Werve & Hers, 1979). In the present studies, hepatocytes incubated with fructose (10 mM) showed a 53% fall in ATP concentrations after 30 s incubation (figure 20). This is another indication of the rapid rate of fructose phosphorylation by ketohexokinase in isolated cells.

The effect of glycerol (10 mM) and dihydroxyacetone (4 and 10 mM) on hepatocyte adenine nucleotide levels was also examined (figure 21). Dihydroxyacetone (4 mM) caused no significant change in ATP levels

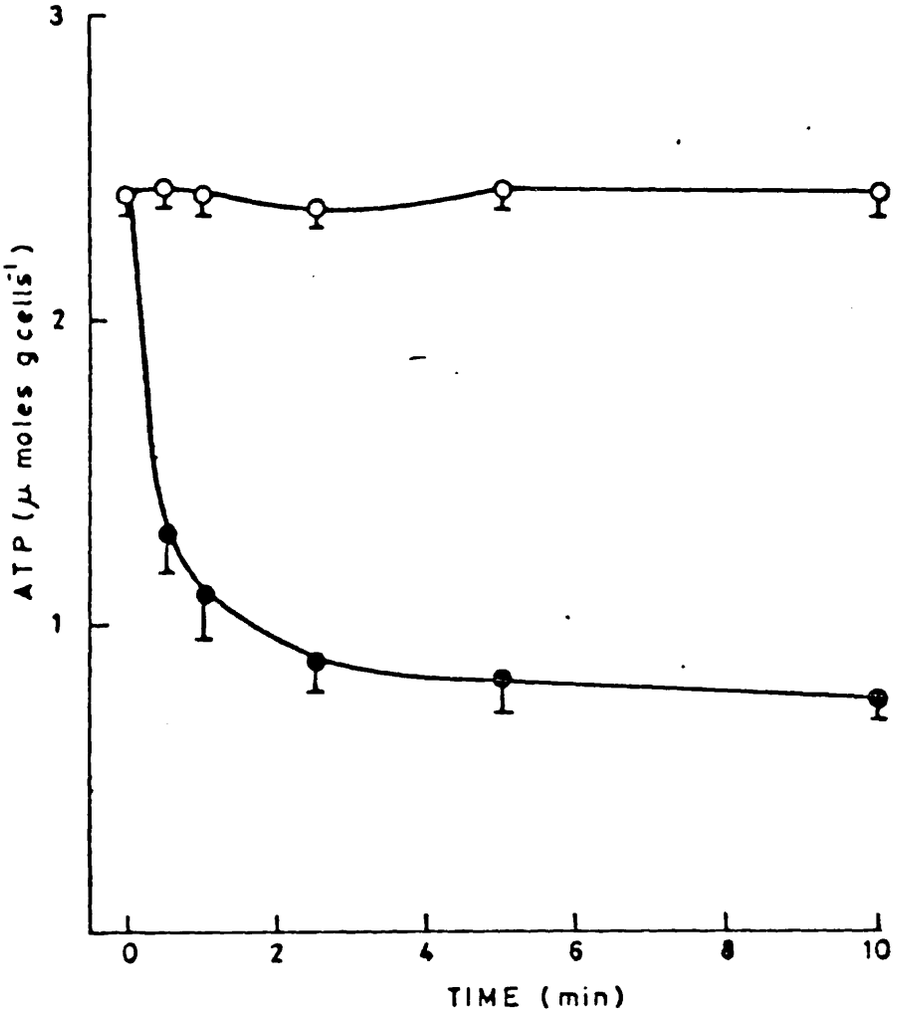


Figure 20. Rapid depletion of ATP in isolated hepatocytes treated with 10 mM fructose (●) compared with no added hexose (O).

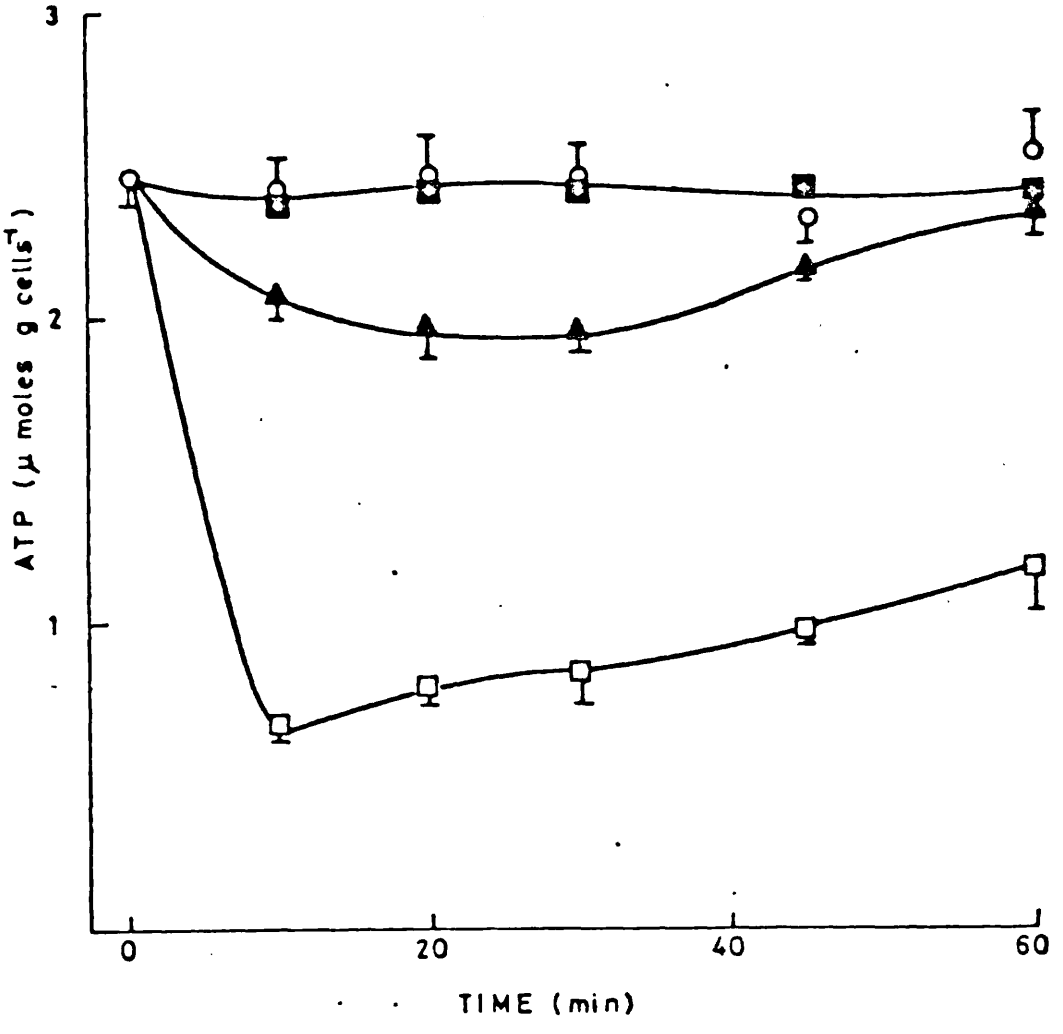


Figure 21. ATP content of isolated hepatocytes following treatment of cells with no addition (○), 4 mM dihydroxyacetone (n=2) (■), 10 mM dihydroxyacetone (▲) or 10 mM glycerol (□).

over a 60 min period, but a higher concentration of the substrate did cause a small but significant ($p<.002$) fall in ATP levels for up to 30 min. This latter finding is in contradiction to the results of Woods & Krebs (1973) who perfused liver for up to 40 min with 10 mM dihydroxyacetone, but the difference is probably the result of using different types of liver preparation. ATP concentrations in hepatocytes incubated for 45 and 60 min were not significantly different from the control values. On the other hand, incubating hepatocytes with 10 mM glycerol caused rapid and profound depletion of ATP during the first 10 min incubation, following which there was a small recovery of hepatocyte ATP concentration (figure 21). In this respect, the effect of the metabolism of glycerol is very similar to that of fructose (figure 19 & 21). This is in agreement with the findings of Woods and co-workers (Woods et al., 1970; Woods & Krebs, 1973) who used perfused liver.

The explanation for the difference between glycerol and dihydroxyacetone with respect to ATP depletion is not immediately clear, since the latter substrate is thought to be phosphorylated more rapidly than glycerol (Woods & Krebs, 1973). The specific activities of triokinase and glycerol kinase are known to be similar although the relative proportions of the two enzymes are dependent on diet and the sex of the animal (Mahmoud, 1979). A possible explanation is that glycolysis from the triose phosphate stage to pyruvate involves the production of two ATP molecules (see figure 1). Following a dihydroxyacetone load the initial step is phosphorylation in a reaction which depletes ATP and is catalysed by triokinase (see figure 3). Thus the glycolysis of dihydroxyacetone to pyruvate involves no net loss of ATP, in fact one molecule of ATP is produced. Gluconeogenesis from the triose phosphate stage also involves no net loss of ATP. On the other hand, glycerol inhibits glycolysis (see figure 30B), so that regeneration of ATP by glycolysis of the substrate can not occur. Therefore, although dihydroxyacetone is phosphorylated more rapidly than glycerol, the former substrate restores ATP levels by virtue of

its metabolism to pyruvate. Such an explanation would require the net glycolysis of 50% of the dihydroxyacetone metabolised.

2. Fructose-1-phosphate

The phosphorylation of fructose causes accumulation of Fru-1-P, *in vivo* (Van den Berghe *et al.*, 1977a) and in perfused liver (Woods *et al.*, 1970), which is an activator of PK at subsaturating substrate concentrations (Eggleston & Woods, 1970). This study was carried out to examine the effects of various substrates of Fru-1-P levels in isolated hepatocytes and to attempt to correlate this with the stimulatory effects of added substrate on PK activity reported in section III.A.

When isolated hepatocytes were incubated with fructose (10 mM), a rapid accumulation of Fru-1-P was observed (figure 22), concomitant with the depletion of ATP (figure 19) and the activation of PK (figure 11B). After the initial burst of Fru-1-P production, there was a more gradual increase in the level of the intermediate for up to 30 min incubation (figure 22). Maximum concentration of Fru-1-P produced (about 36 times the endogenous levels) was reached after 30 min incubation, but high concentrations of Fru-1-P were present throughout the incubation after the initial 30 s (figure 23).

The build up of Fru-1-P after a fructose load indicates that the breakdown of the ketose phosphate by aldolase is much slower than its rate of formation by ketohexokinase. However, since the K_m and V_{max} values of the two enzymes are very similar (Van den Berghe, 1978) then it must be concluded that *in vivo* fructose phosphorylation is much more rapid than aldolytic cleavage. The explanation for this phenomenon is not clear. Woods *et al.*,(1970) concluded that aldolase is inhibited by IMP which accumulates in the liver perfused with fructose but Smith *et al.*, (1977b) and Van den Berghe *et al.*,(1977a), have shown that the accumulation of IMP is subsequent to the accumulation of Fru-1-P. The former authors have suggested that Fru-1-P accumulation is simply the result of the fact that ketohexokinase and

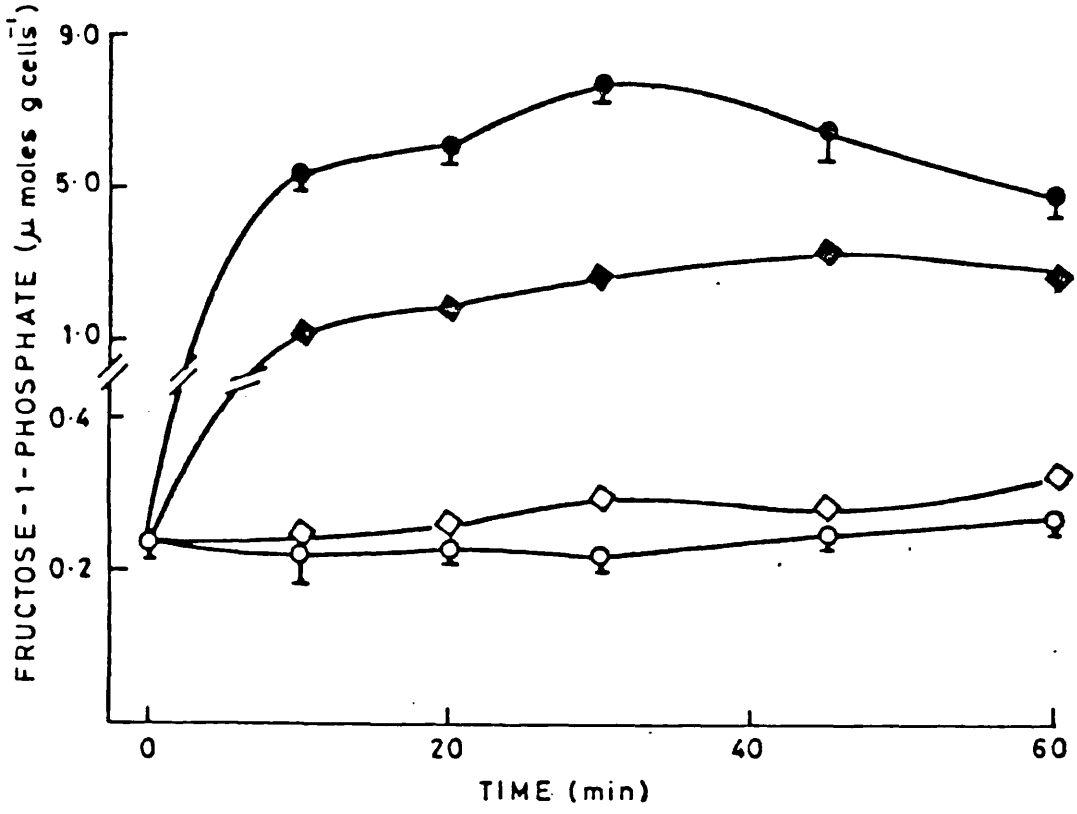


Figure 22. Time course showing changes in fructose-1-phosphate content of isolated hepatocytes incubated with no added hexose (O), 10 mM xylitol (◇), 10 mM sorbitol (◆), or 10 mM fructose (●). Hepatocytes were extracted with perchloric acid and the fructose-1-phosphate determined by an enzymic method (see methods, section IV.B.6e) (for sorbitol and xylitol n=2).

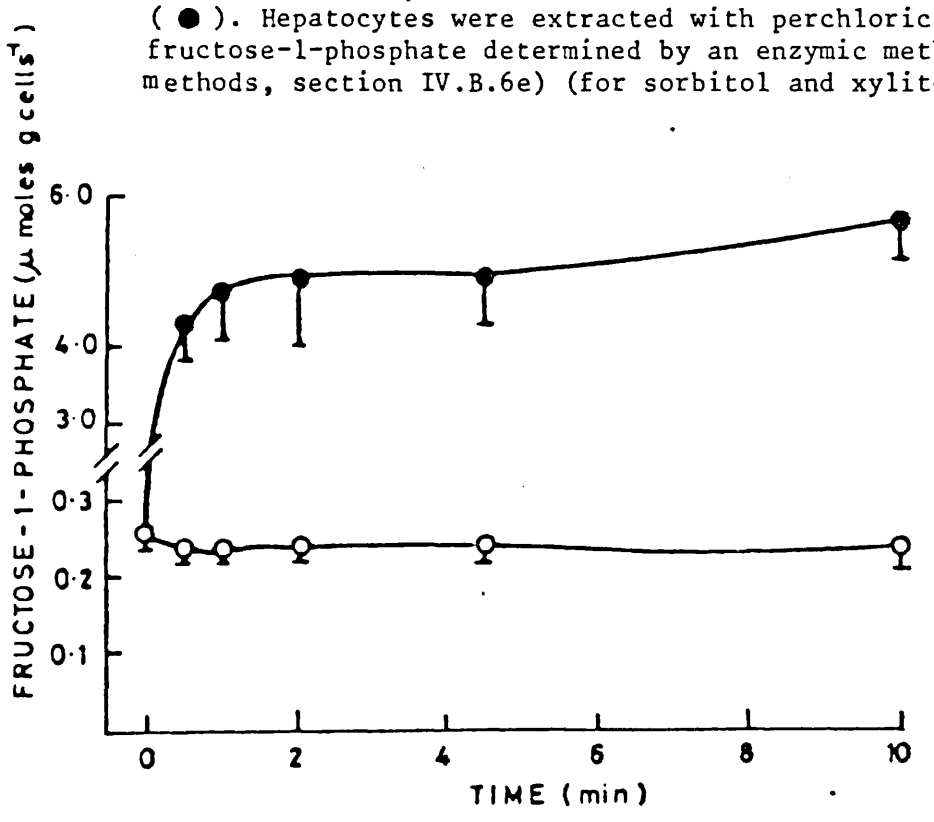


Figure 23. Rapid accumulation of fructose-1-phosphate in isolated hepatocytes treated with no added hexose (O) or 10 mM fructose (●).

aldolase act more rapidly than the enzymes of the metabolic pathways leading to lactate and glucose (Exton & Park, 1969; Van den Berghe et al., 1977a).

Since Fru-1-P is an activator of PK (Eggleston & Woods, 1970), the effects of fructose on hepatocyte Fru-1-P can be compared with PK activity in isolated hepatocytes incubated with fructose (figure 11B). The highest level of Fru-1-P that accumulates after adding fructose (10 mM) is 7.8 μmoles g cells^{-1} or approximately 20 mM, (it is assumed that 1 g of cells contain 380 μl of cytosolic water; Sobell et al., 1976). Taking into account the dilution factor involved in the assay procedure, it is surprising that the stimulatory effect of Fru-1-P is observed, in view of the low affinity of PK for Fru-1-P (see figure 53).

The pattern of rapid effects of fructose (10 mM) on Fru-1-P levels (figure 23) is similar to the activation of the enzyme in washed cells (figure 13B), and the concentration dependency of the enzyme activation by fructose (see figure 12B) is also reflected by Fru-1-P levels presented in figure 24A. From these observations, it seems possible that the activation obtained in washed hepatocytes incubated with fructose may be due to an accumulation of Fru-1-P. On the other hand, it is also probable that $Fru_{16}BP$ also makes a major contribution to the activation observed since the affinity of the enzyme for this effector is much higher than for Fru-1-P (see section III.B.3).

A study of the effects of sorbitol (10 mM) and xylitol (10 mM) on Fru-1-P concentrations in isolated hepatocytes is shown in figure 22. It is of interest to compare the effects of these two substrates because sorbitol is metabolised via fructose in the liver (Förster, 1974), whereas xylitol is first oxidised to xylulose by the NAD^+-dependent xylitol dehydrogenase (Smith, 1962; Williamson et al., 1971) and then phosphorylated to xylulose-5-phosphate which is an intermediate of the pentose phosphate shunt. After a series of transaldolase and transketolase

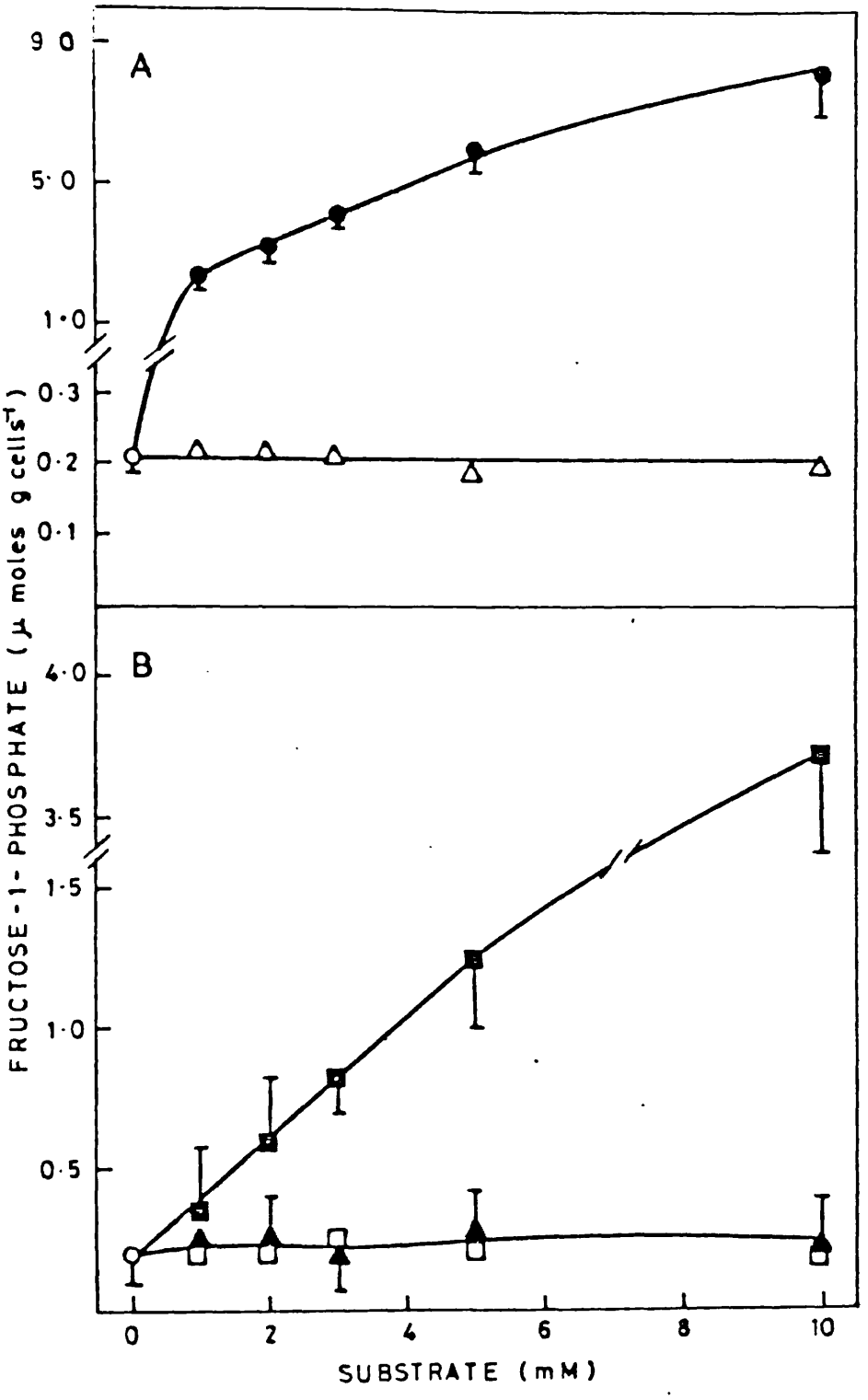


Figure 24. The concentration dependence of the effect of incubating isolated hepatocytes with; glucose (△), fructose (●), dihydroxyacetone (▲), glycerol (□) or D-glyceraldehyde (■) for 20 min, on the fructose-1-phosphate content, (for glucose and glycerol n=2).

reactions xylitol enters the glycolytic sequence as Fru-6-P and glyceraldehyde-3-phosphate (Froesh & Jakob, 1974). As expected, xylitol (10 mM) had no effect on Fru-1-P levels over the 60 min incubation period. In this respect this substrate has the same lack of effect as glucose. Incubating isolated hepatocytes with sorbitol (10 mM) (figure 22), however, caused a time dependent increase in Fru-1-P concentrations. In this case, the levels of Fru-1-P accumulating, are somewhat lower than those observed with 10 mM fructose, presumably because of the limitation of NAD^+ required to convert sorbitol to fructose via sorbitol dehydrogenase. Thus sorbitol also has the effect of increasing $(NADH)/(NAD)^+$ ratio in the hepatocyte (see section III.B.8).

Incubating isolated hepatocytes with glycerol (10 mM) and dihydroxyacetone (10 mM) revealed that these substrates have no effect on Fru-1-P (table 2). Both these trioses are phosphorylated first before any further metabolism (figure 3), thus in order to produce Fru-1-P from the phosphorylated products, a reversal of aldolase fission must occur in the presence of glyceraldehyde. The inability of glycerol and dihydroxyacetone to change hepatocyte Fru-1-P levels was observed at all concentrations of the substrate tested (figure 24B). Thus the activation of PK observed when hepatocytes are incubated with dihydroxyacetone is not due to elevated Fru-1-P concentrations. In addition the possibility that a substantial amount of glycerol or dihydroxyacetone is converted to glyceraldehyde is ruled out.

D-glyceraldehyde increased hepatocyte Fru-1-P levels, an effect which was concentration dependent (figure 24B). In contrast to the effect of fructose (figure 24A), the increase in Fru-1-P observed after incubating hepatocytes with a low D-glyceraldehyde (1 mM) concentration was small, but a substantial level was attained in the presence of 10 mM triose. The formation of Fru-1-P in the presence of D-glyceraldehyde must be the result of a reversal of the aldolase reaction resulting in the production of the ketose phosphate from DHAP and D-glyceraldehyde.

Table 2. The effect of glycerol and dihydroxyacetone on fructose-1-phosphate levels in isolated hepatocytes.

Time (minutes)	Fructose-1-phosphate (μmoles g cells^{-1})		
	Substrate Added		
	No Substrate	Glycerol[a] (10 mM)	Dihydroxyacetone (10 mM)
0	0.24 ± 0.01	-	-
10	0.22 ± 0.04	0.23	0.26 ± 0.03
20	0.23 ± 0.01	0.26	0.26 ± 0.02
30	0.22 ± 0.02	0.23	0.25 ± 0.02
45	0.25 ± 0.02	0.32	0.28 ± 0.02
60	0.26 ± 0.02	0.32	0.28 ± 0.02

Isolated hepatocytes from fed animals were incubated with 10 mM substrate for different time periods (up to 60 min). After the appropriate time interval, the reaction was stopped using perchloric acid and the neutralized supernatant (see methods, section IV.B.6) was used for Fru-1-P determination.

a = results presented are an average of determinations of two different hepatocyte preparations.

However, it is evident from figure 24B, that the Fru-1-P accumulation is not proportional to the triose concentration and that it is not a saturable process under these conditions of the experiment. The increased rate of Fru-1-P accumulation at high D-glyceraldehyde concentration is probably the result of a saturation of the pathways which metabolise the triose, leading to an accumulation of triose phosphates. Under these conditions, the accumulated DHAP combines with glyceraldehyde to form Fru-1-P.

3. Fructose-1, 6-bisphosphate

Another possible mechanism by which PK from isolated hepatocytes is activated after incubating with dihydroxyacetone, D-glyceraldehyde and fructose (section III.A) is the accumulation of $Fru_{16}BP$, a potent activator of the enzyme (Taylor & Bailey, 1967; Llorente et al., 1970; Seubert & Schoner, 1971). The levels of this intermediate in the liver are under hormonal (Pilkis et al., 1976a; 1976b, 1978c) and dietary (Lawson et al., 1976; Claus et al., 1979) regulation. It was therefore of interest to examine the effects of various substrates on $Fru_{16}BP$.

A time course examination of $Fru_{16}BP$ concentrations in untreated isolated hepatocytes (figure 25A) shows that the level of this intermediate are unchanged over the incubation period. Fructose (10 mM) caused a two-fold increase in $Fru_{16}BP$ levels in isolated hepatocytes within 10 min, followed by a small but steady increase of the bisphosphate over the 60 min incubation period (figure 25A). This increase in $Fru_{16}BP$ levels is also concentration dependent, but at physiological fructose concentrations there was only a small increase observed (figure 26A) A closer examination of the fructose (10 mM) effect shows that, there is a short lag of 30 s followed by a rapid increase in $Fru_{16}BP$ during the next 30 s (figure 27). The concentration of $Fru_{16}BP$ accumulating in hepatocytes (36 nmoles g cells^{-1} or 95 µM $Fru_{16}BP$ after 20 min incubation) treated with fructose would not be expected to cause a significant stimulation of the enzyme when the dilution factor is taken into consideration. Nevertheless, it is likely, that the high affinity of the enzyme for the effector results in the retention of the stimulatory

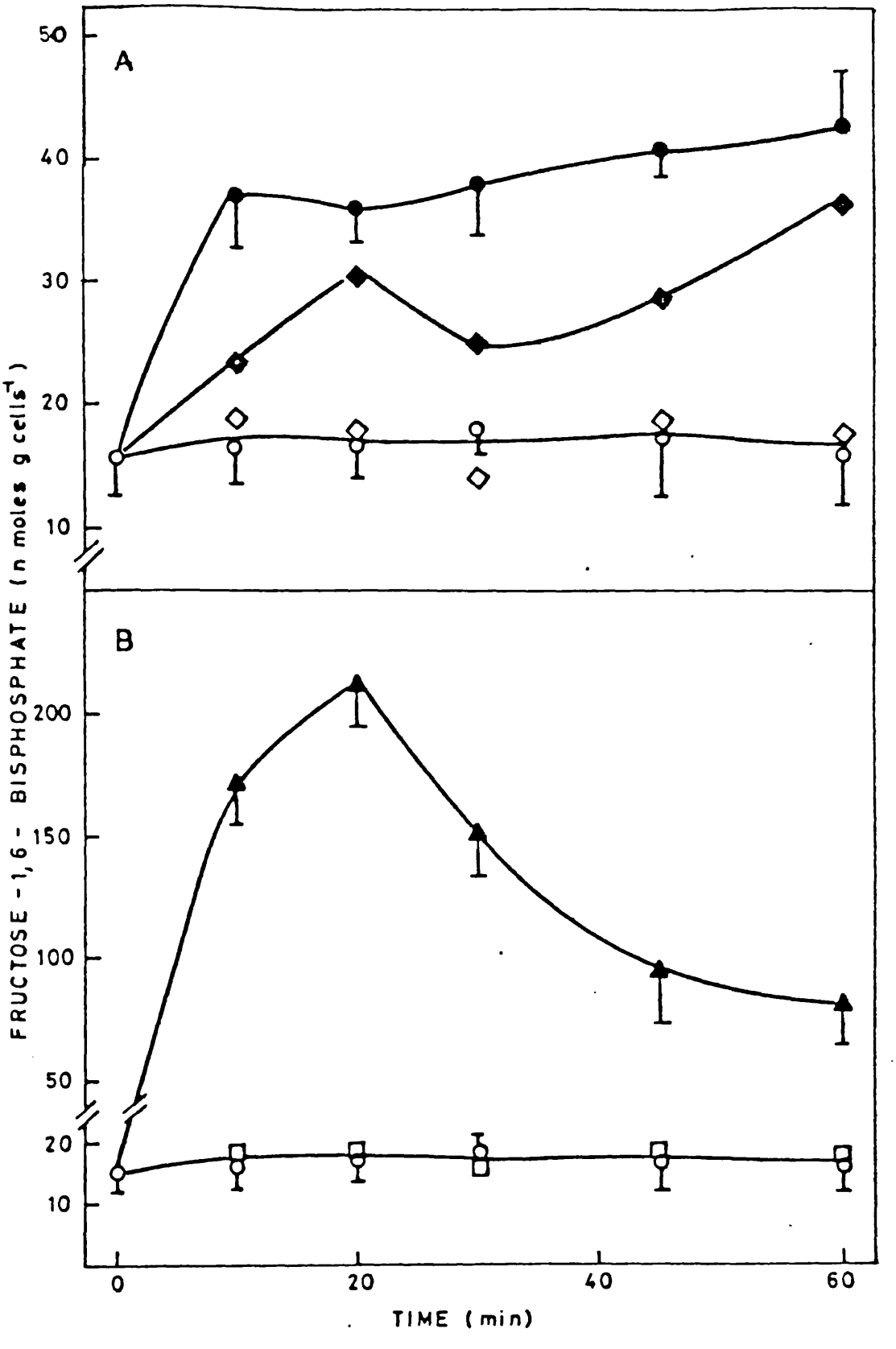


Figure 25. Time course showing changes in fructose-1,6-bisphosphate content of isolated hepatocytes incubated with no added substrate (○), 10 mM xylitol (n=2) (◇), 10 mM sorbitol (n=2)(◆), 10 mM fructose (●), 10 mM glycerol (□) or 10 mM dihydroxyacetone (▲). Hepatocytes were extracted with perchloric acid and the fructose-1,6-bisphosphate determined by an enzymic method (see methods, section IV.B.6d).

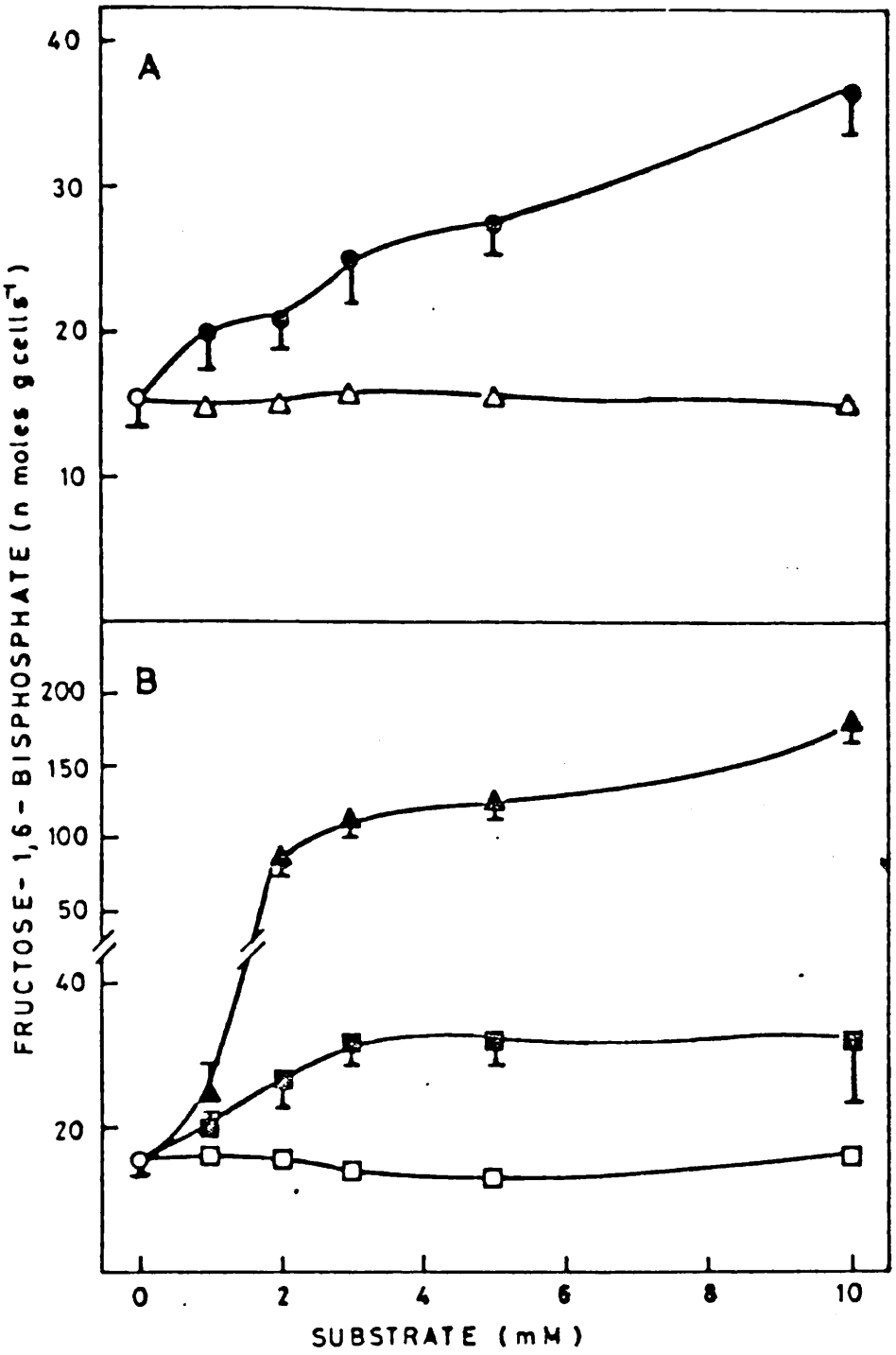


Figure 26. The fructose-1, 6-bisphosphate content in isolated hepatocytes incubated with glucose (n=2) (Δ), fructose (●), dihydroxyacetone (▲), glycerol (n=2) (□) or D-glyceraldehyde (■) for 20 min.

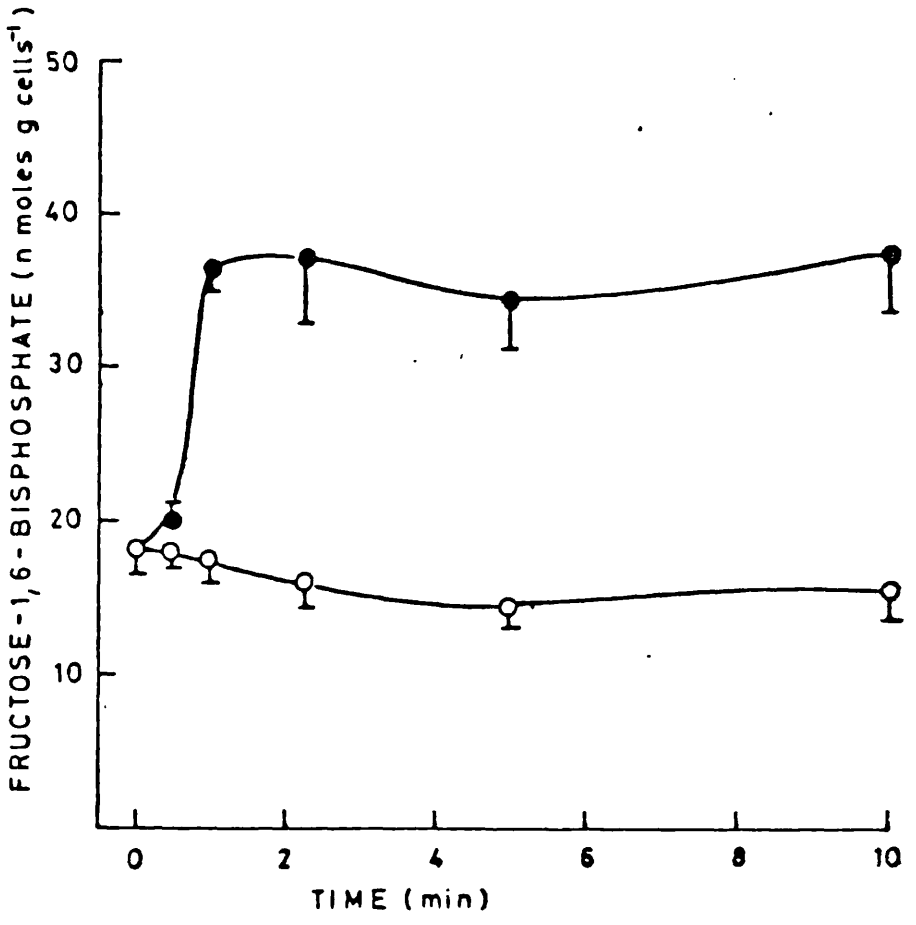


Figure 27. Time course showing the rapid accumulation of fructose-1,6-bisphosphate in isolated hepatocytes incubated with no added hexose (○) or with 10 mM fructose (●).

effect despite the dilution involved. The short time lag observed for the formation of $Fru_{16}BP$ is presumably due to the time taken to increase the triose phosphate pool and, possibly, a slower rate of aldolytic condensation compared to the initial phosphorylation of the ketose.

After the initial metabolism of fructose to the triose phosphates, the products can follow two routes, either gluconeogenesis or glycolysis. In fed animals, glycolysis is the dominant pathway (Pilkis et al., 1978a), hence most of the carbon from fructose would be expected to be channelled towards pyruvate. This is reinforced by some of the properties of the regulatory enzymes concerned (Scrutton & Utter, 1968; Pilkis et al., 1978a). For example, fructose (10 mM) causes a stimulation of PK by $Fru_{16}BP$ and Fru-1-P assuming that the inhibitor accumulating would normally be removed in vivo. In addition 10 mM fructose is known to lower the level of $Fru_{26}BP$, the recently discovered regulator of the Fru-6-P/$Fru_{16}BP$ substrate cycle (Hue, 1981). The result of this would be an inhibition of PFK 1 and a stimulation of $Fru_{16}BPase$ (Claus et al., 1980; Furuya & Uyeda, 1980; Van Schaftingen & Hers, 1980, 1981a; Pilkis et al., 1981; Uyeda et al.,1981a,1981b) resulting in stimulation of gluconeogenesis from triose phosphate. However, at low fructose concentration, $Fru_{26}BP$ concentration is raised resulting in an inhibition of gluconeogenesis. Therefore, it would be expected that at physiological fructose concentration, most of the flux from fructose would be directed to pyruvate and lactate, whereas at high concentration a greater proportion of the flux would be directed to glucose.

From an examination of the effect of 10 mM xylitol and 10 mM sorbitol on $Fru_{16}BP$ concentration (figure 25A) it is clear that xylitol has no significant effect on the levels of this intermediate, whereas sorbitol (10 mM) caused an accumulation of $Fru_{16}BP$. Once again the effect of sorbitol mimicks that of fructose.

A study of the effect of glycerol and dihydroxyacetone indicate that,the former substrate had no effect on $Fru_{16}BP$ level but the latter caused a considerable change in hepatocyte $Fru_{16}BP$ concentration

(figure 25B). The maximum level of $Fru_{16}BP$ accumulating (214 ± 23 n moles g cells^{-1} or 0.6 mM) was observed after 20 min incubation with dihydroxyacetone. This indicates that the activation of PK in isolated hepatocytes,incubated with dihydroxyacetone,observed in the present study (see section III.A) is due to accumulation of this allosteric effector of the enzyme. The stimulation of PK by dihydroxyacetone (see figure 15B) is concentration dependent as is the accumulation of $Fru_{16}BP$ (figure 26B). There is a discrepancy between the relatively small increases in $Fru_{16}BP$ and the observed activation of PK at 1 mM dihydroxyacetone. This accentuates the care that must be exercised in comparing the activation of PK with the concentration of $Fru_{16}BP$ accumulating in the cells. It also suggests that the increased $Fru_{16}BP$ resulting from fructose utilization by hepatocytes may be sufficient to explain the observed activation of PK without invoking a Fru-1-P effect. This is especially true if one considers the high affinity of PK for $Fru_{16}BP$ and the dilution of the effectors during the assay procedure. However, the effect of Fru-1-P on PK in vivo must be considered because of the high levels of the ketose phosphate present in the cytosol of the hepatocyte.

Incubating hepatocytes with D-glyceraldehyde also causes a concentration dependent increase in the $Fru_{16}BP$ (figure 26A). The effect was smaller than that with dihydroxyacetone presumably because of the greater affinity of triokinase for dihydroxyacetone (Veneziale, 1976). An examination of PK activity in hepatocytes incubated with D-glyceraldehyde (figure 16) indicates that maximal activation in washed cells is reached with a low concentration of the substrate. The concentration of $Fru_{16}BP$ present when cells are incubated with 1 mM D-glyceraldehyde (20.4 ± 1.7 n moles g cells^{-1}) is apparently sufficient to cause such activation, if one ignores a possible contribution by Fru-1-P. This is further evidence that the increase in PK activity observed with low levels of fructose is probably due to an elevation of $Fru_{16}BP$ rather than that of Fru-1-P. It also suggests that the changes in $Fru_{16}BP$ levels which occur in vivo must be fairly small

compared to those observed in this study with high substrate concentrations, if these changes are to have any physiological significance.

4. Dihydroxyacetone phosphate

Most of the substrates examined in this study enter the glycolytic pathway at the triose phosphate stage (see figure 3). Thus an examination of the concentration of the triose phosphates in response to various substrates may give some indication of the rate of metabolism of the substrates.

The DHAP concentrations were examined in isolated hepatocytes incubated with 10 mM substrate for different times (Table 3) and at varying concentration of substrates (Table 4). Hepatocytes incubated with fructose showed no changes in DHAP concentration within 20 min at concentrations of fructose up to 10 mM. A longer incubation time resulted in small elevation of the triosephosphate concentration at 10 mM fructose. It is perhaps surprising that no change in DHAP levels was found at the short time intervals, since the levels of $Fru_{16}BP$ (figure 25A); G3P (figure 28A) and pyruvate and lactate (figure 30A) are raised by fructose. The results suggest that once formed, DHAP is rapidly converted to these intermediates. The lack of DHAP accumulation is evidence against the theory put forward by Exton & Park (1967) and Van den Berghe (1978), that Fru-1-P accumulates in response to fructose as a result of high levels of both aldolase and fructokinase activity. On the contrary it is further evidence that aldolase is a rate limiting step in the metabolism of Fru-1-P. Woods et al.,(1970) found an increase in DHAP after 10 min perfusion of the liver with 10 mM fructose. This discrepancy is presumably related to the type of liver preparation used.

In the present study, sorbitol, xylitol and glycerol were also without effect on DHAP levels, whereas dihydroxyacetone rapidly elevated DHAP concentrations (up to about 4-fold), an effect which is concentration dependent (Table 3 & 4). No elevation was observed when

Table 3. The effect of various substrates on DHAP concentrations in isolated hepatocytes

Time (minutes)	Dihydroxyacetone phosphate (nmoles g cells^{-1}) substrate added (10 mM)					
	No substrate	Fructose	Sorbitol	Dihydroxy-acetone	Glycerol	Xylitol
0	39.0 ± 3.3	-	-	-	-	-
10	41.4 ± 2.9	48.2 ± 3.2	37.4 ± 1.3	121.2 ± 33.4	42.7 ± 4.9	47.3 ± 6.9
20	43.4 ± 2.3	45.5 ± 4.8	37.8 ± 2.0	135.3 ± 42.3	46.7 ± 2.0	41.1 ± 1.9
30	43.7 ± 2.7	51.9 ± 3.7	40.8 ± 2.6	121.2 ± 33.9	46.4 ± 4.0	45.5 ± 1.6
45	39.6 ± 3.0	53.2 ± 4.3	45.5 ± 4.0	95.3 ± 29.9	55.8 ± 1.2	44 ± 1.2
60	45.0 ± 3.0	53.8 ± 3.3	49.2 ± 2.1	60.6 ± 16.0	54.6 ± 2.9	63.0 ± 0.52

Isolated hepatocytes from fed animals were incubated for up to 60 min in the presence of various substrates. After the desired incubation period, the reaction was stopped using perchloric acid and the neutralized supernatant (see methods, section IV.B.6) was used for DHAP assay.

Table 4. DHAP levels in isolated hepatocytes incubated with various substrates for 20 min.

Concentration of substrate added (mM)	Dihydroxyacetone phosphate (n moles g cells^{-1})				
	Fructose	Glucose[a]	Glycerol[a]	Dihydroxyacetone	D-Glyceraldehyde
0	42.3 ± 4.6	-	-	-	-
1	40.3 ± 3.0	42.3	42.3	40.0 ± 3.0	41.9 ± 5.0
2	40.0 ± 1.9	45.4	41.4	47.0 ± 3.2	44.3 ± 5.3
3	45.0 ± 3.1	45.4	44.6	96.0 ± 3.0	46.3 ± 3.8
5	45.0 ± 2.5	44.6	41.4	115.5 ± 5.0	23.11± 4.1
10	43.0 ± 1.6	44.6	41.4	139.5 ± 9.3	11.7 ± 2.1

Isolated hepatocytes obtained from fed animals were incubated for 20 min with different substrate concentrations. The reaction was stopped using perchloric acid and the neutralized supernatant (see methods, section IV.B.6) was used to estimate DHAP concentrations.

a - the results presented are an average of two different cell preparations.

hepatocytes are incubated for 20 min with low concentrations of dihydroxyacetone, suggesting that under these conditions, the metabolism of DHAP was not a limiting factor, but that at higher concentrations of the triose, the rate of synthesis of DHAP exceeded the rate of its metabolism, thus leading to an elevation of the triose phosphate. The elevation of DHAP is mirrored by the increase in $Fru_{1,6}BP$ suggesting that the triose phosphate and the hexose diphosphate are in equilibrium. The parallel decrease in the levels of these two metabolites, which occurs after 20 min incubation (figure 25B & Table 3) gives an indication of the rate of metabolism of the triose phosphate pool.

D-glyceraldehyde at concentrations up to 3 mM had no significant effect on DHAP levels (Table 4) despite the fact that this triose is phosphorylated to glyceraldehyde-3-phosphate (by triokinase), which is readily converted to DHAP (figure 3). Unexpectedly the DHAP levels are lowered by high concentrations of D-glyceraldehyde (Table 4). A possible explanation for this effect is that at such high concentrations of D-glyceraldehyde, the reverse aldolase reaction is occurring. Figure 24B shows that at these substrate concentrations Fru-1-P accumulates. Thus it is likely that the high levels of D-glyceraldehyde react with DHAP yielding Fru-1-P and therefore, lowering hepatocyte DHAP levels. At the same time glycolysis is increased by D-glyceraldehyde (figure 32B) contributing to the fall in DHAP concentration.

5. Glycerol-3-Phosphate

Glycerol-3-phosphate (G3P) is another important triose phosphate that may accumulate under various conditions (Burch et al., 1970; Woods & Krebs, 1973). In order for G3P to enter the glycolytic sequence, NAD^+ dependent oxidation to DHAP by glycerol-3-phosphate dehydrogenase must occur. An alternative fate of the G3P is its utilisation in the synthesis of triacylglycerol and phospholipids.

The levels of G3P are unaffected by incubation in the absence of substrate or with glucose (up to 10 mM) (figure 28A & 29A). This is another indication of the viability of hepatocytes since G3P is elevated

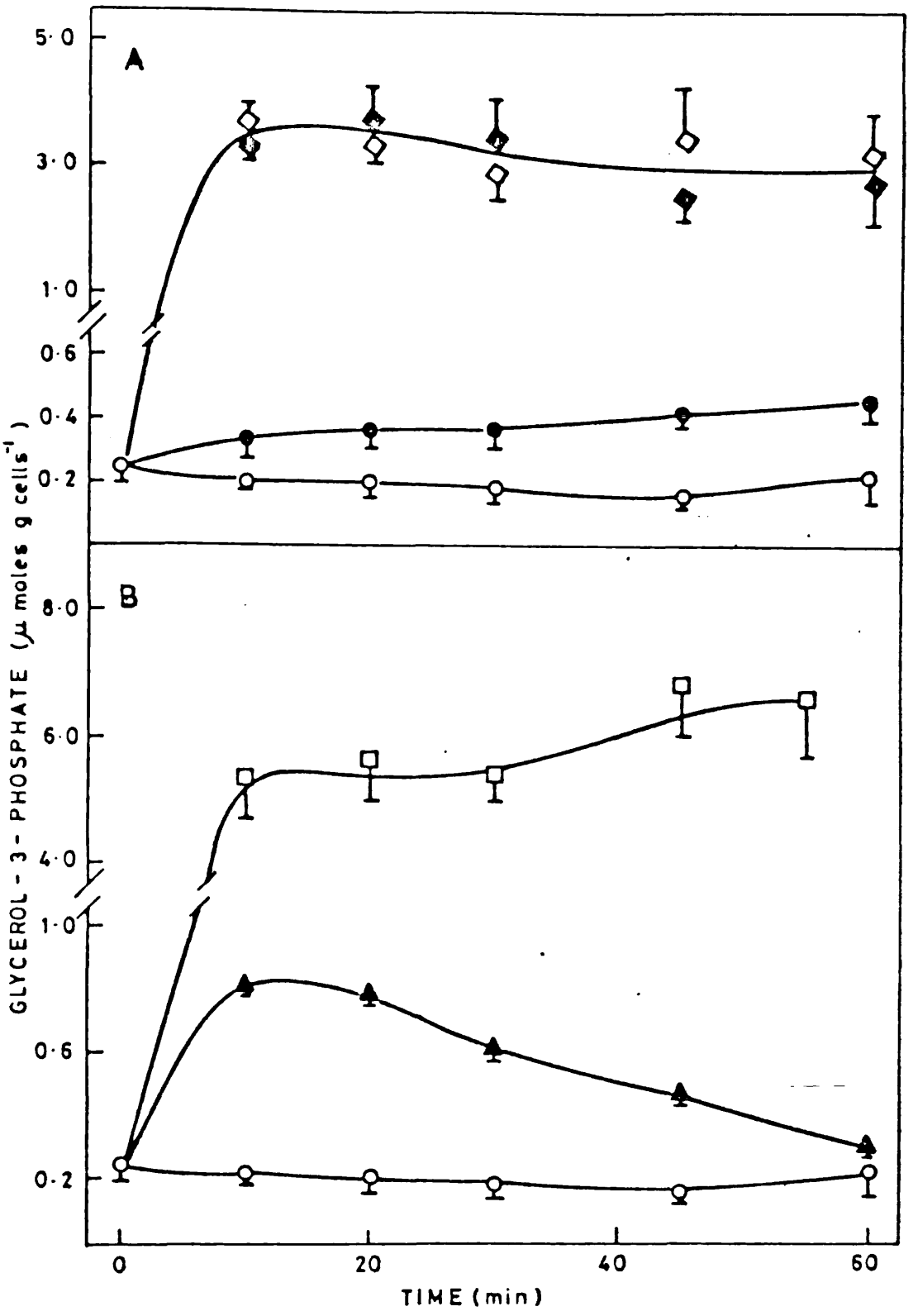


Figure 28. Time course of the changes in the glycerol-3-phosphate content of isolated hepatocytes incubated with no added substrate (○), 10 mM fructose (●), 10 mM xylitol (◇), 10 mM sorbitol (◆), 10 mM glycerol (□) or 10 mM dihydroxyacetone (▲). Hepatocytes were extracted with perchloric acid and the glycerol-3-phosphate determined enzymatically (see methods, section IV.B. 6f).

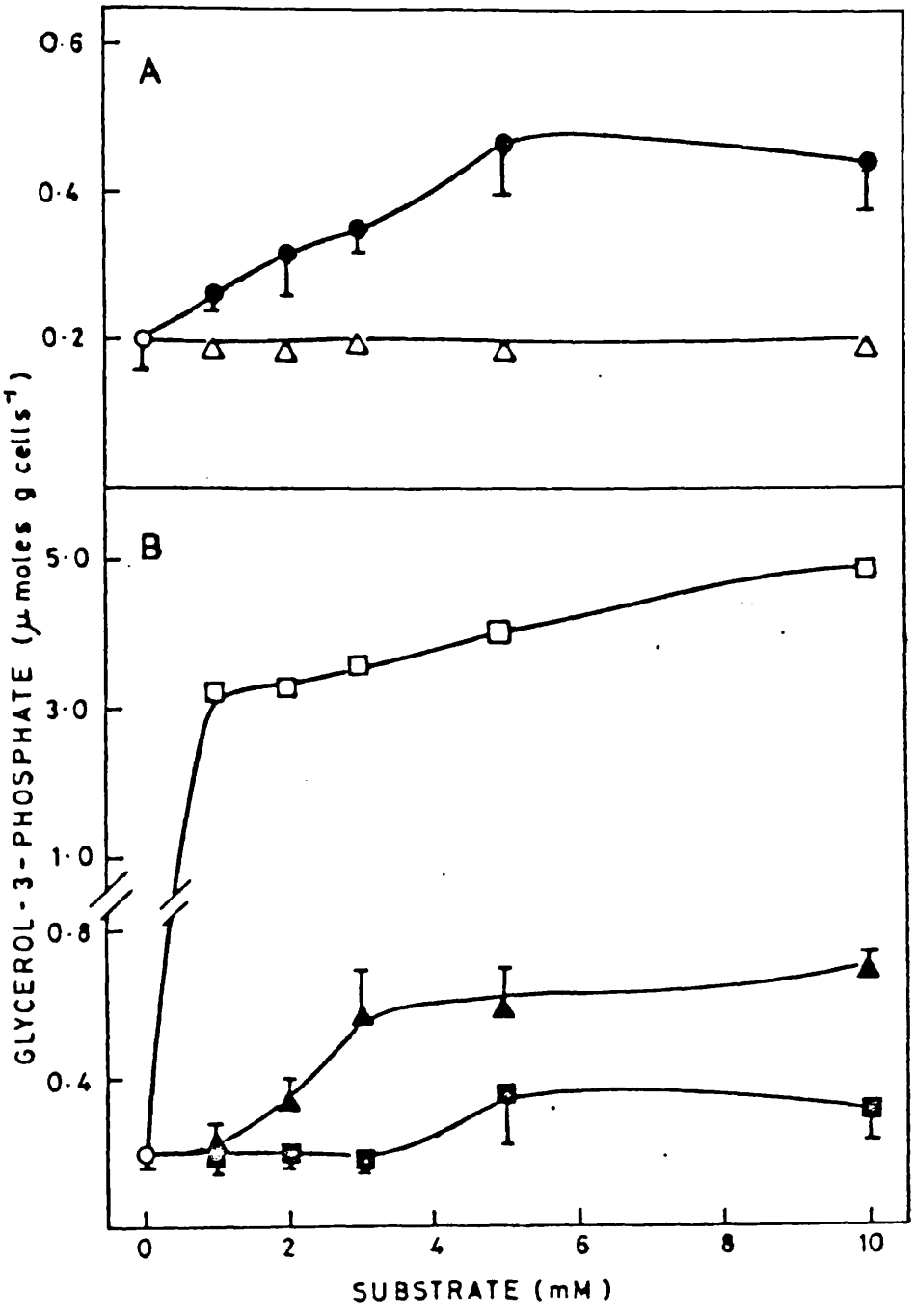


Figure 29. Glycerol-3-phosphate content in isolated hepatocytes incubated with glucose (△), fructose (●), dihydroxyacetone (▲), glycerol (□) or D-glyceraldehyde (■) for 20 min, (for glucose and glycerol n=2).

considerably immediately following the preparation of the hepatocytes (results not shown), but the 10 min preincubation period restores the level of the metabolite to those observed *in vivo* (results not shown).

Hepatocytes incubated with fructose (10 mM) contained elevated levels of G3P, an effect which is time dependent (figure 28A). The increase, although smaller, is also observed at physiological concentrations of fructose (figure 29A). The explanation for the increase in G3P concentrations caused by fructose is not immediately clear since DHAP levels are unaffected (Table 3 & 4). One possible explanation for this phenomenon is that the $(NADH)/(NAD)^+$ ratio is altered by fructose. However, this is not borne out when the change in $(NADH)/(NAD)^+$ ratio was measured using (lactate)/(pyruvate) ratios (see Table 8) and (G3P)/(DHAP) (Table 10) ratios (Hohorst *et al* 1959; Berry, 1980). Another possibility is that an alternative pathway for the metabolism of of glyceraldehyde is in operation in addition to the route involving triokinase. Thus the D-glyceraldehyde formed by the aldolytic cleavage of Fru-1-P, is converted to glycerol by NAD^+ linked glycerol dehydrogenase, and the glycerol formed is metabolised via glycerokinase to G3P and DHAP (Veneziale 1972a; 1972b). This route would not involve a change in $(NADH)/(NAD)^+$ ratio. Evidence for the occurrence of this pathway is also to be found when the effect of D-glyceraldehyde on G3P levels is considered (figure 29B). High concentrations of the triose increase the G3P levels, but at the same time decreasing the DHAP levels, again suggesting that the metabolism of glyceraldehyde via glycerol dehydrogenase is possible. However, the effects of fructose were observed at low concentrations (figure 29B) while D-glyceraldehyde up to 3 mM had no effect on G3P concentrations. The explanation for this discrepancy is not clear.

Dihydroxyacetone (10 mM) caused an initial elevation of G3P concentrations followed by a gradual decline to control levels over the 60 min incubation period (figure 28B). This mirrors the change in DHAP (Table 3) and $Fru_{16}BP$ (figure 25B) during the same period.

The (G3P)/(DHAP) (Table 10) and (lactate)/(pyruvate) (Table 8) ratios are largely unaffected. Therefore the change in G3P observed merely reflects the alteration in the size of the triose phosphate pool. The effect of increasing dihydroxyacetone concentrations on G3P levels (figure 29B) is the same as on DHAP (Table 4) further reinforcing the above hypothesis.

An examination of the time course effect (figure 28B) of glycerol (10 mM) on G3P concentrations in isolated hepatocytes shows that there is a 25-fold increase in the levels of the phosphorylated product within 10 min followed by a gradual increase over the next 50 min. These observations are similar to those made by Woods & Krebs, (1973), using perfused livers from fed rats and by Berry et al.,(1973) using isolated hepatocytes from fasted rats. Glycerol is phosphorylated by glycerokinase to G3P, which in turn can be oxidised to DHAP in a reaction involving NAD^+-dependent glycerol-3-phosphate dehydrogenase. Considering the vast difference detected in G3P levels accumulating in hepatocytes after a dihydroxyacetone (10 mM) load (0.8 $\pm$ 0.1 µ moles g $cells^{-1}$) and a glycerol (10 mM) load (5.3 $\pm$ 0.9 µmoles g $cells^{-1}$), it is evident that the oxidation of G3P to DHAP is the limiting step in glycerol metabolism. Thus it is clear that the formation of G3P is more rapid than its catabolism, which is somewhat surprising because the activity in liver of glycerol-3-phosphate dehydrogenase is higher than that of glycerokinase (Burch et al., 1970; Mahmoud, 1979). It thus seems probable that under these conditions the increase in G3P content of hepatocytes is observed because of the limited availability of NAD^+ in the hepatocyte. A possible mechanism for the regeneration of NAD^+ from NADH is the conversion of pyruvate to lactate, which is also limiting because of the low levels of pyruvate available in the hepatocyte due to a slow rate of glycolysis from glycerol (figure 30B). Therefore the overall $(NADH)/(NAD^+)$ ratio in the hepatocyte is increased by a glycerol load and this is reflected by the (lactate)/(pyruvate) ratio (table 8) and (G3P)/(DHAP) ratio (Table 10). The concentration of G3P in the hepatocyte was considerably increased at all concentrations of substrate examined (figure 29B). A concentration

of 1 mM substrate elevated the concentration of the phosphorylated intermediate 17-fold, thus the mechanism described above is also valid at lower concentrations of the substrate.

When isolated hepatocytes were incubated with sorbitol (10 mM) and xylitol (10 mM) there was a rapid increase in G3P concentrations after 10 min incubation (figure 28A). Since the metabolism of these substrates involves an initial oxidation step coupled to NAD^+ reduction, it seems probable that the elevated G3P levels occur when DHAP is reduced by NADH-dependent glycerol-3-phosphate dehydrogenase in order to restore NAD^+ levels. Further evidence that these substrates are oxidised in a reaction involving reduction of NAD^+ is shown by the (lactate)/(pyruvate) ratios and (G3P)/(DHAP) ratios (Table 8 & Table 10 respectively). The (G3P)/(DHAP) ratios are higher when hepatocytes are incubated with glycerol (10 mM) than when incubated with sorbitol or xylitol. This is presumably because in glycerol treated cells, there is a greater overall reduction of NAD^+ because the initial phosphorylation step elevates G3P concentrations, whereas, in hepatocytes treated with the latter substrates, there is a certain amount of regeneration of NAD^+ during the reduction of DHAP to G3P. However, the reason why the (lactate)/(pyruvate) ratios for glycerol (10 mM) was lower than those for sorbitol (10 mM) and xylitol (10 mM) is not clear, since it might be expected that the former substrate would deplete the cell of NAD^+ more effectively than sorbitol and xylitol.

6. Phosphoenolpyruvate

It is evident from the observations made on metabolite levels in isolated hepatocytes incubated with various substrates that, the activation of PK by these substrates in washed cells (see section III.A) is probably due to the accumulation of Fru-1-P, $Fru_{16}BP$ or both. On the other hand, when PK activity is measured in unwashed cells incubated with high fructose (10 mM) concentrations, an inhibition of the enzyme is observed (see figure 11A). Thus, in an attempt to obtain a clearer picture of the flux through PK under these conditions, the concentrations of PEP (a substrate for the enzyme)in hepatocytes were measured.

An examination of the time course dependency of PEP concentrations in isolated hepatocytes reveals that, in untreated cells as well as cells treated with glucose (up to 10 mM) there is no significant change in the levels of the phosphorylated substrate (Table 5 & 6). This result is not surprising because glucose has no effect on the PK activity in either washed or unwashed cells (figure 12A & B), suggesting that all the glycolytic reactions, including that of PK, are in a steady state and the rate of glycolysis to PEP is matched by the PK activity. Hepatocytes incubated with fructose (10 mM) showed no significant change in PEP concentrations during the same time period (Table 5), indicating that, although there is increased glycolysis from fructose, PK is not rate limiting, presumably because of the activation of the enzyme by Fru-1-P and $Fru_{16}BP$.

Unexpectedly,dihydroxyacetone caused an elevation of PEP levels (Table 5) despite the fact that $Fru_{16}BP$ levels are raised by this substrate (figure 25B). This suggests that the rate of glycolysis to PEP from dihydroxyacetone is greater than that from fructose, and that in the case of the triose, PK is a rate limiting step in its metabolism despite the high levels of $Fru_{16}BP$ in the cell. However, when the rates of glycolysis, as measured by lactate and pyruvate (figure 30), from dihydroxyacetone and fructose are compared, there is initially a greater output of lactate from the former substrate, consistent with the above explanation, which slows down with time and is considerably less than that from fructose over a 60 min incubation period (figure 30A and figure 30B). The explanation for this phenomenon is not clear. It is possible that another inhibitor of PK is accumulating inside the hepatocytes incubated with dihydroxyacetone (10 mM), but there is little evidence to support this hypothesis. Another possible explanation for the differential effect of fructose and dihydroxyacetone can be explained by the inhibitory action of ATP on PK activity (Lorente et al., 1970; Seubert & Schoner, 1971; Imamura et al., 1972). When hepatocytes are incubated with 10 mM dihydroxyacetone there is an increase in PEP levels, an effect not observed with 10 mM fructose (Table 5), and this is presumably related to the profound depletion of ATP in the case of the latter substrate (figure 19), which should result in releasing the inhibition of PK by the nucleotide triphosphate.

Table 5. Phosphoenolpyruvate levels in isolated hepatocytes incubated with various substrates.

Time (minutes)	Phosphoenolpyruvate (μmoles g cells^{-1}) Substrate added (10 mM)				
	No Substrate	Fructose	Glucose	Glycerol	Dihydroxyacetone
0	0.15 ± 0.02	-	-	-	-
10	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.12 ± 0.02*	0.18 ± 0.06^{NS}
20	0.15 ± 0.01	0.15 ± 0.02	0.14 ± 0.02	0.10 ± 0.01***	0.19 ± 0.03*
30	0.14 ± 0.02	0.13 ± 0.02	0.15 ± 0.03	0.10 ± 0.01*	0.23 ± 0.05**
45	0.14 ± 0.01	0.18 ± 0.02	0.13 ± 0.02	0.11 ± 0.01**	0.2 ± 0.06^{NS}
60	0.14 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.09 ± 0.01***	0.2 ± 0.06^{NS}

Isolated hepatocytes prepared from fed rats were incubated with 10 mM substrate for up to 60 min. The reaction was stopped using perchloric acid and the supernatant was neutralized with K_2CO_3 and used to determine PEP concentrations (see methods, section IV.B.6).

Substrate versus control: NS - not significant; * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.

Table 6. Phosphoenolpyruvate levels in isolated hepatocytes incubated for 20 min with various substrates.

Concentration of Substrate added (mM)	Phosphoenolpyruvate (μmoles g cells^{-1}				
	Fructose	Glucose	Glycerol	Dihydroxyacetone	D-Glyceraldehyde[a]
0	0.16 ± 0.03	-	-	-	-
1	0.20 ± 0.04^{NS}	0.17 ± 0.03	0.13 ± 0.003	0.16 ± 0.01	0.15
2	0.26 ± 0.03***	0.15 ± 0.01	0.13 ± 0.003	0.14 ± 0.02	0.17
3	0.23 ± 0.05*	0.16 ± 0.01	0.12 ± 0.005	0.20 ± 0.02^{NS}	0.16
5	0.12 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	0.23 ± 0.03*	0.10
10	0.17 ± 0.01	0.16 ± 0.01	0.12 ± 0.02	0.26 ± 0.03**	0.10

Isolated hepatocytes from fed animals were incubated for 20 min with different concentrations (up to 10 mM) of substrate. After the incubation period, the reaction was stopped with perchloric acid and the neutralized supernatant used to estimate PEP concentrations.

a = results presented are an average of two different cell preparations.

substrate versus control: NS = not significant; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

A further examination of the PEP levels in isolated hepatocytes incubated for 20 min with various substrates (Table 6), shows that all concentrations of glucose (up to 10 mM) have no significant effect on hepatocyte PEP concentrations. However, although high fructose concentrations (5 and 10 mM) had no significant effect on the concentration of PEP, physiological concentrations of the hexose caused a small but significant increase in PEP concentrations (Table 6). This seems to indicate that at these concentrations of fructose (i.e. 2 and 3 mM) the rate of synthesis of PEP is not matched by PK activity. Nevertheless, from the results shown in section III.A (see also figure 12), low ketose concentrations are sufficient to cause activation of PK, which would seem to indicate that the rate of fructolysis to PEP is greater than the PK activity. There is no evidence in the literature that the levels of the other substrate for PK (ADP) are significantly altered by fructose (Mäenpää et al., 1968; Van den Berghe et al., 1977a; Yip & Lardy, 1981). There is no significant effect of low dihydroxyacetone concentrations on hepatocyte PEP levels, but higher concentrations of the triose caused a significant elevation of this metabolite (Table 6). This may be related to a higher rate of glycolysis from fructose than from dihydroxyacetone. However, the depletion of ATP by low concentrations of fructose (2 mM) is much less than at higher concentrations (5 and 10 mM) of the substrate (figure 19) and this probably results in a significant inhibition of PK by the ATP at low fructose concentrations leading to an elevation of PEP. On the other hand, after an initial burst of lactate plus pyruvate production, glycolysis from dihydroxyacetone proceeds at a much slower rate, ATP is not depleted, and as a result of these two factors PEP is elevated. Rates of glycolysis from D-glyceraldehyde are similar to those from dihydroxyacetone (figure 32B) yet PEP levels are lowered by high concentrations of D-glyceraldehyde (Table 6). This difference between dihydroxyacetone and D-glyceraldehyde may be related to the raised Fru-1-P levels in the case of the latter substrate (figure 24B).

Glycerol (10 mM) caused a decrease in PEP levels within 10 min (Table 5) despite the profound depletion of ATP (figure 21) in this

time. The decrease in PEP was found at all concentrations of the substrate examined (Table 6). Since there is no net lactate plus pyruvate production from glycerol (10 mM) indeed the basal rate of glycolysis is inhibited by the substrate (figure 30B), the lowering of PEP by glycerol can simply be explained by the change in $(NADH)/(NAD^+)$ ratio which results in the inhibition of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase (Furfine & Velick, 1965)step. This is borne out by the evidence that while PEP levels drop in response to a glycerol load, G3P and DHAP levels rise (figure 28 & 29). Since glyceraldehyde-3-phosphate is in equilibrium with DHAP and 1, 3 PGA is in equilibrium with PEP (Jakob et al., 1971) this suggests that the control is imposed at the glyceraldehyde-3-phosphate dehydrogenase step.Such an explanation is consistent with the effect of xylitol in perfused liver (Jakob et al., 1971) where PEP levels are severely lowered by this substrate whereas triose phosphate and G3P levels are raised.

7. Total glycolytic flux in isolated hepatocytes incubated with various substrates

The substrates studied in this thesis are mainly metabolised via the glycolytic and gluconeogenic sequences (see introduction section I, also see figure 3). The rate of accumulation of the major end products of glycolysis (i.e. pyruvate and lactate) gives an estimation of the glycolytic flux, and thus the flux through PK. However, it must be noted that the contribution of gluconeogenesis from lactate, pyruvate dehydrogenase activity and alanine aminotransferase activity, would result in an underestimation of the glycolytic flux.

An examination of the time course of total pyruvate plus lactate produced by isolated hepatocytes shows that, there is a slow but significant rate of glycolysis in glucose (10 mM) treated cells as well as in untreated cells (figure 30) over a 60 min incubation period. There is no statistical difference between the rate of glycolysis in the presence and absence of added glucose (up to 10 mM) (figure 30A and 32A). Presumably the pyruvate and lactate arises from the breakdown

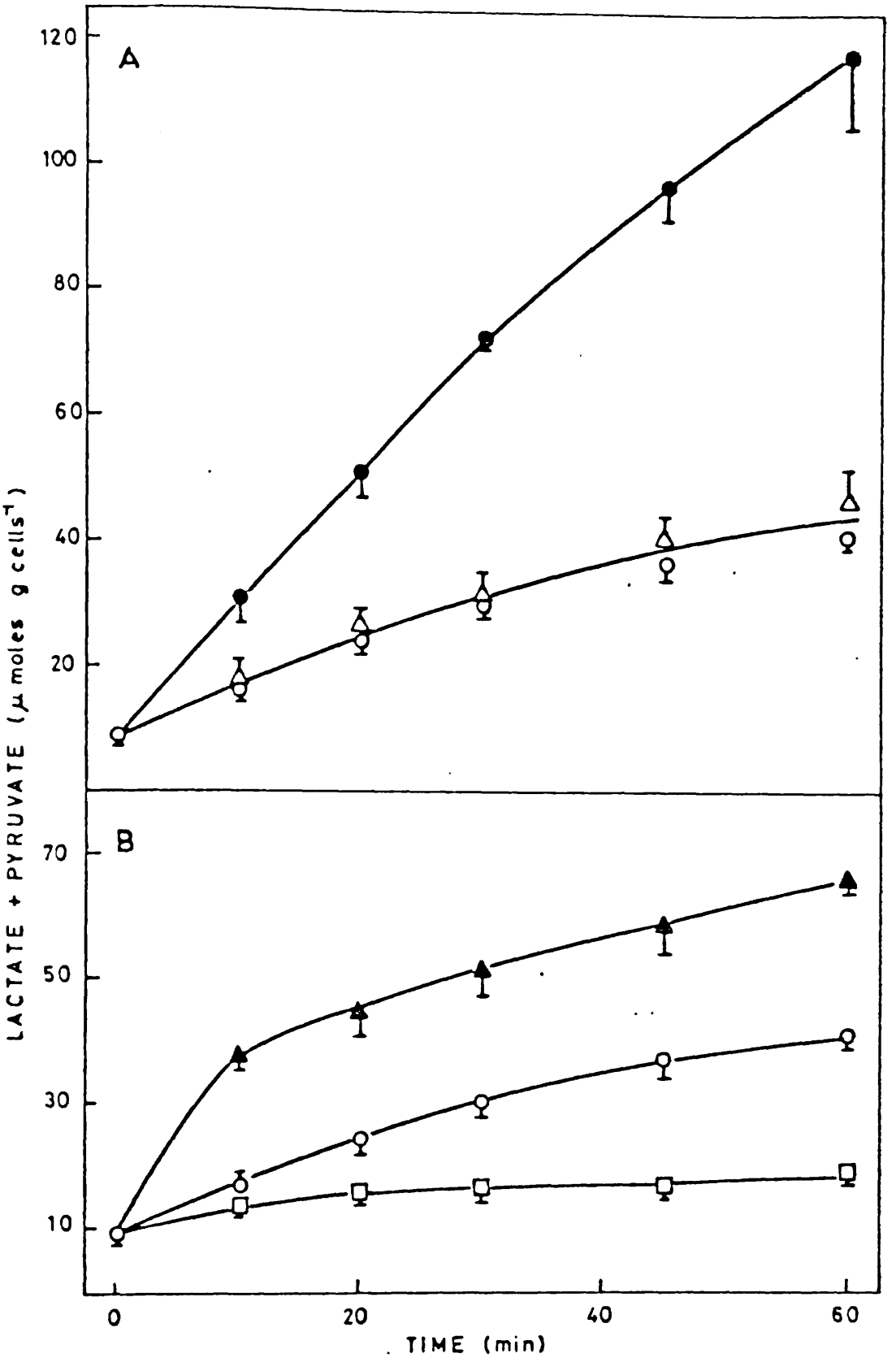


Figure 30. Time course of the total lactate plus pyruvate content in isolated hepatocytes incubated with no added substrate (○), 10 mM glucose (△), 10 mM fructose (●), 10 mM glycerol (□) or 10 mM dihydroxyacetone (▲). A perchloric acid extract was obtained after the incubation period and pyruvate and lactate were determined enzymatically (see methods, section IV.B.6h and IV.B.6i).

of glycogen since the addition of glucose has no effect on the output of the glycolytic products. Therefore, this must mean that glucose at these concentrations (approximately 12 mM)makes no significant contribution to the glycolytic flux or that the limiting step is subsequent to the hexose phosphate pool. One of the important rate limiting steps in glycolysis is thought to be the PFK 1 reaction (Stadtman, 1966; Mansour, 1972; Bloxham & Lardy, 1973; Ramaih, 1974; Hofmann, 1976; Uyeda, 1979), now known to be regulated by $Fru_{26}BP$ (Van Schaftingen et al., 1980b, 1980c; Claus et al., 1980) which in turn is regulated by the size of the hexose phosphate pool and the hormonal state of the animal (Van Schaftingen et al., 1980b; Van Schaftingen & Hers, 1981b). Presumably the $Fru_{26}BP$ levels in the hepatocytes treated with glucose (10 mM) are not altered sufficiently to result in a modification of PFK 1 activity in the cell (Van Schaftingen et al., 1980b, observed elevated levels of the stimulator of PFK 1 after incubating hepatocytes with 20 mM glucose).

The result of incubating isolated hepatocytes with 10 mM fructose, is to increase (by 4-fold compared to the control) the production of pyruvate and lactate over a 60 min incubation period (figure 30A). The simplest explanation for this phenomenon is that fructose metabolism bypasses the main regulatory step in glycolysis (see figure 3) and it can be postulated that the maximum flux through PK is 4-fold greater than through PFK 1. It is possible, however, that flux through PFK 1 is also increased because of the increased glycogenolysis known to occur in response to fructose (Van de Werve & Hers, 1979), and also the lowering of ATP levels (figure 19) by the ketose would be expected to relieve the inhibition of PFK 1 (Brand & Söling, 1974). However, fructose (10 mM) is also known to lower $Fru_{26}BP$ levels (Hue, 1981) which would also compensate for this effect. A similar effect on total glycolytic flux is also observed in hepatocytes from fasted animals (figure 31), with a minimal rate of pyruvate and lactate output in the control and glucose (10 mM) treated hepatocytes whereas the glycolytic rate in the presence of fructose (10 mM) is comparable to that

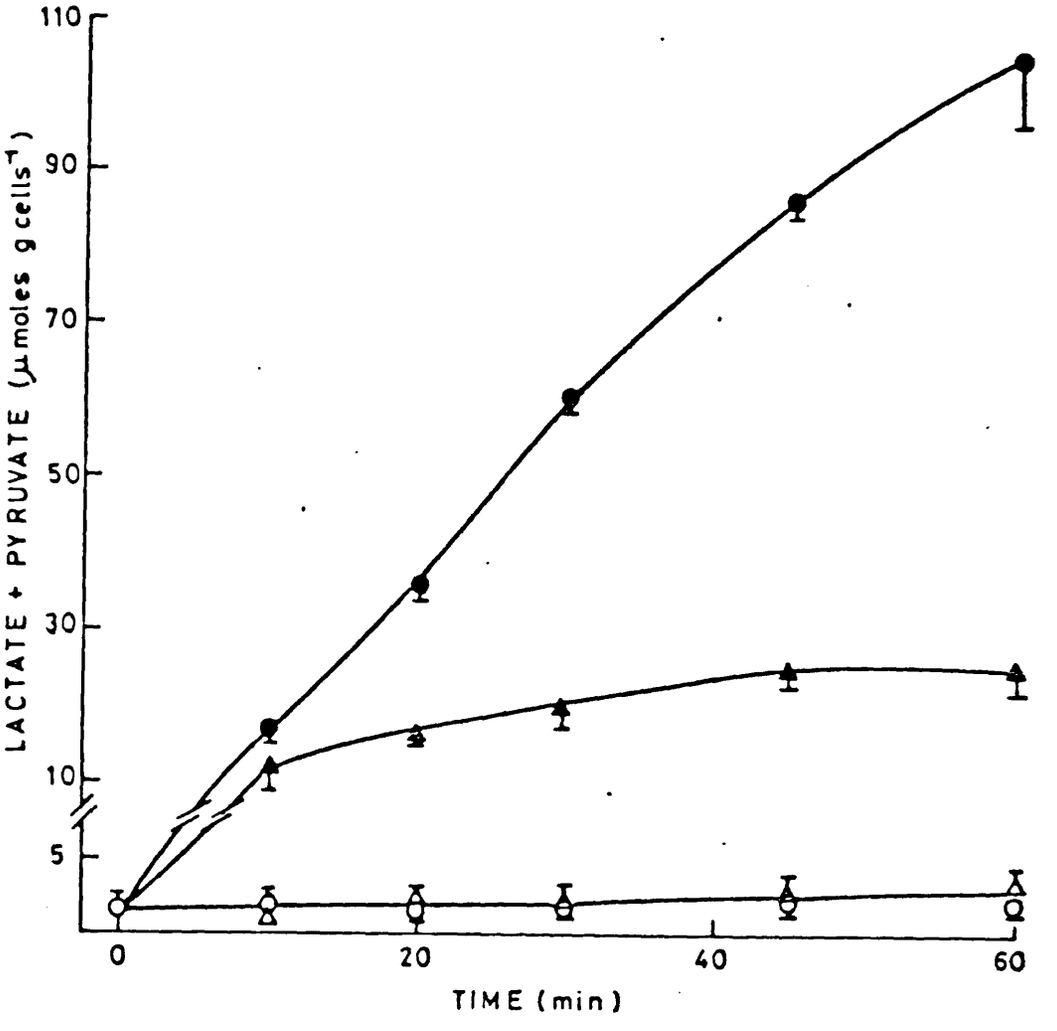


Figure 31. Time course showing the total lactate plus pyruvate content in hepatocytes isolated from 24 h fasted rats. The cells were incubated with no added substrate (○), 10 mM glucose (△), 10 mM dihydroxyacetone (▲) or 10 mM fructose (●).

in the fed animals (compare figure 30A and figure 31). This is further evidence that the pyruvate and lactate output reflects the activity of PK in the hepatocyte. There was a concentration dependent increase in pyruvate and lactate output by hepatocytes from fed animals in response to fructose (figure 32A). However, the curve appears to be biphasic, this correlates well with the PEP levels in the hepatocytes under identical conditions (see Table 6). Thus, at low concentration of fructose, glycolysis to PEP is more rapid than flux through PK as a result PEP accumulates, but with increasing fructose concentrations, the increase in $Fru_{1,6}BP$ (figure 26A) and more notably the lowering of ATP concentrations (figure 19) would result in the deinhibition of PK, and thus a more rapid output of pyruvate and lactate. This is accompanied by diminished PEP concentrations (Table 6).

An initial rapid output of pyruvate and lactate (equivalent to that observed with fructose) is evident after incubation of isolated hepatocytes with dihydroxyacetone (10 mM), but after this time there is a return to the rate of output observed in control hepatocytes (figure 30B). This effect was also observed in hepatocytes from fasted rats (figure 31). The explanation for this phenomenon is not clear since glycolysis to PEP is not inhibited because elevated PEP levels are observed in the presence of 10 mM dihydroxyacetone after 20 min (Table 6). This suggests that PK is rate limiting under these conditions despite the elevated $Fru_{1,6}BP$ levels found (figure 26B). In contrast to fructose, dihydroxyacetone (10 mM) depletes the hepatocyte ATP level by only a small amount which would result in the inhibitor reducing flux through PK. This mechanism would explain the diminished pyruvate plus lactate output after 10 min but does not explain the initial rapid flux of carbon with the triose.

At low concentration of substrate, the pyruvate and lactate output in response to dihydroxyacetone, fructose and D-glyceraldehyde is very similar (figure 32A & B), but at a concentration of fructose of 5 mM and above there is a further increase in the output of pyruvate plus lactate whereas this does not occur with the trioses. This is possibly related to the fact that dihydroxyacetone does not deplete

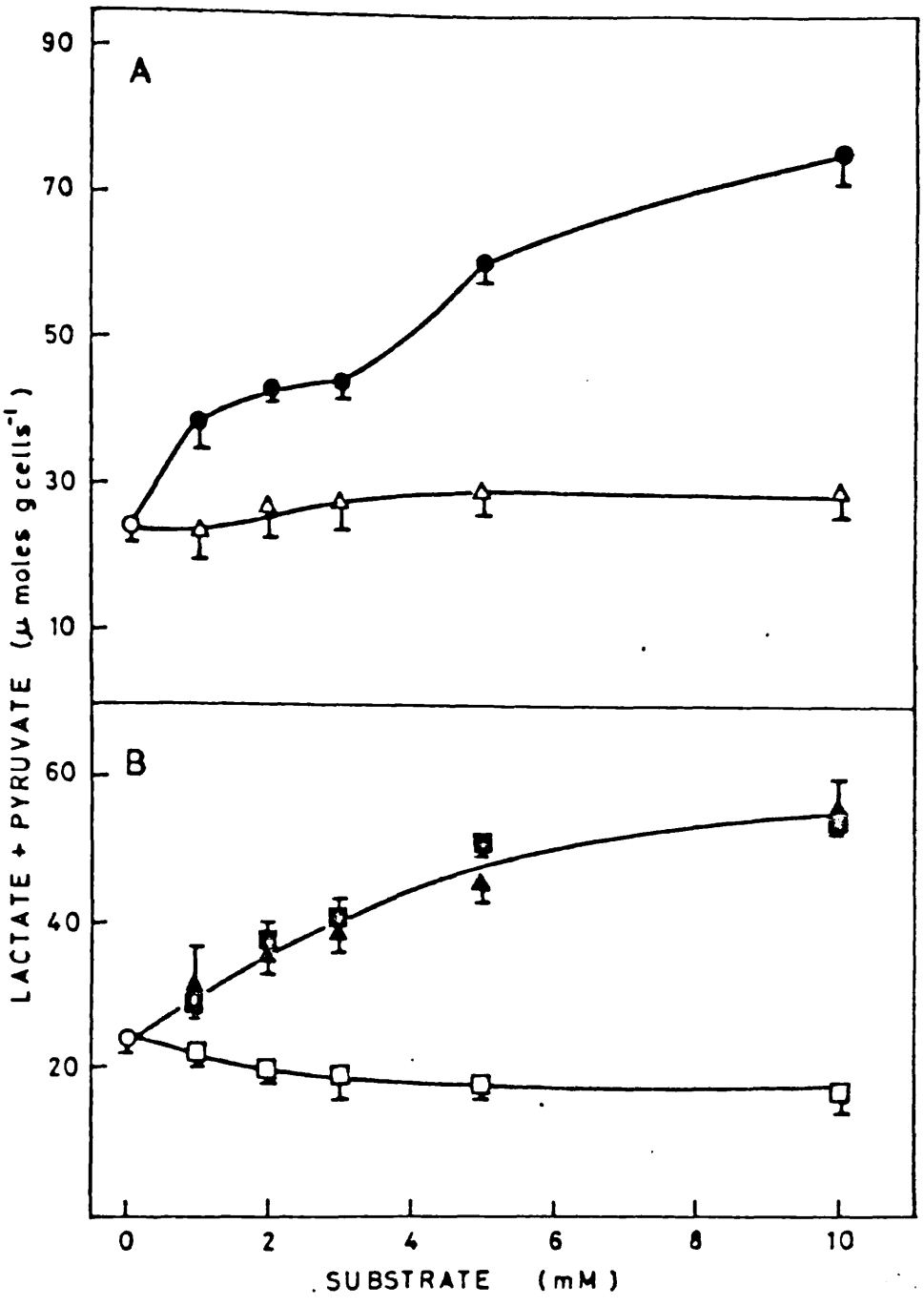


Figure 32. Total lactate plus pyruvate output by isolated hepatocytes incubated with varying concentrations of glucose (△), fructose (●), glycerol (□), D-glyceraldehyde (■) or dihydroxyacetone (▲). The hepatocytes were incubated with substrate for 20 min.

ATP levels. Presumably D-glyceraldehyde does not deplete ATP to the same extent as fructose either, since the K_m and the V_{max} of triokinase would favour phosphorylation of dihydroxyacetone rather than D-glyceraldehyde (Frandsen & Grunnet, 1971). The effect of D-glyceraldehyde on pyruvate and lactate output is virtually identical to that of dihydroxyacetone which is not surprising since the trioses are metabolised via triokinase and the same glycolytic enzymes. In general, none of these substrates altered the $(NADH)/(NAD^+)$ ratio as estimated by the (lactate)/(pyruvate) ratio (Table 8). Therefore, the effects observed cannot be attributed to changes in cytoplasmic redox potential which could affect the rate of glycolysis (Furfine & Velick, 1965).

In contrast to the substrates discussed above, incubation of hepatocytes with glycerol (10 mM) caused no net formation of the glycolytic products compared to the control (figure 30B). Indeed, when compared to the control the pyruvate plus lactate output was significantly inhibited. This suggests that basal glycolysis is inhibited by this substrate, and this is borne out by an examination of the concentration dependence of the effect of glycerol (figure 32B). The mechanism of this inhibition is probably related to the change in $(NADH)/(NAD^+)$ ratio as indicated by (lactate)/(pyruvate) ratios (Table 8). The accumulation of G3P in response to a glycerol load results in severe depletion of NAD^+ which is also required by glycerol-3-phosphate dehydrogenase. Since there is a limited supply of NAD^+ in the cell, there are various mechanisms in operation in order to restore the level of the coenzyme (see introduction I.E). Further confirmation that glyceraldehyde-3-phosphate dehydrogenase is inhibited by a glycerol load comes from the fact that PEP levels are lowered (PEP is in equilibrium with 1, 3 PGA) and the metabolites of the triose phosphate pool are increased.

The reduced substrate xylitol (10 mM) had similar effects to that of glycerol (Table 7). The initial step in the metabolism of xylitol involves its conversion to D-xylulose in an NAD^+ dependent reaction (see figure 6). This substrate increased the $(NADH)/(NAD^+)$

Table 7. The effect of sorbitol and xylitol on total lactate plus pyruvate in isolated hepatocytes.

Time (minutes)	Lactate plus Pyruvate (μmoles g cells^{-1})		
	Substrate added (10 mM)		
	No Substrate	Sorbitol	Xylitol
0	8.9 ± 0.9	-	-
10	16.5 ± 1.2	17.3 ± 0.8^{NS}	12.8 ± 1.0**
20	24.4 ± 1.6	21.0 ± 2.7^{NS}	16.4 ± 0.9***
30	29.9 ± 1.5	26.8 ± 1.4^{NS}	19.1 ± 1.5***
45	36.8 ± 2.9	50.0 ± 0.9***	20.2 ± 2.2***
60	41.1 ± 2.4	72.6 ± 1.6***	27.8 ± 1.7***

Hepatocytes isolated from fed animals were incubated with 10 mM substrate for varying time periods. After the incubation period, the reaction was stopped using perchloric acid and the neutralized extracts used to estimate lactate and pyruvate concentrations as described in methods (Section IV.B.6).

Substrate versus control: NS = not significant;
** = $p<0.01$; *** = $p<0.001$.

ratio (Table 8 & 10) as well as inhibiting pyruvate plus lactate output (Table 7). This observation is consistent with the results of Jakob et al., (1971) who found similar effects of this substrate on metabolite levels in perfused liver. Furthermore, xylitol has been reported to cause the accumulation of oxalate (see figure 6) (Rofe et al., 1977; Barngrover et al., 1981), a potent inhibitor of hepatic PK (Buc et al., 1978, 1981). Another possible explanation for the apparent inhibition of glycolysis by xylitol and glycerol is an increased rate of gluconeogenesis from lactate, but Jakob et al., (1971) found that xylitol is an inhibitor of gluconeogenesis.

The results obtained with sorbitol are an interesting contrast to those observed with xylitol and glycerol (Table 7). Sorbitol is metabolised via the NAD^+-dependent sorbitol dehydrogenase, to fructose. The $(NADH)/(NAD^+)$ ratio, as estimated by (lactate)/(pyruvate) ratio is increased initially to a greater extent by sorbitol (10 mM) than by glycerol and xylitol (Table 8). After 45 and 60 min there is little difference, in (lactate)/(pyruvate) ratio, between the effect of sorbitol and xylitol but the effect of sorbitol is more profound than that of glycerol at all times indicated (Table 8). Thus the pyruvate plus lactate output would be expected to be inhibited to below control values if the $(NADH)/(NAD^+)$ ratio is critical as suggested previously. However, in the case of this substrate there is apparently no stimulation or inhibition of glycolysis up to 30 min, but after 30 min there is a stimulation so that the rate of pyruvate and lactate output approach that observed with fructose.

It is well known that sorbitol is rapidly converted to fructose in hepatocytes (Berry et al., 1973), and the fructose is rapidly phosphorylated by ketohexokinase, resulting in the high levels of Fru-1-P observed using sorbitol as a substrate (figure 25A). Thus it would be expected that ATP would be depleted by this substrate and in addition $(NADH)/(NAD^+)$ would be increased. Therefore, the situation might be expected to be analagous to that with glycerol and an inhibition of glycolysis might be expected. However, in contrast to glycerol, sorbitol raises Fru-1-P and $Fru_{1,6}BP$ levels in the cell and this may

account for the increased glycolytic flux. The depletion of ATP and the increase in Fru-1-P and $Fru_{1,6}BP$ may activate PK sufficiently to convert PEP, present at low concentrations, to pyruvate. This, coupled to the regeneration of NAD^+ by the LDH reaction and by the conversion of DHAP to G3P may be sufficient to relieve the inhibition of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase step. This hypothesis is further reinforced by the observation that as (lactate)/(pyruvate)and (G3P)/(DHAP) ratios (Table 8 & Table 10 respectively) fall there is an increased rate of pyruvate and lactate output from sorbitol (Table 7).

8. An estimation of hepatocyte 'redox state'

Glycolysis to lactate from the triose phosphates involves an NAD^+ utilizing and an NAD^+ producing step (figure 3), thus there is no net depletion of NAD^+. However, upon subjecting the liver to a reduced substrate load this steady state ceases to exist (because these substrates are initially oxidised by NAD^+ coupled enzymes), resulting in a raised (NADH)/(NAD^+) ratio. Thus, a net decrease in NAD^+ may cause inhibition of glycolysis at the glyceraldehyde-3-phosphate dehydrogenase step resulting in decreased pyruvate and lactate production (see previous section). This study was carried out in an attempt to correlate the metabolic effects of various substrates with the changes in the cytoplasmic 'redox state' in the hepatocyte.

The (NADH)/(NAD^+) ratios in the cytoplasm have been measured using various methods, one of the oldest methods being the assay of (G3P)/(DHAP) ratio (Hohorst et al., 1959), which is generally considered to give valid results because of the high activity of glycerol-3-phosphate dehydrogenase. Williamson et al.,(1967a) also reported that the components of the LDH system in the liver are maintained in a constant state apparently close to thermodynamic equilibrium, thus (lactate)/(pyruvate) ratios are also considered to be a true reflection of the 'redox state', the ratio of free (NADH)/(NAD^+) within the hepatocyte (Williamson et al., 1967a; Bücher et al., 1972). The other redox couple that has been used as a measure of (NADH)/(NAD^+)

ratio is sorbitol and fructose because although there is considerably less sorbitol dehydrogenase activity than LDH in the liver (Gibson et al., 1953; Leissing & McGuinness, 1978), fructose can also be reduced to sorbitol at substantial rates (Berry & Werner, 1973), thus making (sorbitol)/(fructose) a valid indicator of (NADH)/(NAD^+) ratios. In this study (G3P)/(DHAP) and (lactate)/(pyruvate) were used as a measure of (NADH)/(NAD^+) ratios since sorbitol and fructose were used as substrates.

A study of the (lactate)/(pyruvate) ratios shows that the ratios are not altered in untreated hepatocytes over a 60 min incubation period (Table 8A). The values obtained are in agreement with those obtained by various workers (Woods & Krebs, 1973; Förster, 1974) which is another indication that the hepatocyte preparations used in this study were viable. Incubating hepatocytes with glucose, fructose, dihydroxyacetone or D-glyceraldehyde (up to 10 mM of each substrate) had no significant effect on the (lactate)/(pyruvate) ratios (Table 8A & B). This lack of effect is also apparent in cells from 24 h fasted animals treated with dihydroxyacetone, glucose or fructose (Table 9). This phenomenon is easily explained by the fact that these substrates are in the oxidised form and therefore their metabolism does not involve a net utilization of NAD^+ (see also figure 3). Incubation of isolated hepatocytes with 10 mM of the reduced substrates glycerol, sorbitol or xylitol caused a rapid elevation of (lactate)/(pyruvate) ratios within 10 min (Table 8A), followed by a gradual decrease over the next 50 min in the case of glycerol and sorbitol. The effect of glycerol on (lactate)/(pyruvate) ratios was concentration dependent, and this effect was apparent at all concentrations of substrate (up to 10 mM) tested (Table 8B). An explanation for the elevation of (lactate)/(pyruvate) ratios caused by reduced substrates can be offered by a study of the metabolism of these substrates (figure 3 & 6). In order to enter the glycolytic-gluconeogenic pathway, the above substrates are oxidised in reactions coupled to NAD^+, thus resulting in depleted NAD^+ in the hepatocytes. In an attempt to restore NAD^+ levels, reduction of pyruvate to lactate occurs. However, since there is limited glycolysis with these substrates (see figure 30 B & Table 7), the cell is unable to completely compensate

Table 8. (Lactate)/(Pyruvate) ratios in isolated hepatocytes incubated with various substrates

A - Time course of the effect of incubating hepatocytes with 10 mM substrate.

Time (minutes)	(Lactate)/(Pyruvate) ratios					
	Substrate added (10 mM)					
	No Substrate	Fructose	Sorbitol	Dihydroxyacetone	Glycerol	Xylitol
0	10.3 ± 1.1	-	-	-	-	-
10	9.7 ± 0.9	10.6 ± 0.1	91.1 ± 11.0	12.2 ± 1.8	46.0 ± 1.5	66.8 ± 7.8
20	9.8 ± 0.6	10.3 ± 1.7	80.0 ± 8.5	12.2 ± 1.5	41.4 ± 1.4	77.7 ± 7.5
30	10.7 ± 1.0	9.8 ± 0.3	88.2 ± 14	9.3 ± 1.1	32.3 ± 2.6	69.0 ± 5.2
45	9.9 ± 0.8	10.1 ± 0.5	63.1 ± 7.3	10.0 ± 1.6	31.9 ± 1.1	85.1 ± 8.1
60	9.4 ± 0.3	10.1 ± 0.3	52.9 ± 4.5	9.5 ± 0.9	26.2 ± 1.1	54.1 ± 9.1

B - Effect of incubating hepatocytes for 20 min with increasing substrate concentration.

Concentration of Substrate added (mM)	(Lactate)/(Pyruvate) ratios				
	Fructose	Glucose[a]	Glycerol	Dihydroxyacetone	D-Glyceraldehyde
0	9.5 ± 0.3	-	-	-	-
1	10.3 ± 1.5	9.9	18.0 ± 1.1	9.3 ± 0.3	9.1 ± 0.9
2	10.7 ± 2.0	9.6	19.7 ± 1.1	10.7 ± 1.3	9.8 ± 1.2
3	10.1 ± 1.1	10.8	21.5 ± 1.0	8.6 ± 0.7	9.9 ± 0.8
5	10.6 ± 0.8	10.7	27.6 ± 5.0	10.2 ± 1.4	10.1 ± 0.8
10	10.8 ± 0.8	9.5	31.7 ± 7.0	11.7 ± 1.9	10.7 ± 0.8

a = results shown are an average obtained from two different cell preparations.

Table 9. (Lactate)/(pyruvate) ratios in isolated hepatocytes from 24 h fasted rats incubated with various substrates.

Time (minutes)	(Lactate)/(pyruvate) ratios			
	Substrate added (10 mM)			
	No Substrate	Fructose	Glucose	Dihydroxyacetone
0	10.7 ± 0.9	-	-	-
10	11.5 ± 2.1	9.0 ± 0.9	10.4 ± 1.1	10.9 ± 1.3
20	11.5 ± 1.3	10.1 ± 0.4	11.4 ± 1.4	10.9 ± 1.1
30	11.2 ± 0.8	9.7 ± 0.5	10.0 ± 0.5	10.3 ± 0.4
45	12.0 ± 1.8	10.4 ± 0.5	10.0 ± 0.9	10.2 ± 0.4
60	10.1 ± 0.6	11.2 ± 0.7	9.3 ± 1.1	10.4 ± 0.7

for the depleted NAD^+ and hence the (lactate)/(pyruvate) ratio is increased. The reduction of (lactate)/(pyruvate) ratios observed with longer incubation time is presumably due to slow restoration of NAD^+ concentrations in the hepatocytes by the shuttle mechanism described in section I.E.

A time course study of (G3P)/(DHAP) ratios shows that in untreated hepatocytes there was no significant change over a 60 min incubation period (Table 10). Incubating isolated hepatocytes with up to 10 mM dihydroxyacetone or glucose caused no significant changes in (G3P)/(DHAP) ratios (Table 10 A & B). This lack of effect of the substrates was similar to that observed when (lactate)/(pyruvate) ratios were examined. However, high concentrations of fructose (Table 10 A & B) caused a significant elevation of (G3P)/(DHAP) ratios which was not mirrored by parallel changes in (lactate)/(pyruvate) ratios. A further study of (G3P)/(DHAP) ratios reveals that low D-glyceraldehyde (up to 3 mM) concentrations also have no effect whereas 5 and 10 mM substrate caused substantial increases in the (G3P)/(DHAP) ratios (Table 10B). Once again this effect is not reflected by a similar effect on (lactate)/(pyruvate) ratios.

Berry (1980) also found discrepancies between (G3P)/(DHAP) and (lactate)/(pyruvate) ratios in isolated hepatocytes. He concluded from his observations that (G3P)/(DHAP) are not good indicators of the cytoplasmic (NADH)/(NAD^+) ratios, possibly because the two enzymes involved, i.e. LDH and glycerol-3-phosphate dehydrogenase do not equilibriate with the same cytoplasmic pool of NAD^+ and NADH. The effects on (G3P)/(DHAP) of fructose and D-glyceraldehyde may be related since the latter is a product of the metabolism of the former. Nevertheless, it must be noted that in the case of the triose the level of G3P is increased (figure 29B) and the DHAP levels are decreased (Table 4). A possible explanation of the effect of D-glyceraldehyde is that at low concentrations, the triose is metabolised via glyceraldehyde-3-phosphate and DHAP whereas at high concentrations, the metabolism via glycerol, G3P and DHAP may become significant. The involvement of the latter pathway may result in the elevation of G3P at the same time

Table 10. (G3P)/(DHAP) ratios in isolated hepatocytes incubated with various substrates.

A - Time course of the effect of incubating hepatocytes with 10 mM substrate.

Time (minutes)	(Glycerol-3-phosphate)/(Dihydroxyacetone phosphate) Ratios					
	Substrate added (10 mM)					
	No Substrate	Fructose	Sorbitol	Dihydroxyacetone	Glycerol	Xylitol
0	6.3 ± 2.0	-	-	-	-	-
10	5.0 ± 1.0	7.1 ± 2.3^{NS}	93.6 ± 6.2	6.9 ± 6.2^{NS}	133.7 ± 23.0	83.9 ± 12.9
20	5.6 ± 0.7	8.3 ± 3.0^{NS}	97.8 ± 11.5	6.0 ± 0.9^{NS}	125.2 ± 8.7	77.9 ± 9.0
30	4.3 ± 1.0	7.1 ± 1.8^{NS}	86.9 ± 12.0	5.2 ± 1.1^{NS}	120.1 ± 5.0	63.6 ± 15.0
45	4.1 ± 0.5	7.6 ± 1.7*	57.9 ± 6.8	5.1 ± 1.7^{NS}	121.8 ± 4.5	78.4 ± 8.8
60	4.9 ± 1.3	8.5 ± 2.4^{NS}	57.8 ± 7.5	5.3 ± 1.4^{NS}	118.3 ± 11.5	60.1 ± 9.1

B - Effect of incubating hepatocytes for 20 min with increasing substrate concentration.

Concentration of Substrate added (mM)	(Glycerol-3-phosphate)/(Dihydroxyacetone phosphate) Ratios				
	Fructose	Glucose[a]	Glycerol[a]	Dihydroxyacetone	D-Glyceraldehyde
0	4.6 ± 0.6	-	-	-	-
1	6.6 ± 1.2^{NS}	4.3	75.0	6.0 ± 1.0^{NS}	4.7 ± 1.6
2	8.0 ± 1.5*	3.7	73.1	7.4 ± 1.8^{NS}	4.8 ± 1.0
3	7.9 ± 0.8**	4.2	79.2	6.1 ± 1.4^{NS}	4.0 ± 0.9
5	10.4 ± 2.8*	4.2	90.1	5.3 ± 1.8^{NS}	15.8 ± 3.5 **
10	10.0 ± 2.5*	4.3	118.4	5.1 ± 0.9^{NS}	27.3 ± 4.8 **

a = results presented are an average of two different cell preparations.

substrate versus control: NS - Not Significant; * = $p<0.05$; ** $p<0.01$.

as DHAP. becomes depleted as the result of Fru-1-P synthesis by the reverse aldolase reaction. This may result in a lack of equilibrium state of glycerol-3-phosphate dehydrogenase despite the availability of NAD^+. This is supported by the evidence that the K_m of triokinase for D-glyceraldehyde is much lower (0.019 mM) than the K_m of NAD^+ linked glycerol dehydrogenase (11 mM) for the same substrate (Leuthardt and Wolf, 1954; Sillero et al., 1969; Frandsen & Grunnet, 1971; Veneziale, 1972b). Alternatively it is possible that Fru-1-P accumulating with both D-glyceraldehyde and fructose is affecting the level of G3P via an effect of one of the enzymes involved in its metabolism. The reduced substrates, glycerol, sorbitol and xylitol tested raised (G3P)/(DHAP) ratios at all concentrations (Table 10). The explanation for this phenomenon is discussed above.

9. Glucose

The evidence available from the studies in previous chapters indicates that fructose activates PK in washed cells, in addition increasing the net glycolytic flux. However, a closer examination of the glycolytic flux (figure 30) from various substrates suggest that fructolysis is occurring at a linear rate over the 60 min incubation period, whereas after dihydroxyacetone administration there is a burst of glycolysis followed by a slower rate of pyruvate plus lactate production comparable to the control rate. It was thus of interest to study glucose output in the presence of these two substrates.

An examination of the time course for glucose production in isolated hepatocytes from fed animals (figure 33) shows that there is a slow rate of glucose production in untreated cells (0.6 μmoles min^{-1} g $cells^{-1}$). Incubating with dihydroxyacetone (10 mM) and fructose (10 mM) caused a significant increase in glucose production during the first 30 min incubation, but there was no significant difference in the rate of gluconeogenesis from either substrate (figure 33). Further incubation (for up to 60 min) resulted in a decrease in the rate of glucose output with both substrates. The decrease in the

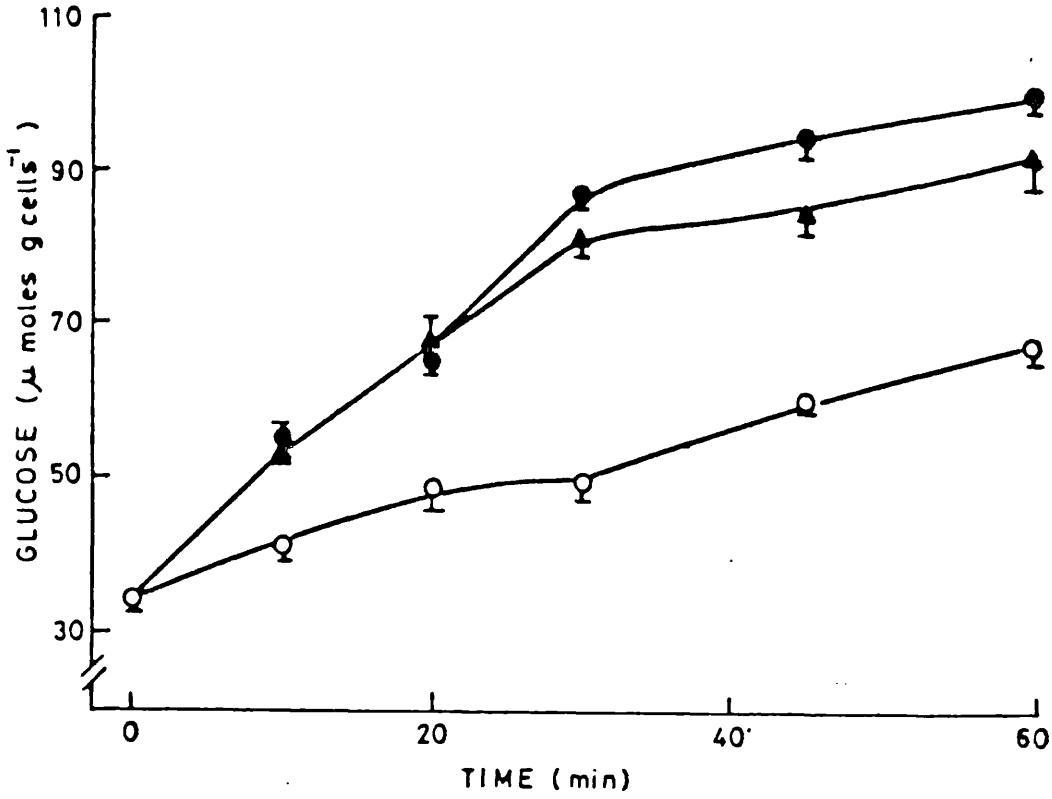


Figure 33. Time course showing changes in the glucose level of isolated hepatocytes incubated with no added substrate (○), 10 mM dihydroxyacetone (▲) or 10 mM fructose (●). Hepatocytes were extracted with perchloric acid and the glucose determed by an enzymatic method (see methods, section IV.B.6g).

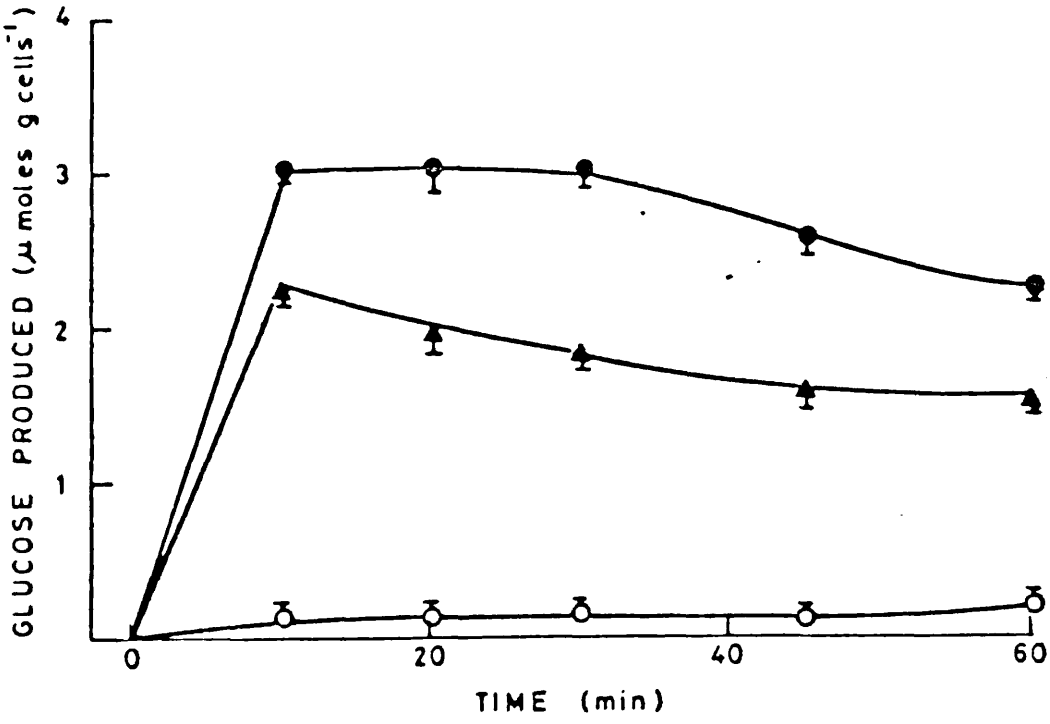


Figure 34. Time course showing the glucose produced when hepatocytes isolated from 24 h fasted rats were incubated with 10 mM fructose or dihydroxyacetone (symbols as in figure 33).

rate of glucose output observed with both substrates may be related to the depletion of added substrate especially in the case of dihydroxyacetone. The total glucose produced from dihydroxyacetone during the first 30 min incubation period is approximately 3.5 mM, and since 2 moles of dihydroxyacetone are used to form 1 mole of glucose, this amount of glucose produced is equivalent to 7 mM substrate. Considering the dihydroxyacetone is also metabolised to pyruvate plus lactate (figure 30B) it is not surprising that the rate of glucose production from this substrate slows down with time. It must be noted that fructose effects cannot be easily explained because this substrate also stimulates glycogenolysis by activating phosphorylase, secondary to the depletion of ATP (Van de Werve & Hers, 1979). Thus the effect of fructose observed above may be due to increased glycogenolysis, increased gluconeogenesis or both, whereas the effects observed with dihydroxyacetone are probably due to increased gluconeogenesis. Thus the burst of glycolysis, followed by a slower rate observed with dihydroxyacetone (figure 30B), may also be due to limited substrate since a considerable amount is converted to glucose. With fructose no such effect is apparent (figure 30A) presumably because glycogenolysis is also stimulated.

There is no significant production of glucose in untreated hepatocytes from 24 h fasted rats (figure 34). The basal glucose concentration is also considerably less than that observed in fed animals presumably due to lack of glycogen. Incubating with 10 mM dihydroxyacetone and fructose (figure 34) reveals that both these substrates cause increased glucose production, the latter substrate producing more glucose over the whole incubation period. After the initial burst of glucose production, there is a gradual decrease of glucose concentrations with time. A possible explanation for this effect is that the glucose produced is used to restore glycogen depleted during fasting or alternatively it undergoes glycolysis.

In agreement with the reports by Foster & Blair (1978) and Claus et al., (1979); who used hepatocytes from fasted rats, the major products of dihydroxyacetone metabolism in hepatocytes from fed animals were glucose, lactate and pyruvate. The former authors reported that dihydroxyacetone (10 mM) doubled the output of lactate by cells from fed animals while increasing glucose output by 33%. The former finding is in line with the results in the present study but a much greater stimulation of glucose output was observed.

C. INVESTIGATION INTO POSSIBLE INHIBITORS OF PYRUVATE KINASE

Incubation of isolated hepatocytes with high fructose (10 mM) concentrations causes inhibition of PK in unwashed cells. Experiments from the previous section (III.A) provide strong evidence that the inhibition caused by fructose is reversible by either salting out the enzyme with $(NH_4)_2SO_4$ or removing the hepatocyte medium. Thus, this rules out inhibition by a change in the phosphorylation state of the enzyme. A wide variety of substances have been reported (see section II.C) to inhibit PK, and such inhibition is of physiological importance (section II.F) since this enzyme occupies an important strategic position in glycolysis. This section discusses the inhibition of PK in isolated hepatocytes incubated with fructose and investigates the possible role played by some of the inhibitors discussed in the introduction (see section II.C). The role of other as yet undiscovered inhibitors and their physiological importance is also discussed.

1. Effect of pH on pyruvate kinase activity

Evidence that PK is strongly affected by pH has been previously discussed (see section II.C.3). The intracellular pH of perfused liver has been quoted as 7.24 while the perfusate pH was 7.4 (Cohen et al.,1971; Lloyd et al., 1973; Cohen & Iles, 1975). A further study of intracellular pH by Iles et al., (1980) showed that the fall in intracellular pH after addition of fructose is rapid and

follows a similar time course to the fall in ATP. Perfusion with 10 mM substrate caused a decrease in pH (0.1 - 0.2 units) within 6 min. Therefore, it was of interest to study the pH in the final assay conditions.

An examination of the pH of the reaction mixture, either before or after the assay, reveals that the pH is not altered significantly under these conditions. This finding is not surprising since the buffers used are both at pH 7.4 and the cell preparation is diluted 120 times during the preparation of the reaction mixture. These findings rule out the possibility that a change in pH is causing the inhibition observed.

2. Effects of adenine nucleotide catabolism

Intravenous injection of fructose in normal humans significantly increases uric acid concentration in the plasma, with concomitant increases in urinary excretion of urate (Perheentupa & Raivo, 1967). Although there is evidence of de novo purine synthesis, (Emmerson, 1974; Raivo et al., 1975), the major sources of the extra uric acid seem to be preformed purine nucleotides, which, following fructose administration are known to undergo extensive breakdown in the liver (Mäenpää et al., 1968; Burch et al., 1969, 1970). Increased formation of the end product of purine catabolism, allantoin (Raivo et al., 1968), and the depletion of adenine nucleotides (Woods et al., 1970) have also been reported to occur in the perfused rat liver system. Smith et al., (1977b) reported that incubating hepatocytes with 28 mM fructose causes an accumulation of allantoin which is not observed in the presence of glucose. Thus a study was carried out to observe the effects of the products of adenine nucleotide catabolism on PK activity.

i) Effect of AMP metabolic products on pyruvate kinase activity

High fructose concentrations cause inhibition of PK in unwashed cells and this effect is not apparent after incubating with low fructose concentrations (section III.A). The inhibition caused by

high fructose concentrations is probably due to the accumulation of an inhibitor. As a consequence of depletion of ATP by fructose the rate of AMP catabolism is known to increase. Thus a number of AMP catabolic products were tested for their effects on the activity of a partially purified PK preparation.

The results obtained (Table 11) indicate that the only adenine nucleotide catabolic products that affect PK activity significantly were uric acid and allantoin. Of the metabolites which had no effect on PK activity, the use of higher concentrations (up to 4 mM) had no significant effect on PK activity (results not shown). A closer examination of the uric acid and allantoin effects was carried out as shown in Table 11B. The results obtained provide further evidence that the end products of AMP catabolism inhibit PK whereas the intermediary metabolites do not.

ii) Effect of allopurinol on fructose induced inhibition of pyruvate kinase

Xanthine oxidase the enzyme which produces uric acid in the catabolism of AMP, is inhibited by allopurinol (Massey et al., 1970a; Spector & Johns, 1970; Fain & Shepherd, 1977; Ní fhaoláin & Coughlan, 1978). Allopurinol is an analogue of hypoxanthine which is converted to oxipurinol (4, 6-dihydroxypyraxolo (3,4-d) pyrimidine) by xanthine oxidase (Elion et al., 1966; Massey et al., 1970a, 1970b).

The results presented in table 11 seem to indicate that uric acid and allantoin may be causing the inhibition of PK activity observed in unwashed hepatocytes after incubating with high fructose concentrations. It was, therefore, of interest to examine the combined effects of fructose and allopurinol on PK activity in unwashed cells. The results shown in table 12 give the indication that allopurinol (20 μM) does in fact reverse the inhibition observed with high fructose concentrations to some extent, but the stimulation observed in washed cells (figure 12B) was not apparent. This reversal of the effect of fructose by allopurinol appeared to strengthen the hypothesis that the end products of AMP catabolism inhibit PK activity.

Table 11. The effect of AMP breakdown products on partially purified pyruvate kinase from isolated hepatocytes. The v/V_{max} for the control is assumed to represent 100% activity (n = 2)

A - Effect of various metabolites (* n =7)

Metabolite	Concentration mM	Enzyme Activity %	Inhibition %
Control*	-	100	-
Adenosine	1.0	97	-
AMP	1.0	98	-
Allantoin*	1.0	69 ± 2.4	31
Hypoxanthine	0.5	97	-
IMP	1.0	103	-
Urea	1.0	99	-
Uric Acid*	0.5	78 ± 4.0	22
Xanthine	0.5	98	-

B - Effect of different concentrations of uric acid and allantoin.

Metabolite			
Uric Acid		Allantoin	
Concentration mM	Enzyme Activity %	Concentration mM	Enzyme Activity %
0	100	0	100
0.1	92	0.5	97
0.2	90	1.0	73
0.3	88	2.0	70
0.4	85	3.0	70
0.5	75	4.0	65

Table 12. Pyruvate kinase activity in isolated hepatocytes incubated for 20 minutes with increasing fructose concentrations in the presence and absence of 20 μM allopurinol. The enzyme was assayed at 0.2 mM PEP. (n=2).

Fructose mM	Pyruvate Kinase Activity	
	μmoles min^{-1} g $cells^{-1}$	
		+ 20μM Allopurinol
0	24.7 ± 0.8	25.0 ± 0.6
1.0	29.8 ± 1.0	29.8 ± 2.1
2.5	27.1 ± 0.9	27.9 ± 1.2
5.0	20.2 ± 1.2	25.5 ± 1.1
10.0	16.7 ± 0.5	24.8 ± 1.6
20.0	15.6 ± 1.3	25.2 ± 0.5
50.0	15.73± 2.6	25.5 ± 0.8

(V_{max} (62.5 ± 0.5) was unaffected under these conditions.

There are a number of other possible explanations for the lack of enzyme activation in the unwashed hepatocytes incubated with allopurinol and high fructose concentrations. For example, allopurinol may prevent the accumulation of the activator, but a study of the effect of low concentrations of fructose (figure 35) on the activation of PK shows that allopurinol had no effect. Another possibility is that allopurinol is affecting PK activity, but it was found that concentrations of up to 10 μM allopurinol had no effect on the enzyme (results not shown). A third possibility is that the concentration of allopurinol used is not sufficient to prevent accumulation of uric acid and allantoin. A study of allopurinol effects reveals that higher concentrations (up to 500 μM) of the xanthine oxidase inhibitor did not reverse the inhibition caused by fructose to a greater extent than that observed with 10 μM allopurinol (results not shown). This is in agreement with the data of Fain & Shepherd,(1977), who reported a 98% inhibition of xanthine oxidase with 10 μM allopurinol. A fourth possibility is that allopurinol prevents the accumulation of uric acid and allantoin, but not the accumulation of another inhibitor which may also have some effects on the stimulation of PK by endogenous $Fru_{16}BP$.

A comparison of the effects on PK of glycerol (10 mM), fructose (5 mM) and hypoxanthine (1.75 mM) in the presence and absence of allopurinol is shown in Table 13. The inhibitory effect of fructose was partially reversed by allopurinol at short time intervals, but the activity observed after 60 min incubation was identical. Glycerol mimicked the effect of fructose but with this substrate the reversal by allopurinol was only observed after 10 min incubation. The similarities between glycerol and fructose effects presumably reflects the depletion of ATP by these substrates, but the fact that the allopurinol effect was only observed at shorter time intervals, suggests that there are two factors involved, one being the accumulation of allantoin and uric acid which is blocked by allopurinol, and another factor which is unaffected by the drug. However, incubating isolated hepatocytes with hypoxanthine (1.75 mM) did not

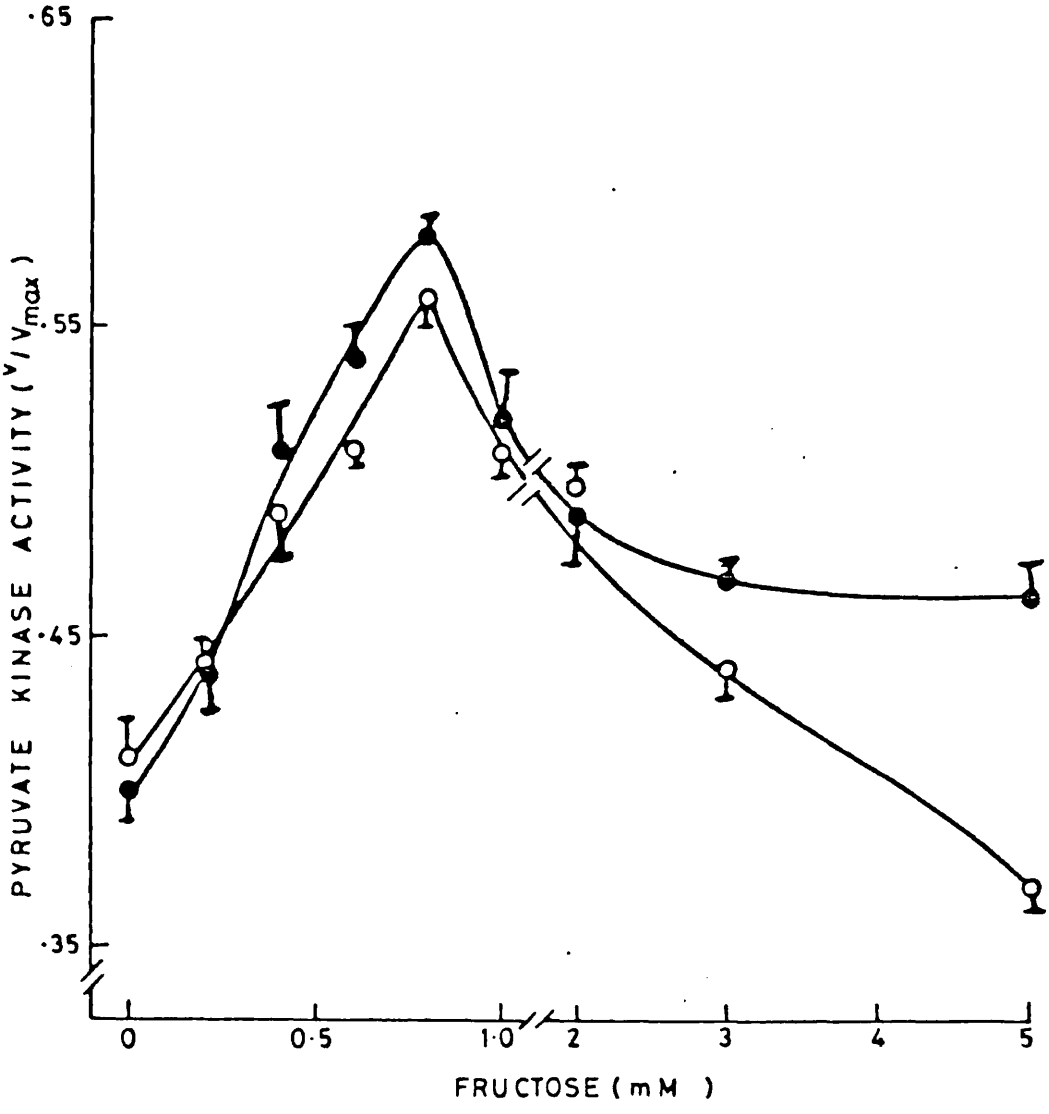


Figure 35. The effect of 20 μM allopurinol (●) on pyruvate kinase activity extracted from unwashed hepatocytes following incubation with fructose for 15 min. Controls, without allopurinol (○).

Table 13. The effect of glycerol, fructose and hypoxanthine on PK activity in isolated hepatocytes incubated with or without 50 μM allopurinol.

Time (minutes)	Pyruvate Kinase Activity (v/V_{max})							
	Substrate added							
	No Substrate		10 mM Glycerol		5 mM Fructose		1.75 mM[a] Hypoxanthine	
		+ 50 μM Allopurinol		+ 50 μM Allopurinol		+ 50 μM Allopurinol		+ 50 μM Allopurinol
0	0.42	0.44	-	-	-	-	-	-
10	0.43	0.46	0.32	0.40	0.34	0.39	0.45	0.44
30	0.44	0.42	0.29	0.31	0.33	0.40	0.41	0.39
60	0.40	0.40	0.30	0.33	0.33	0.33	0.41	0.41

a = 1.75 mM substrate was used because above these concentrations hypoxanthine is not soluble

Isolated hepatocytes were incubated with glycerol, fructose or hypoxanthine for varying time periods in the presence and absence of 50 μM allopurinol and the reaction stopped by freezing in liquid N_2 (see methods, section IV.B.3).

significantly affect PK activity (Table 13). This lack of effect was also apparent when the cells were incubated with 1 mM xanthine (results not shown), despite the fact that this is an immediate precursor of uric acid and allantoin (figure 4). The conclusions drawn from this experiment were that either hypoxanthine and xanthine do not raise the uric acid and allantoin levels in isolated hepatocytes or that the allantoin and uric acid accumulating in response to glycerol and fructose are not sufficient to explain the inhibition of PK by these substrates.

iii) Uric acid and allantoin concentrations in isolated hepatocytes

This was tested by examining the allantoin and uric acid levels in isolated hepatocytes incubated with 10 mM fructose, which depletes ATP severely,or 10 mM dihydroxyacetone, which depletes ATP to a much lesser extent (figure 19 & 21). Uric acid levels in isolated hepatocytes were assayed by both the colorimetric and the U.V. method, but no urate could be detected under any conditions. This is in agreement with the results obtained by Van den Berghe et al., (1980) and is presumably the result of a high uricase activity present in the rat liver.

Allantoin levels in the extrahepatocyte medium were also measured in the presence and absence of added fructose and dihydroxyacetone (figure 36). Relatively high levels of allantoin (0.16 mM) were found with the preincubated cells, presumably arising from the catabolism of adenine nucleotides during the isolation procedure and in the preincubation period. Incubation with 10 mM fructose, resulted in a rapid output of allantoin within the first 10 min (corresponding to the rapid degradation of ATP) followed by a more gradual increase over the next 60 min (figure 19). A lesser but substantial increase in allantoin levels was also observed in hepatocytes incubated with dihydroxyacetone (10 mM). This output was comparable to that observed in the control cells (figure 36). Similar results were also obtained when the total allantoin, in both the hepatocyte and

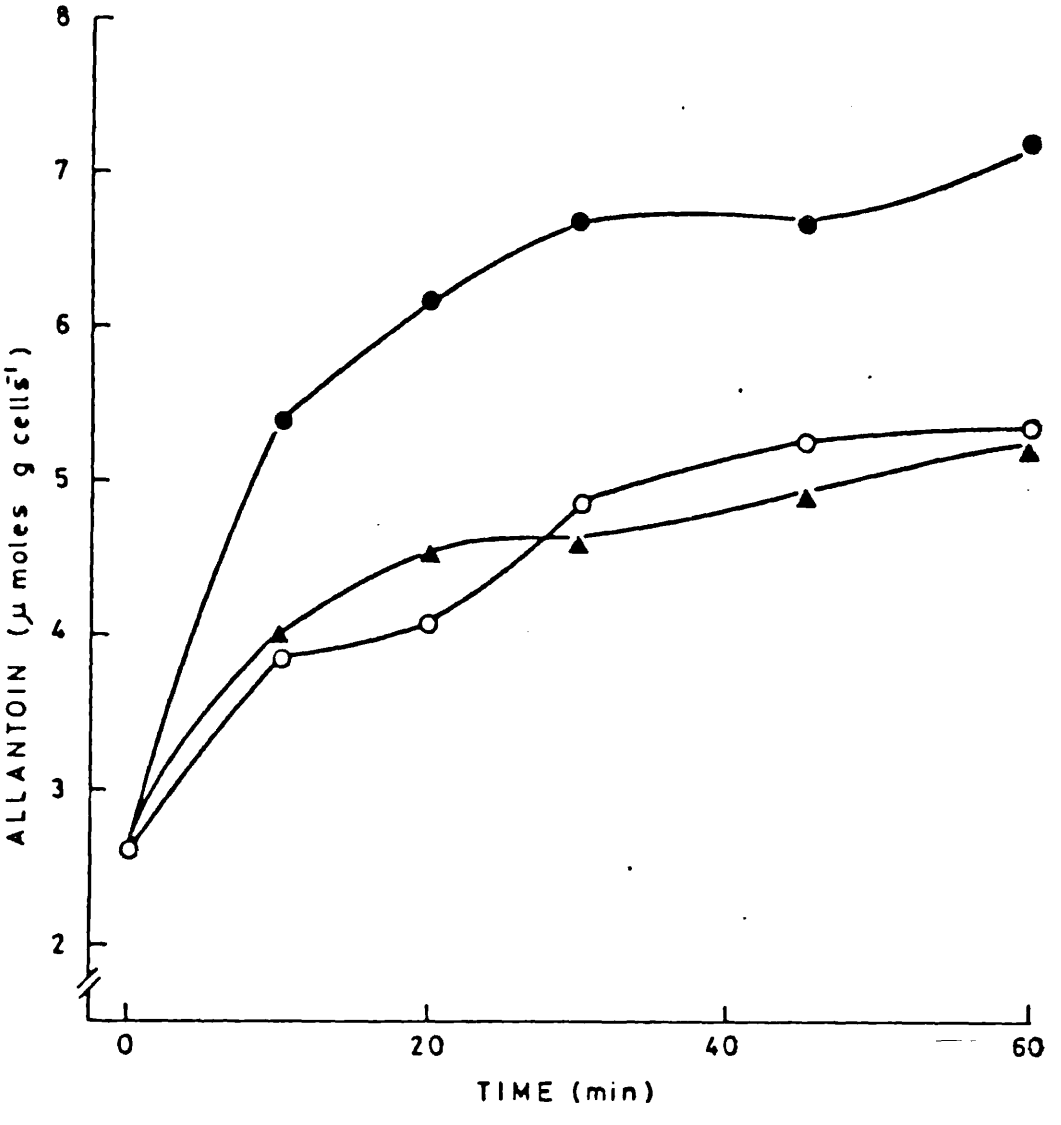


Figure 36. Allantoin output by isolated hepatocytes incubated with 10 mM fructose (●), 10 mM dihydroxyacetone (▲) or no added substrate (○). Allantoin was determined colorimetrically in neutralized perchloric acid extracts of the incubation mixture (see methods, section IV.B.6c). (n=2).

the medium was assayed, suggesting that the plasma membrane is freely permeable to allantoin.

Thus, it appears that treatment with fructose does cause an increase in allantoin output by the isolated hepatocytes and that the hepatocyte membrane is permeable to this end product. Therefore, it could be a candidate for the inhibition of PK observed with fructose in unwashed cells. However, a consideration of the concentration of allantoin attained in these experiments (figure 36) and the concentration dependence of the inhibitory effect of allantoin on PK (Table 11B) render it unlikely that the inhibition is due directly to allantoin accumulation. However, when the procedure used is examined, another possible explanation can be considered. PK activity as assayed by the spectrophotometric method is raised by fructose and dihydroxyacetone in washed cells despite the large dilution of the intracellular factors involved. This suggests that $FRu_{16}BP$ is tightly bound to the PK during the extraction and assay procedure. Allantoin may cause some dissociation of the $Fru_{16}BP$ from the PK at the stage where the hepatocyte preparation is frozen and thawed prior to its dilution and assay.

3. Alanine

The free amino acids are key gluconeogenic precursors. Alanine and glutamine have been shown to be the predominant amino acids released from rat skeletal muscle (Ishikawa et al., (1972; Ruderman & Lund, 1972; Snell, 1976; Snell & Duff, 1977), where they are synthesized de novo (Ruderman & Berger, 1974; Garber et al., 1976). The uptake of alanine by the liver exceeds that of other amino acids (Ishikawa, 1976) and the carbon of alanine is rapidly incorporated into blood glucose making this amino acid an effective gluconeogenic carrier (Mallette et al., 1969a; Snell, 1979). Changes in the steady state concentrations of intermediates in the gluconeogenic pathway observed using perfused liver, suggest that glucagon acts to enhance gluconeogenesis from alanine by increasing the transport of this amino

acid into the hepatic cell and by stimulating the conversion of pyruvate to PEP(Mallette et al., 1969a; 1969b). Other factors that affect hepatic alanine levels are; insulin, which inhibits gluconeogenesis from this substrate by reducing its uptake by the liver (Felig, 1973) and steroid hormones, which have been shown to increase hepatic alanine concentrations (Betheil et al., 1965).

Hepatic alanine levels are important because this amino acid plays a major role in maintaining blood glucose homeostasis; inhibition of PK being one of its important regulatory functions (see section II.C.2). A low alanine concentration (0.1 mM) is sufficient to produce a 50% inhibition of PK, when the enzyme is assayed at 0.3 mM PEP and 1 mM Mg ADP in the absence of other effectors (Seubert & Schoner, 1971). This concentration of alanine is far below the values (0.5 - 1.5 mM) calculated for physiological conditions (Williamson et al.,1967b ; Brosnan et al., 1970; Schoner et al., 1970). It was of interest, therefore, to study the effect of various substrates on the levels of alanine in isolated hepatocytes in order to examine the possibility that the inhibition of PK observed with fructose (section III.A) was the result of alanine accumulation.

i) Effect of various substrates on alanine concentration in isolated hepatocytes

The de novo synthesis of alanine in muscle involves the transfer of amino groups to the carbon skeleton of pyruvate (Snell, 1979). Thus, the availability of pyruvate may determine the rate of alanine formation in the liver. Decreasing the availability of pyruvate by dichloroacetate (which promotes pyruvate oxidation by activating pyruvate dehydrogenase)diminishes alanine release in vivo (Blackshear et al., 1975), and in vitro (Snell, 1976; Snell & Duff , 1977). Conversely, increasing the availability of pyruvate by perfusing rat hind limb preparations with glucose (Pozefsky & Tancredi, 1972) or lactate (Ruderman & Berger, 1974) or by incubating isolated muscle preparations with glucose (Odessey et al., 1974; Snell, 1976; Snell & Duff, 1977) or pyruvate (Garber et al., 1976) increases alanine release. These observations were all made using muscle

preparations. The present study was done to find out if fructose and other pyruvate-producing substrates would have the same effect in isolated hepatocytes.

An examination of alanine concentrations in isolated hepatocytes, reveals that there was a basal rate (0.02 μmoles min^{-1} g $cells^{-1}$) of alanine formation in the hepatocytes with no added substrate (figure 37), presumably as a result of endogenous glycolysis. Cell preparations incubated with glucose (10 mM) showed a small increase in alanine levels when compared to the control. However, incubating with fructose (10 mM) caused a 3-fold increase in alanine levels during the first 10 min, followed by a gradual increase (figure 37A).

The hepatic synthesis of alanine from pyruvate involves a transamination from glutamate catalysed by alanine aminotransferase. The glutamate required for transamination reaction presumably arises from several sources. Firstly, from a pool of free glutamate in the liver; secondly, by transamination reactions involving other amino acids and α-ketoglutarate and thirdly, as a result of the direct amination of α-ketoglutarate in a reaction catalysed by glutamate dehydrogenase. The latter enzyme can utilize either NAD^+ or $NADP^+$ and is inhibited by GTP (Dieter et al., 1981). Since GTP levels are reported to be low in isolated hepatocytes and GTP is depleted by a fructose load (Van den Berghe et al., 1980) this should result in a higher activity of the enzyme in fructose treated cells.

The ammonium ions required for the direct amination by glutamate dehydrogenase can arise from various sources. For example, adenine nucleotide catabolism involves a deamination reaction which would result in release of free NH_4^+. Thus the increased rate of pyruvate accumulation in the case of fructose (figure 38A) is accompanied by the release of NH_4^+ as a result of the AMP deaminase reaction. A second possibility postulated by Sainsbury (1980), is that, normally NH_4^+ in

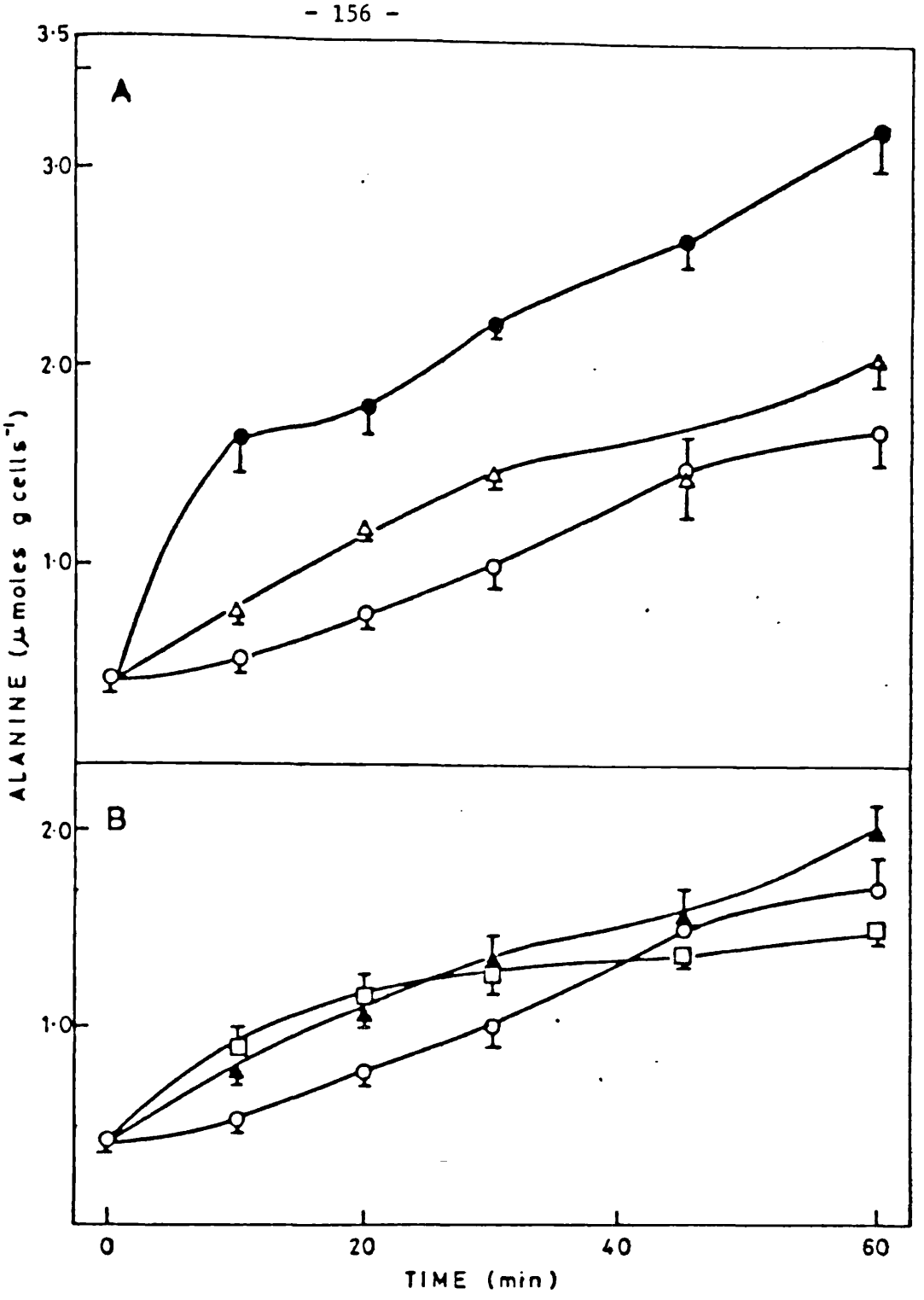


Figure 37. Alanine output by isolated hepatocytes incubated with no added substrate (○), 10 mM glucose (△), 10 mM fructose (●), 10 mM glycerol (□) or 10 mM dihydroxyacetone (▲). Hepatocytes were extracted with perchloric acid and alanine determined colorimetrically (see methods, section IV.B.6b).

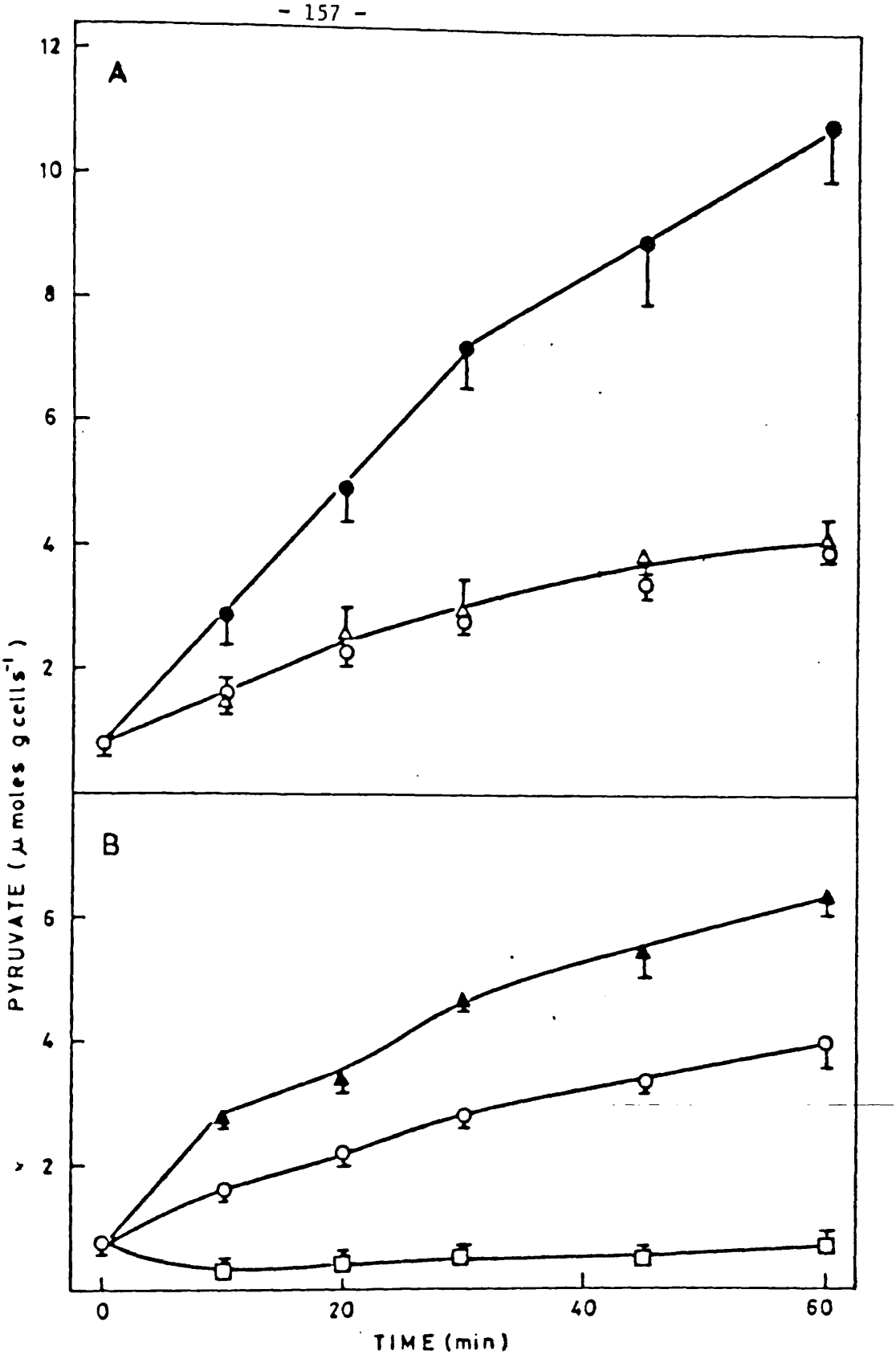


Figure 38. Pyruvate output by isolated hepatocytes incubated with 10 mM glucose, 10 mM fructose, 10 mM glycerol or 10 mM dihydroxyacetone (symbols as in figure 37).

hepatocytes is present in the form of NH_4MgPO_4. Thus the effect of a fructose load would be to decrease Pi, which in turn would lead to the dissociation of the complex and an increase in the free NH_4^+. This seems to suggest that the increase in alanine observed with fructose (figure 37A) is the result of increased pyruvate accumulation (figure 38A) and increased NH_4^+ availability. This is borne out by the evidence in Table 14, which shows that the effects of fructose on alanine output are only observed at 5 and 10 mM. Since stimulation of pyruvate output is observed at low concentrations of fructose (figure 39A), this suggests that NH_4^+ may be a limiting factor in alanine production at these concentrations.

Further evidence for this hypothesis was obtained from the results on a study of the effect of added NH_4Cl(10 mM) on the output of alanine. In the absence of NH_4Cl, the usual stimulation of alanine output was observed, but on addition of NH_4Cl both the basal and the fructose induced increase in the output of alanine were considerably elevated (figure 40). This increase is probably due to the increased availability of glutamate as the result of glutamate dehydrogenase activity. These observations are in accordance with the results of Hems et al., (1975), who found that lactate raised alanine concentrations in hepatocytes from 48 h - starved animals, and at the same time glutamate levels were increased. The addition of NH_4Cl resulted in an increase of both glutamate and alanine. Thus the rapid rise in alanine concentrations occurring after fructose administration is the result of pyruvate accumulation. The much smaller rise observed in the absence of NH_4Cl suggests that the level of intracellular NH_4^+ may also be a limiting factor.

An examination of the fructose-induced alanine production over a 10 min incubation period (figure 41) revealed that a significant effect of fructose (10 mM) was observed after 1 min, whereas pyruvate output was only significantly increased after 5 min (figure 42). This observation coupled to the fact that ATP levels are significantly

Table 14. Alanine levels in isolated hepatocytes incubated for 20 min with varying substrate concentrations.

Concentration of substrate (mM) added	Alanine (μmoles g cells^{-1})			
	Fructose	Glucose	Dihydroxyacetone	Glycerol
0	0.70 ± 0.04	-	-	-
1	0.89 ± 0.08	0.85 ± 0.02	0.84 ± 0.04	0.89 ± 0.06
2	0.91 ± 0.09	0.94 ± 0.003	0.90 ± 0.04	0.96 ± 0.03
3	1.08 ± 0.12	0.98 ± 0.02	1.01 ± 0.02	0.97 ± 0.03
5	1.40 ± 0.09	1.02 ± 0.03	1.06 ± 0.03	1.01 ± 0.04
10	1.82 ± 0.01	1.11 ± 0.02	1.07 ± 0.03	1.14 ± 0.07

Isolated hepatocytes from fed animals were incubated for 20 min with increasing substrate concentrations (up to 10 mM). After the incubation period, the reaction was stopped using perchloric acid and the neutralized extracts used for alanine assays as described in methods (section IV.B.6).

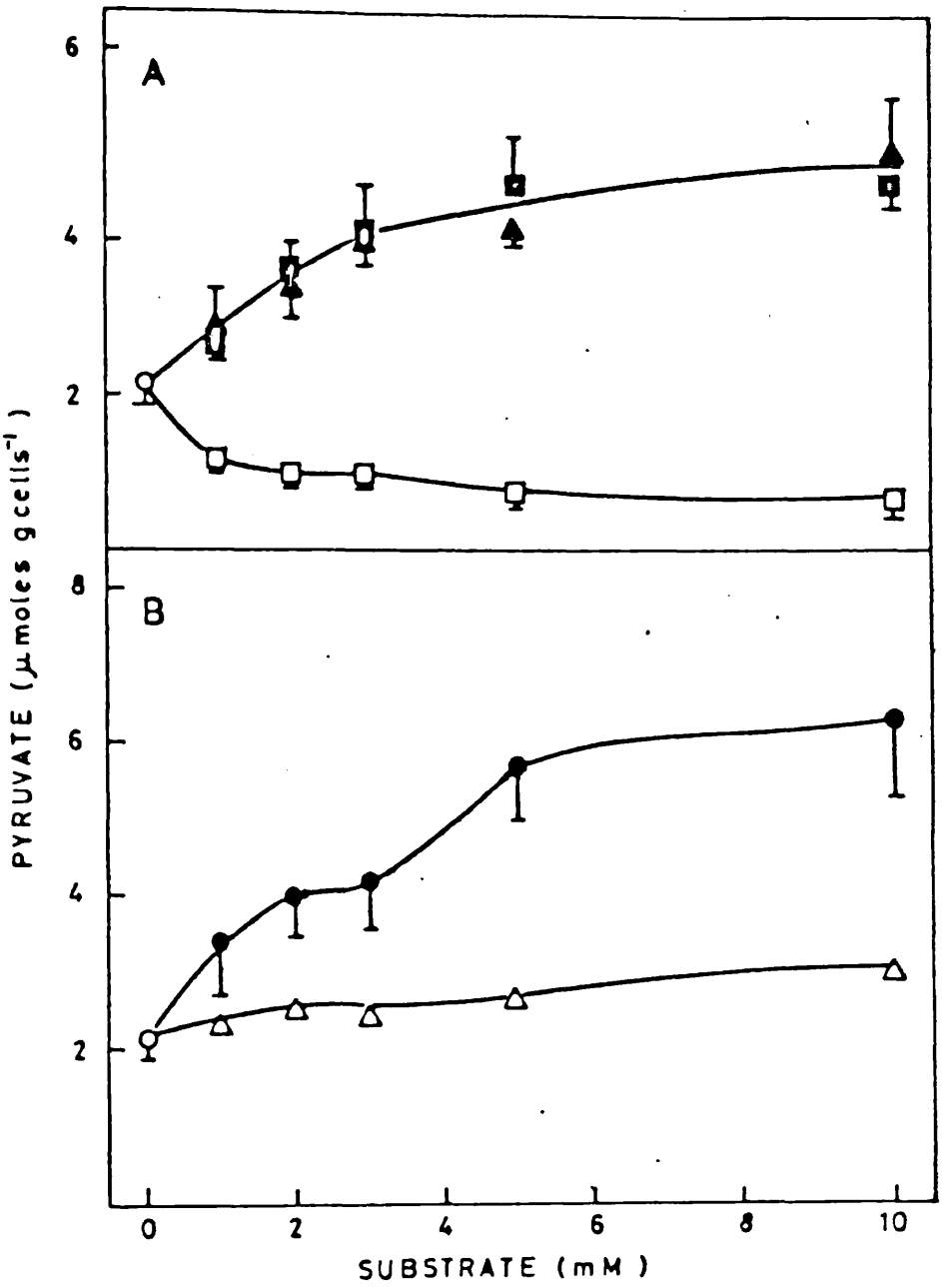


Figure 39. Pyruvate output by isolated hepatocytes incubated with glucose (△), fructose (●), glycerol (□), dihydroxyacetone (▲) or D-glyceraldehyde (■) for 20 min (glucose n=2).

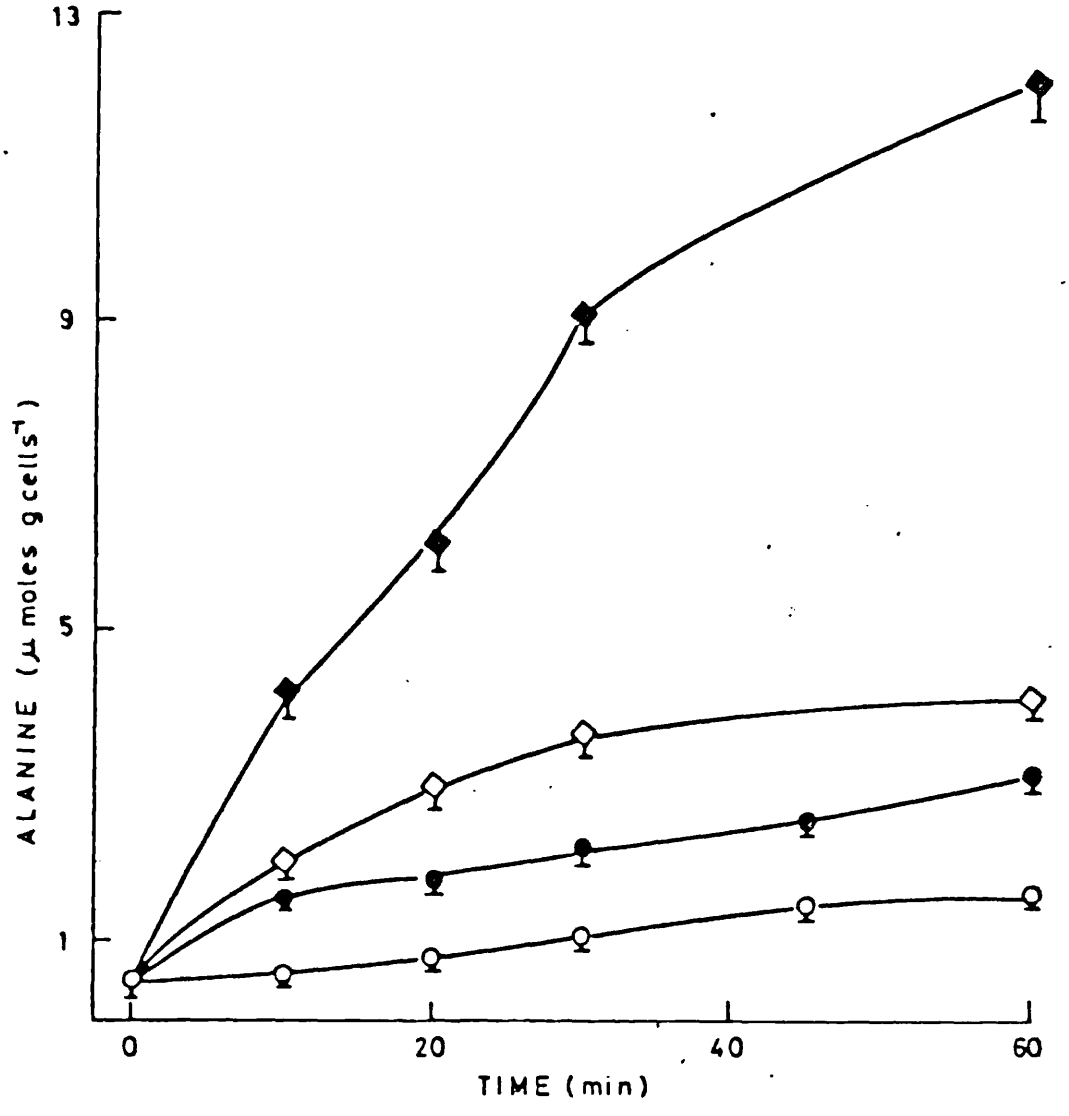


Figure 40. Alanine output by isolated hepatocytes treated with no added substrate (○), 10 mM fructose (●), 10 mM NH_4Cl (◇) or 10 mM fructose and 10 mM NH_4Cl (◆).

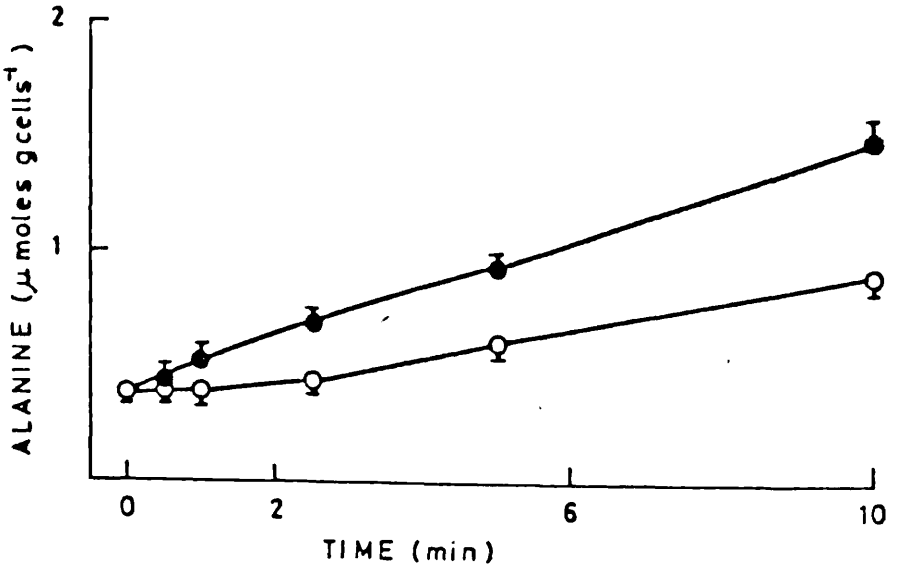


Figure 41. Rapid alanine output by isolated hepatocytes incubated with (●) or without (O) added fructose (10 mM).

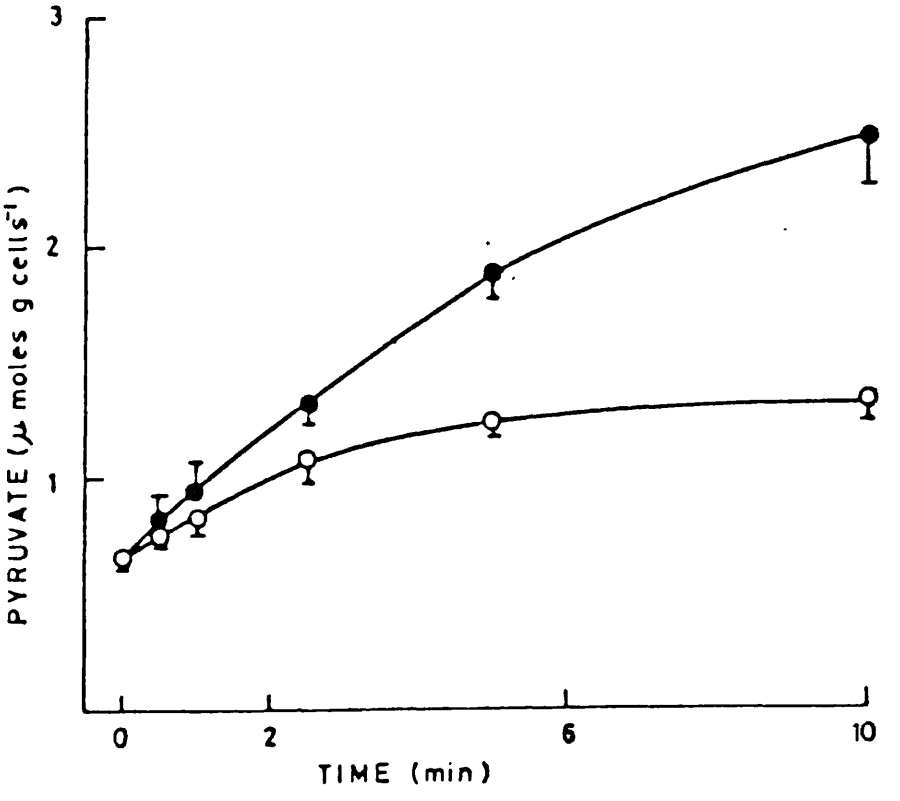


Figure 42. Rapid pyruvate output by isolated hepatocytes incubated with or without 10 mM fructose (symbols as in figure 41).

reduced within 30 s (figure 20) is further evidence for the involvement of an increased NH_4^+ availability during synthesis of alanine. In general, however, the time course for pyruvate accumulation parallels that for alanine production suggesting that the level of pyruvate is also an important factor.

This hypothesis was tested further by comparing the effects of glycerol (which causes a substantial depletion of hepatocyte ATP (figure 21), but inhibits pyruvate production (figure 38B)) and dihydroxyacetone (which does not deplete hepatocyte ATP severely (figure 21) but increases pyruvate formation (figure 38B)), on alanine production. A time course examination of the effect of these substrates on hepatocyte alanine concentration (figure 37B) revealed that both the trioses caused a small but significant increase in alanine production during the first 20 min of incubation, but this was much less than that observed with fructose. Initially there was no difference in the rate of alanine output in the presence of dihydroxyacetone (10 mM) and glycerol (10 mM), but after 20 min the rate of alanine output in the presence of the latter substrate was much reduced compared to dihydroxyacetone. An explanation for this phenomenon can be put forward by considering the effects of the two substrates on ATP and pyruvate levels. Glycerol depletes the hepatocyte ATP (figure 21), decreases the pyruvate levels (figure 38B) and inhibits the formation of total lactate plus pyruvate (figure 30B). The elevation of NH_4^+ caused by the phosphorylation of glycerol would cause a rapid conversion of pre-existing pyruvate to alanine initially; but then the supply of pyruvate would become limiting because of the inhibition of glycolysis. In the case of dihydroxyacetone, pyruvate output is increased (figure 38B), but in this case NH_4^+ is the limiting factor in alanine production. With fructose, the ATP depletion and pyruvate accumulation would result in the high rate of alanine production observed. The effect of dihydroxyacetone and glycerol on alanine production was also found to be concentration dependent (Table 14). There appears to be no significant difference in the rate of alanine output in the presence of either glycerol or

dihydroxyacetone. However, it is likely that in the case of the former substrate pyruvate accumulation is limiting, whereas in the case of the latter substrate, NH_4^+ concentration is limiting.

ii) Effect of aminooxyacetate on alanine concentrations in isolated hepatocytes

It is evident from the above results that fructose (10 mM) causes an increase in alanine production by hepatocytes. The proposed mechanism for this effect is that fructose increases the concentration of both NH_4^+ and pyruvate in the hepatocyte and this leads to transamination of glutamate and pyruvate to yield alanine. It was of interest, therefore, to study the effect of aminooxyacetate, an inhibitor of aminotransferases (Hopper & Segal,1962; Otto, 1965; Blackshear et al., 1975; Garber et al.,1976; John et al., 1978) on alanine levels in isolated hepatocytes and also to examine PK activity under these conditions.

An examination of alanine levels in isolated hepatocytes reveals that aminooxyacetate inhibits the basal alanine production by untreated hepatocytes (Table 15). Similarly the raised alanine output due to fructose is also inhibited by aminooxyacetate. The simplest explanation for this phenomenon is the inhibition of pyruvate transamination, but an examination of the lactate and pyruvate levels in the aminooxyacetate treated cells revealed that, while the rate of glycolysis as estimated by lactate and pyruvate output was unaffected, the pyruvate levels fell considerably following treatment of the cells with the inhibitor (Table 16). This is a consequence of a change in the (lactate)/(pyruvate) ratio. An explanation for the change in the (lactate)/(pyruvate) ratio can be found in the work of Rognstad & Clark,(1974) who suggested that aminooxyacetate may inhibit the malate-aspartate cycle which serves to remove excess cytosolic NADH. From the results in Table 16, it would appear that basal glycolysis from glycogen and glycolysis from fructose to pyruvate would generate

Table 15. The effect of aminooxyacetate on alanine levels in isolated hepatocytes incubated for 20 min in the presence and absence of 10 mM fructose.

Concentration of Aminooxyacetate (mM)	Alanine (μmoles g cells^{-1})	
	No Substrate	Fructose
0	0.75 ± 0.09	1.75 ± 0.15
0.25	0.62 ± 0.08	0.91 ± 0.09
0.5	0.65 ± 0.04	0.75 ± 0.07
1.0	0.61 ± 0.04	0.81 ± 0.03
2.0	0.57 ± 0.02	0.77 ± 0.14

Isolated hepatocytes were incubated with or without fructose and aminooxyacetate for 20 min. The reaction was stopped using perchloric acid and the neutral extracts used for alanine estimation as described in methods (section IV.B.6).

Table 16. Lactate and pyruvate levels in isolated hepatocytes incubated with aminooxyacetate for 20 min in the presence and absence of 10 mM fructose.

Concentration of Aminooxyacetate	Lactate(μmoles g cells^{-1})		Pyruvate(μmoles g cells^{-1})		(Lactate)/(pyruvate)ratios	
	No Substrate	Fructose	No Substrate	Fructose	No Substrate	Fructose
0	23.8 ± 3.7	43.0 ± 4.4	2.4 ± 0.04	4.7 ± 0.9	10.5 ± 1.3	9.3 ± 2.0
0.25	22.9 ± 2.2	44.2 ± 4.0	0.9 ± 0.1	2.2 ± 0.5	27.8 ± 3.4	20.0 ± 1.5
0.5	23.7 ± 3.2	47.7 ± 3.3	0.7 ± 0.07	1.7 ± 0.1	34.9 ± 2.5	29.1 ± 3.6
1.0	24.1 ± 2.1	42.2 ± 4.0	0.6 ± 0.06	1.1 ± 0.2	39.9 ± 1.9	40.1 ± 1.7
2.0	25.1 ± 2.6	44.9 ± 4.1	0.6 ± 0.01	0.6 ± 0.1	42.8 ± 3.5	73.9 ± 3.4

Hepatocytes isolated from fed animals were incubated in the presence of aminooxyacetate (up to 2.0 mM). The reaction was stopped using perchloric acid, and the neutralized supernatant was used to determine lactate and pyruvate concentrations (see methods, section IV.B.6).

excess NADH at the glyceraldehyde-3-phosphate dehydrogenase step. Some of the NADH is removed by the action of LDH, but since some pyruvate is converted to alanine, then an excess NADH is produced. Normally this is removed by malate-aspartate shuttle (figure 5B), but if this is inhibited by aminooxyacetate then excess NADH will alter the (lactate)/(pyruvate) ratio in the direction shown in Table 16. Such a mechanism, however, would imply that the pyruvate-glutamate transaminase is not completely inhibited by aminooxyacetate and that the effect of the inhibitor on alanine output is the result of a lack of availability of pyruvate.

An examination of the effect of aminooxyacetate on the inhibition of PK caused by incubation of hepatocytes with 10 mM fructose (Table 17), showed that although the transaminase inhibitor reversed the inhibition due to fructose, no stimulation was observed as might be expected from the accumulation of Fru_{16} BP and Fru-1-P and the depletion of ATP in the hepatocytes exposed to fructose (see section III.B). This is evidence that alanine accumulation may account for some of the inhibition of PK observed after a fructose load, but other factor(s) must also be involved.

iii) Alanine concentrations in isolated hepatocytes from fasted rats

A study of the PK activity in isolated hepatocytes from rats fasted for 24 h (figures 17 & 18) indicated that fructose (10 mM) and dihydroxyacetone (10 mM) caused activation of the enzyme in unwashed cell preparations. There are a number of possible explanations for this phenomenon. One possibility is that in hepatocytes from fasted animals the inhibitor(s) do not accumulate to the same extent after administration of a high fructose load as in cells isolated from fed animals. Alternatively, the concentration of the other effectors may change upon fasting such that the enzyme is rendered less susceptible to the inhibitor. Another possibility is that the phosphorylated

Table 17. The effect of aminooxyacetate on pyruvate kinase activity in isolated hepatocytes incubated with or without 10 mM fructose for 20 min (n = 2).

Concentration of Aminooxyacetate (mM)	Pyruvate Kinase Activity (v/V_{max})	
	No Substrate	Fructose
0	0.42	0.32
0.25	0.39	0.36
0.5	0.39	0.35
1.0	0.40	0.40
2.0	0.41	0.40

Isolated hepatocytes were incubated in the presence and absence of 10 mM fructose and increasing concentrations of aminooxyacetate. The reaction was stopped by freezing samples in liquid N_2 and PK assayed as described in methods (see section IV.B.3).

enzyme,which is found upon fasting, is inhibited to a lesser extent by the inhibitor. Phosphorylated PK is known to be more sensitive to ATP and alanine inhibition (Ekman et al., 1976; Ljungström et al., 1976). This suggests that the latter possibility is invalid or that the inhibition has a totally different effect to that of ATP and alanine. Williamson et al.,(1967b) have reported a 65% decrease in hepatic alanine levels as the result of fasting. It was, therefore, of interest to examine the effects of various substrates on alanine levels in hepatocytes from fasted rats in an attempt to further examine the possibility that the inhibition of PK is caused by alanine.

A time course study revealed that in the absence of substrate, very little alanine accumulation occurred in hepatocytes from fasted animals (figure 43). This can be correlated with the low rate of glycolysis by these cells and is a further indication that alanine output is dependent on pyruvate availability. Glucose (10 mM) caused very little accumulation of alanine (figure 43) or pyruvate (figure 44) in cells from fasted animals, but fructose (10 mM) again stimulated alanine output to the same extent as it did in the cells from fed rats (figures 37A & 43). This stimulation is again reflected by the rate of lactate and pyruvate output observed under these conditions (figure 31). There was no significant difference in the elevation of alanine production caused by dihydroxyacetone (10 mM) in hepatocytes from fed and fasted animals over a 60 min incubation period (figure 43). Again the changes in alanine levels mirror the changes in pyruvate (figure 44).

The lack of difference in the effect of fructose on alanine output in fed and fasted animals is evidence against the involvement of alanine in the inhibition of PK which occurs when hepatocytes from fed animals were incubated with 10 mM fructose.

iv) Intrahepatocyte alanine concentrations

The observations made above indicate clearly that alanine accumulation in isolated hepatocytes from both fed and fasted animals occurs as a consequence of NH_4^+ and pyruvate availability. These studies also

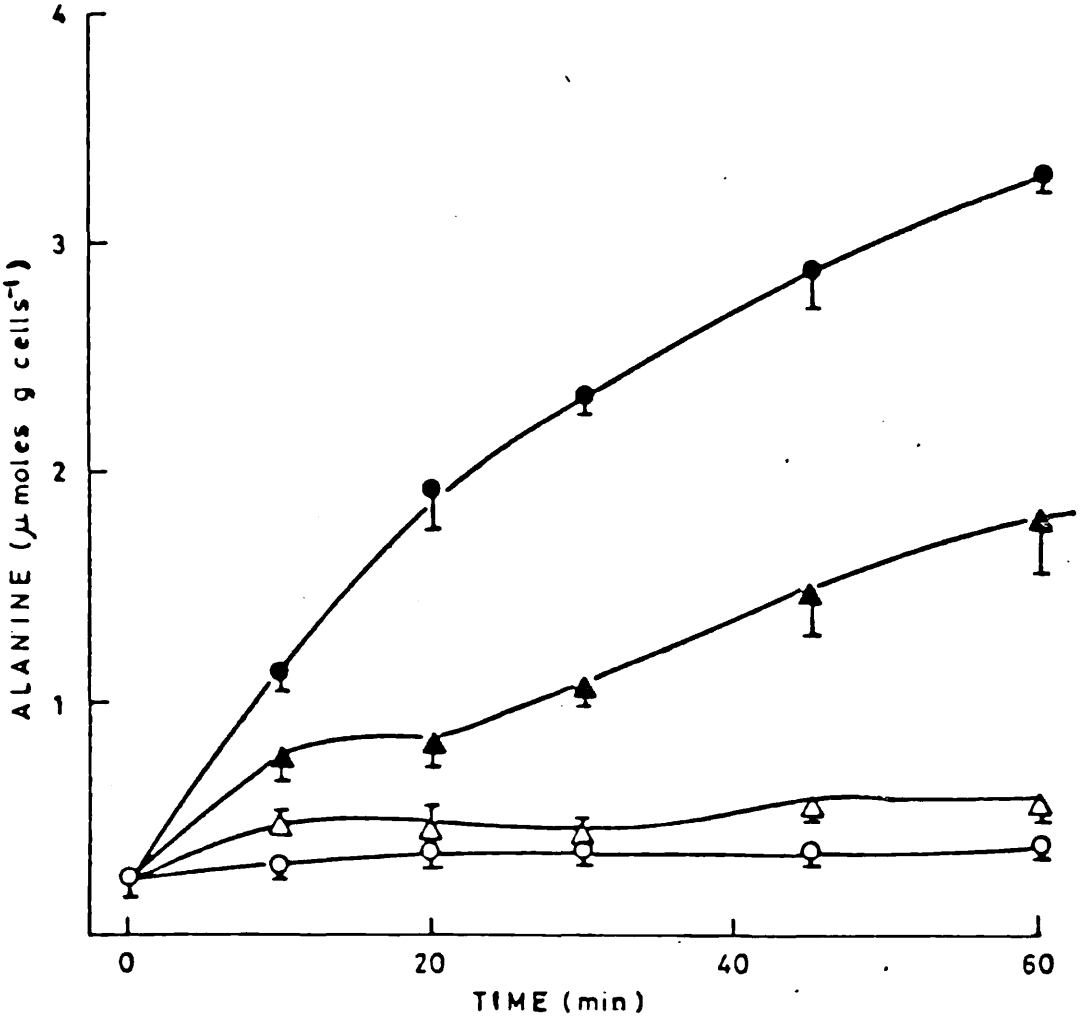


Figure 43. Time course showing the alanine content of hepatocytes isolated from 24 h fasted rats and incubated with no added substrate (○), 10 mM glucose (△), 10 mM dihydroxyacetone (▲) or 10 mM fructose (●).

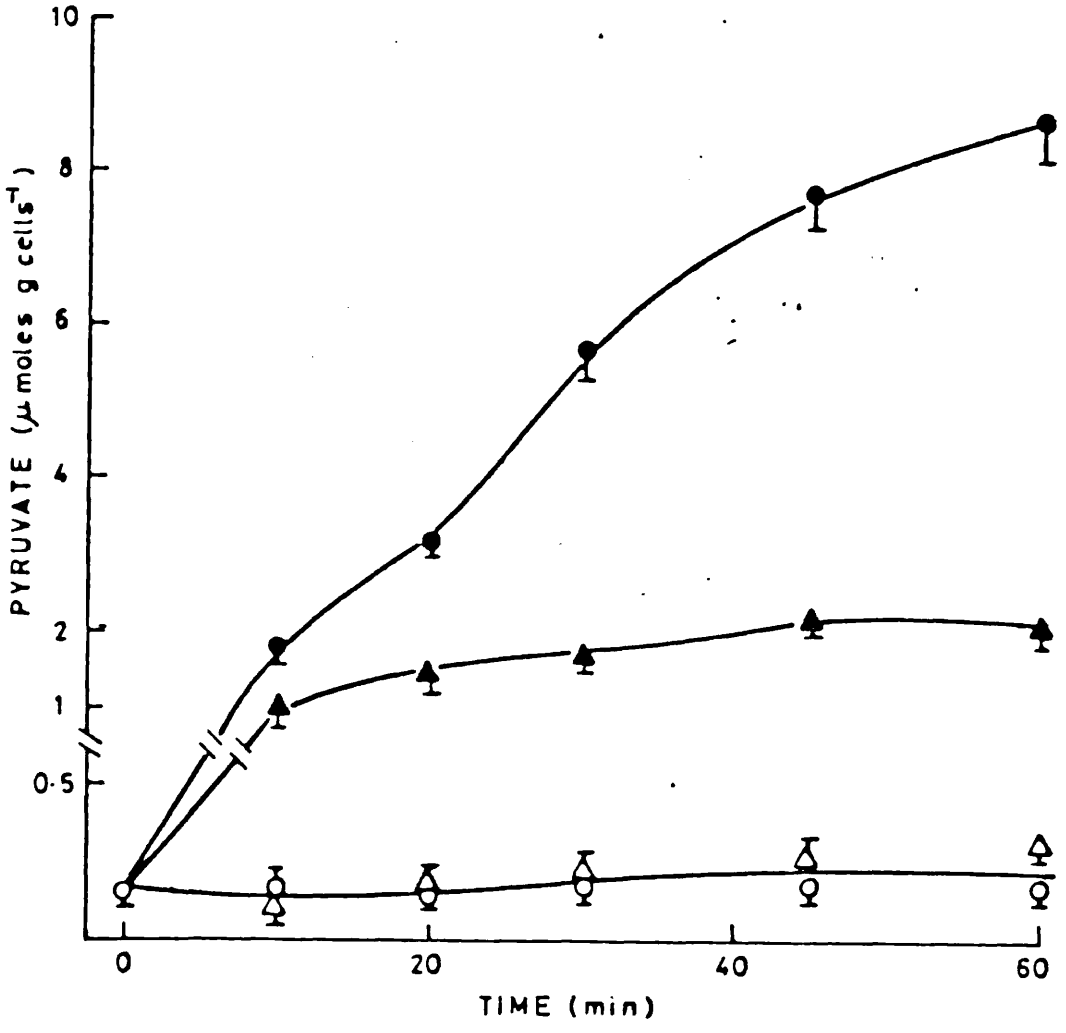


Figure 44. Time course showing the pyruvate content of hepatocytes isolated from 24 h fasted rats and incubated with 10 mM glucose, 10 mM dihydroxyacetone, 10 mM fructose or no added substrate (symbols as in figure 43).

show that the greatest output of alanine occurs in the presence of fructose and that elevation of this amino acid is inhibited by aminooxyacetate. From the results in section III.A , it is clear that the inhibitor which accumulates in isolated hepatocytes after a high fructose load is removed by discarding the extrahepatocyte medium. Since alanine was considered a possible candidate for the inhibition of PK, the distribution of the alanine between the hepatocyte and the medium was investigated.

A comparison of the total alanine accumulating and alanine concentrations inside the hepatocyte from fed rats (Table 18) shows that some of the alanine formed leaves the cell. The amount of alanine inside the untreated hepatocytes is about 40% of the total alanine. The uptake and subsequent metabolism of amino acids in liver parenchymal cells has been subjected to intensive research (Ishikawa, 1976). The presence of specific amino acid transport systems in the parenchymal cell membrane has been demonstrated both in whole cells (Edmondson et al., 1977;1979; Le Cam & Freychet, 1977; Sips et al., 1980b) and in isolated plasma membrane vesicles (Van Amelsvoort et al., 1978;1980; Sips et al., 1980a). Thus there is a distribution of alanine against a concentration gradient by the hepatocytes as evidenced by the results in this study (Table 18). A rough calculation of the ratio of the concentration of alanine inside and outside the cell yields a result of approximately 30, which is in agreement with the evidence of Hems et al., (1975) for alanine levels in isolated hepatocytes incubated with lactate.

A quantitative study of the alanine levels in isolated hepatocytes shows that the total alanine in both the cells and medium increases from 0.03 to 0.10 mM in the 60 min incubation period. However, if the alanine levels inside the hepatocytes are examined (0.55 to 1.42 mM - assuming 1 g cells contains 380 µl cytosolic water - Sobell et al., 1976) it seems that the intracellular alanine concentrations are similar to those reported in the literature (0.5 - 1.5 mM) (Brosnan et al., 1970; Schoner et al., 1970). In the case of fructose total alanine increases from 0.03 to 0.11 mM whereas the intracellular alanine changes from

Table 18. Alanine concentrations in isolated hepatocytes incubated with various substrates.

Time (minutes)	Alanine (μmoles g cells^{-1})									
	Substrate added (10 mM)									
	No Substrate		Glucose		Glycerol		Dihydroxyacetone		Fructose	
	Intra-hepato-cyte	Total	Intra-hepato-cyte	Total	Intra-hepato-cyte	Total	Intra-hepato-cyte	Total	Intra-hepato-cyte	Total
0	0.21	0.48±0.05	-	-	-	-	-	-	-	-
10	0.29	0.57±0.06	0.25	0.88±0.03	0.23	0.89±0.1	0.23	0.79±0.05	0.42	1.64±0.16
20	0.39	0.79±0.08	0.35	1.24±0.07	0.40	1.15±0.10	0.46	1.06±0.07	0.74	1.80±0.12
30	0.41	1.07±0.11	0.54	1.50±0.06	0.50	1.27±0.07	0.61	1.30±0.12	0.68	2.20±0.06
45	0.68	1.50±0.18	0.68	1.55±0.20	0.69	1.38±0.04	0.76	1.54±0.10	0.99	2.64±0.16
60	0.54	1.73±0.18	0.72	2.15±0.13	0.80	1.48±0.02	0.77	2.01±0.12	1.20	3.20±0.22

Isolated hepatocytes obtained from fed animals were incubated with various substrates for different time periods (up to 60 min). The intrahepatocyte alanine concentrations were obtained by first washing the cells (see methods, section IV.B.3) and then the reaction was stopped by adding perchloric acid to the cells. The total alanine concentrations were obtained by stopping the reaction in unwashed cells. Alanine was assayed and described in methods (see section IV.B.6). The intrahepatocyte alanine concentrations presented are an average of two different hepatocyte preparations.

0.55 to 1.95 mM in 20 min. Thus when the dilution factors are taken into account the inhibition of PK by high concentrations of fructose cannot be accounted for by changes in total alanine concentrations. The lack of change in the medium alanine concentrations in response to fructose is evidence against the involvement of the amino acid in the inhibition of PK, since the evidence points to the presence of the inhibitor in the extrahepatocyte medium. However, it is possible that the localized alanine level inside the hepatocyte may extert a regulatory effect on PK in situ and thus alter the glycolytic flux.

D. KINETIC STUDIES ON PYRUVATE KINASE EXTRACTED FROM ISOLATED HEPATOCYTES

The results from the previous section(III.B) indicate that a high concentration of fructose leads to a depletion of intracellular ATP and an accumulation of $Fru_{1,6}BP$ and Fru-1-P within the cell. Taken together this should result in the activation of PK in situ as measured by glycolytic flux and the activation of the enzyme as measured in a cuvette. However, while it is evident that total glycolytic flux is increased by fructose (figure 30A), the activation of PK is only apparent in hepatocytes washed after treatment with fructose (figure 11B). This seems to suggest that an inhibitor is present in the extrahepatocyte medium, which causes a profound inhibition of PK. Two possible candidates to explain this inhibition are alanine and allantoin. The following experiments were performed to study the kinetic properties of PK in order to examine if the changes detailed above could be explained by the kinetic properties of the enzyme.

1. The effect of fructose treatment on the kinetic parameters of a crude pyruvate kinase preparation isolated from hepatocytes

A comparison of the kinetic properties of PK from hepatocytes incubated with fructose (10 mM) with those of PK from untreated hepatocytes clearly shows the inhibition observed in unwashed cells (figure 45A) and

the stimulation which occurs when the washed cells were assayed (figure 45B). These effects were only apparent at subsaturating PEP concentrations, while V_{max} is unchanged by fructose treatment. A kinetic analysis of PK activity (using Hill plots) from washed or unwashed cells, treated or not treated with fructose (10 mM), showed that the changes in activity observed were mainly as a result of the changes in the affinity of the enzyme for PEP, as estimated by the $S_{0.5}$ values (figure 46). The $S_{0.5}$ values in the control cells were unaffected by the washing treatment (unwashed cells $S_{0.5}$ = 0.25 mM, washed cells $S_{0.5}$ = 0.29 mM) but both values were lower in the crude hepatocyte preparation (figure 46A & 46B) than in the $(NH_4)_2SO_4$ purified enzyme ($S_{0.5}$ = 0.59 mM, figure 46C). This is presumably because $(NH_4)_2SO_4$ treatment removes the tightly bound $Fru_{16}BP$, resulting in the inactivation of the enzyme. This effect is also apparent when the $S_{0.5}$ value for PK from washed cells treated with fructose ($S_{0.5}$ = 0.16 mM) is compared to the $(NH_4)_2SO_4$ purified enzyme ($S_{0.5}$ = 0.69 mM). However, it is apparent that the $S_{0.5}$ value for the enzyme from unwashed cells ($S_{0.5} \simeq$ 0.50 mM) treated with 10 mM fructose is higher than the value for the comparable enzyme from washed cells ($S_{0.5} \simeq$ 0.15 mM).

The kinetic analysis of the crude enzyme preparation from hepatocytes indicates that the changes in affinity for PEP observed when hepatocytes are treated with fructose are due to changes in the level of activator(s) and inhibitor(s) since $(NH_4)_2SO_4$ treatment abolishes the difference between the fructose-treated and the untreated cells. Thus the changes in enzyme activity occurring as a result of fructose treatment are not due to a stable change in the enzyme. The fact that in the control, the $(NH_4)_2SO_4$ treatment results in an increase in the $S_{0.5}$ value is evidence that there is a carry-over of activator(s) (presumably $Fru_{16}BP$) in the extraction and assay procedure. This must be borne in mind when interpreting results obtained with the assay of PK from hepatocytes. The effect of $Fru_{16}BP$ and Fru-1-P are also apparent in figure 45B. The difference between the fructose-treated

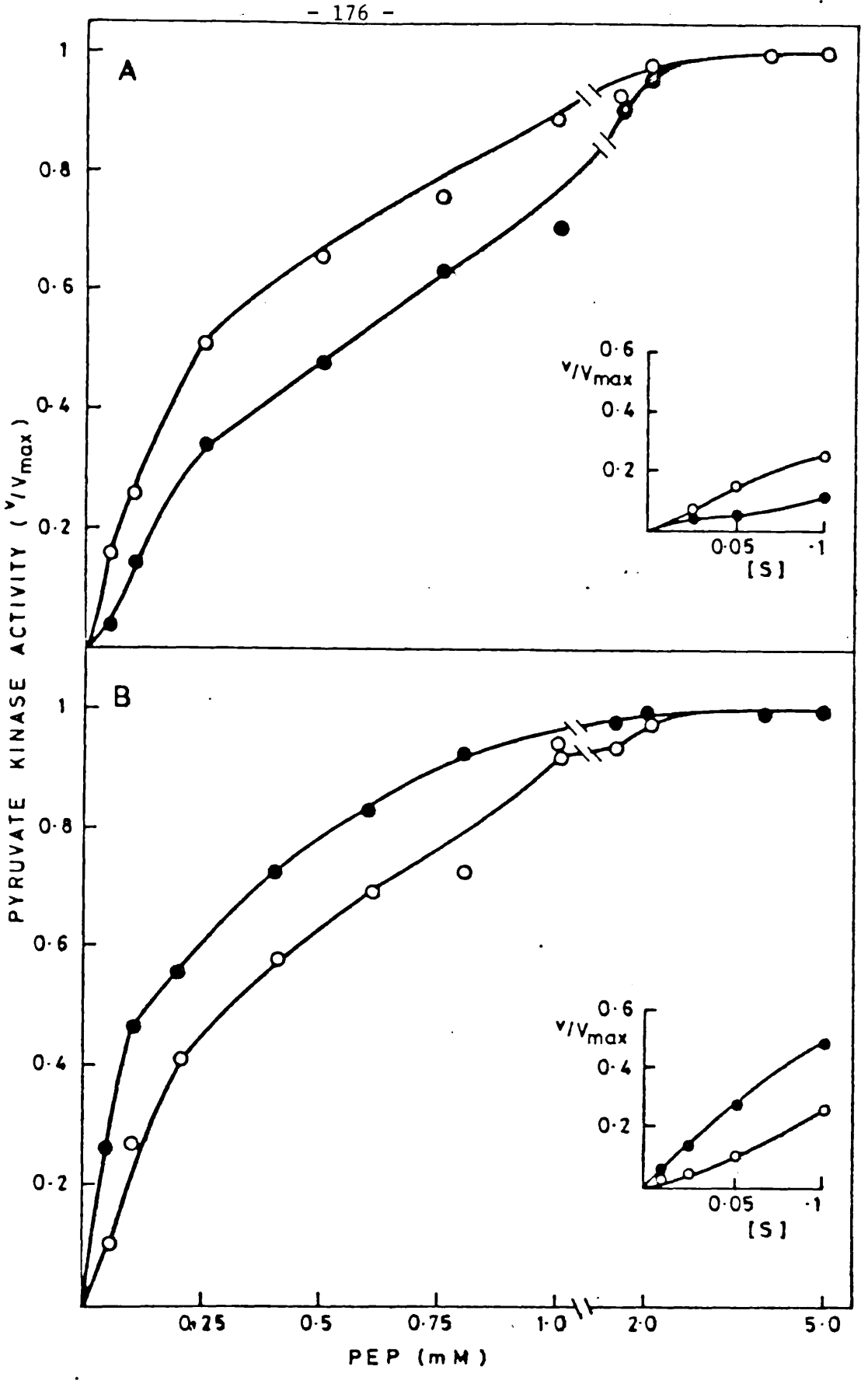


Figure 45. Effect of increasing PEP concentrations on a crude extract of cells extracted from isolated hepatocytes incubated with 10 mM fructose (●) or no added substrate (O) for 20 min. A, unwashed hepatocytes; B, washed hepatocytes. Insert shows the data obtained with low PEP concentrations.

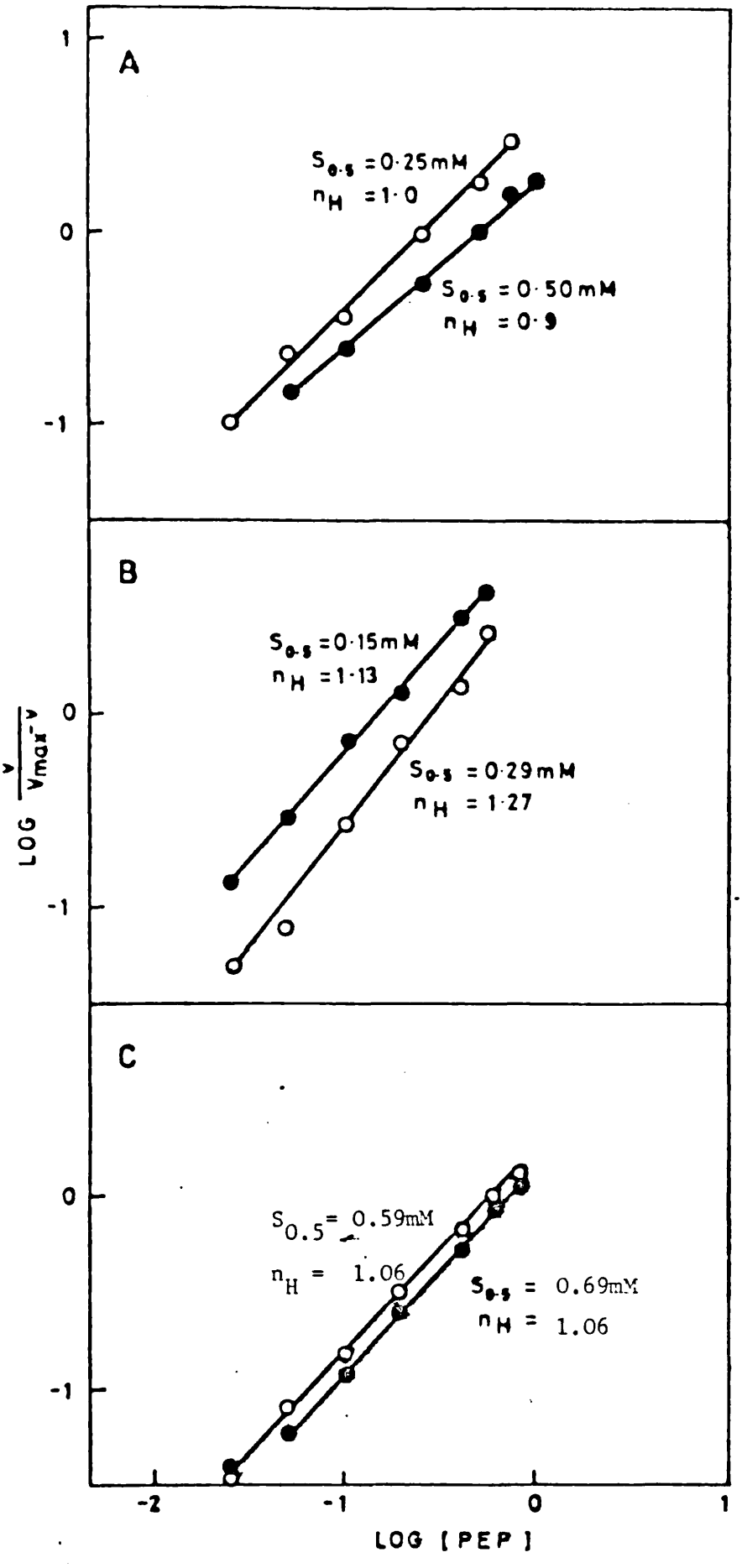


Figure 46. A & B, Hill plots of data in figure 45.

C, Effect of $(NH_4)_2SO_4$ precipitation on the kinetic properties of pyruvate kinase extracted from hepatocytes treated with (●) or without (O) 10 mM fructose for 20 min.

and untreated hepatocytes is presumably related to the high level of these effectors found in the treated cells (see figures 22 & 25). Similar effects were also found with hepatocytes from fasted rats (results not shown).

The explanation for the inhibition of the PK in the extracts of cells from fed rats incubated with fructose is more problematical. The simplest explanation is that an inhibitor has accumulated which overcomes the stimulatory effect of $Fru_{16}BP$ and Fru-1-P. Alternatively, it is possible that the reversal of the stimulatory effect is due to a dissociation of the tightly bound activator from PK during the extraction and assay procedure. Thus the carry-over of $Fru_{16}BP$ may be much reduced in the presence of the increased concentration of alanine and/or allantoin. The fact that the $S_{0.5}$ value for the fructose-treated enzyme from unwashed cells is lower than the $S_{0.5}$ value for the $(NH_4)_2SO_4$ treated enzyme lends further support for this mechanism. Therefore, it is possible that the inhibition of PK activity observed with fructose is the result of a decreased carry-over of $Fru_{16}BP$ rather than the accumulation of an inhibitor since the changes in the known inhibitors are not sufficient to explain this inhibition (see section III.C).

2. Effect of magnesium ions on partially purified pyruvate kinase

It has been reported that free Mg^{2+} levels are increased by a fructose dependent depletion of ATP-Mg in the liver (Levin et al., 1963; Van de Werve & Hers, 1979; Sainsbury, 1980). Thus the effect of Mg^{2+} on a partially purified PK preparation was studied and presented in figure 47. The Mg^{2+} concentrations shown are those of added $MgCl_2$. It is evident from these results that PK has an absolute requirement for this divalent ion. The maximum activity was obtained with 10 mM Mg^{2+} whereas higher concentrations were inhibitory as shown by changes in both v and V_{max}. Normally 10 mM Mg^{2+} ions were used in the assay medium (see methods, IV.B.5). The ratio of

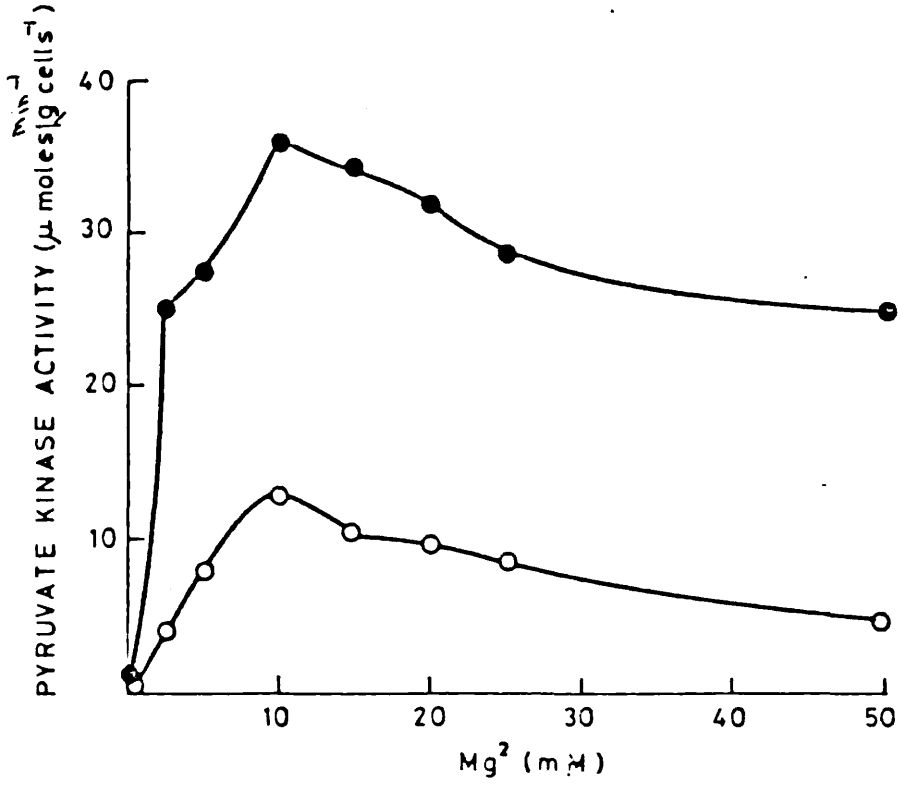


Figure 47. Effect of Mg^{2+} ions on the activity of a partially purified pyruvate kinase preparation. The enzyme activity was assayed with 0.2 mM (○) and 4.0 mM (●) PEP.

v/V_{max} is unaffected by Mg^{2+} and the possible change in Mg^{2+} level caused by fructose in the isolated hepatocytes is very small compared to the total Mg^{2+} in the assay. Thus it is very unlikely that the inhibition of PK caused by fructose is due to changes in free Mg^{2+}. The presence of $Fru_{16}BP$ and Fru-1-P did not normally change the effect of Mg^{2+} on the PK activity (results not shown).

3. Effect of PEP and ADP concentrations on partially purified pyruvate kinase

The effect of varying PEP concentrations on the $(NH_4)_2SO_4$ purified enzyme is shown in figure 48 . The sigmoidal relationship observed by other workers is evident. The $S_{0.5}$ values for PEP as determined by a Hill plot, for the enzyme from fed animals is 0.58 mM (figure 48B). This is within the range of value, reported in the literature for phosphorylated and unphosphorylated PK (Ekman et al., 1976; Claus et al., 1979). The $S_{0.5}$ for ADP was 0.2 mM (cf Ekman et al., 1976) and V_{max} was obtained at 1 mM ADP (figure 49). Thus throughout the present study PK has been assayed at saturating ADP concentrations.

$Fru_{16}BP$ decreases the $S_{0.5}$ value of the enzyme for PEP to 45 µM (figure 48). This compares well with the values obtained by Ekman et al., (1976) ($S_{0.5}$ = 40 µM) and Claus et al., (1979) ($S_{0.5}$ = 50 µM). Similarly, Fru-1-P is a stimulator of PK (cf Eggleston & Woods, 1970), in this case the $S_{0.5}$ of the enzyme is reduced to 240 µM in the presence of 1 mM Fru-1-P (figure 48). The possibility that the effect of Fru-1-P could be due to contamination by $Fru_{16}BP$ was tested by purifying the hexose phosphate by paper chromatography and no significant change in the stimulating power of Fru-1-P was observed after this treatment.

4. Inhibitors of pyruvate kinase

Alanine is a known inhibitor of PK (Seubert & Schoner, 1971) and this was confirmed in the present study by examining the activity of the partially purified enzyme in the presence and absence of $Fru_{16}BP$

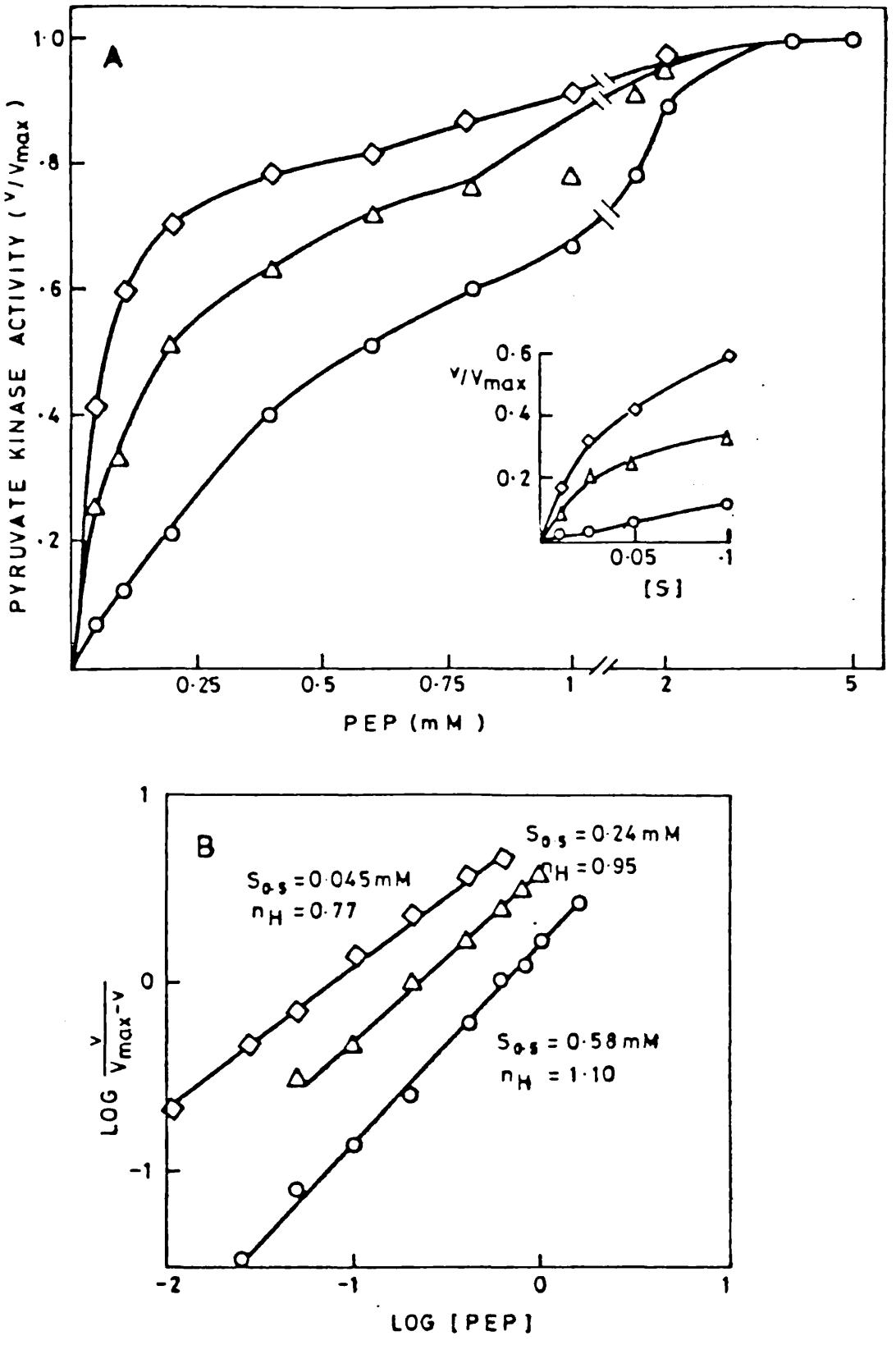
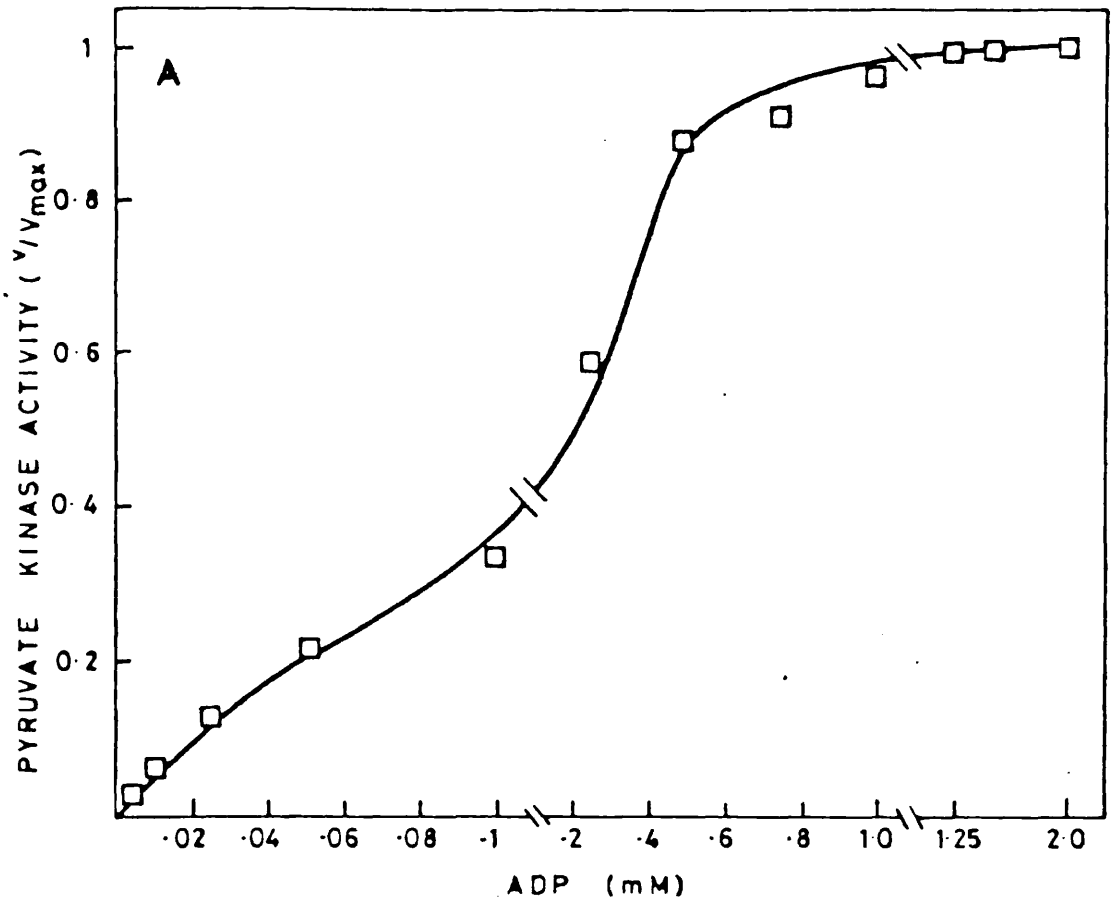


Figure 48. A, The effect of 50 µM $Fru_{1,6}BP$ (◇), 1 mM Fru-1-P (△) or no added effector (○) on the activity of a partially purified pyruvate kinase preparation assayed at various PEP concentrations. Insert shows the data obtained with low PEP concentrations.

B, Hill plots of data in figure 48A.



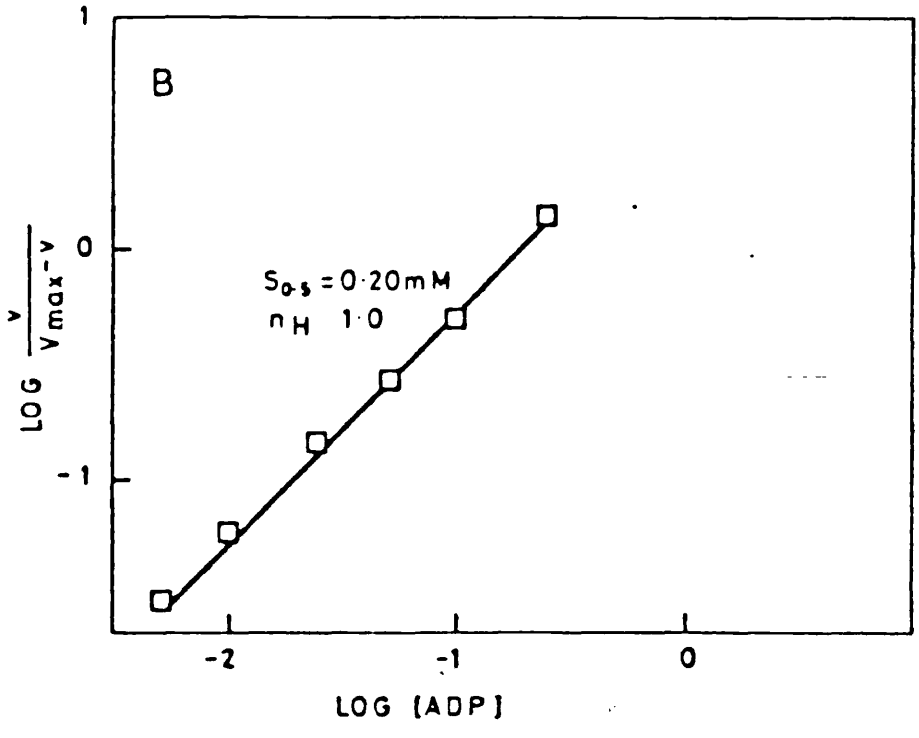


Figure 49. A, Effect of increasing ADP concentrations on the activity of a partially purified pyruvate kinase preparation.
B, Hill plots of data in figure 49A.

(figure 50A) and Fru-1-P (figure 50B). Physiological concentrations of alanine (0.5 - 1.5 mM) caused a substantial inhibition of the enzyme in the presence and absence of $Fru_{16}BP$. Alanine (1 mM) increased the half maximal stimulation of the enzyme from 0.4 µM to 2.0 µM $Fru_{16}BP$ (figure 51). However, the concentration of $Fru_{16}BP$ used is much less than that observed *in vivo* suggesting that PK may be maximally activated *in vivo*. However, it is possible that the enzyme *in situ* is more sensitive to the allosteric inhibitors than the purified enzyme (Llorente *et al.*, 1970; Seubert & Schoner, 1971). The evidence in the present study (see section III.B) indicates that while $Fru_{16}BP$ may regulate flux through PK, the depletion of ATP may play a more important role in the increased glycolytic flux observed with fructose. In this case the increase in alanine levels in the isolated hepatocytes do not substantially affect the enzyme activity, since this would be expected to inhibit glycolytic flux.

The inhibitory effect of alanine is a result of a change in the $S_{0.5}$ for PEP with no corresponding change in V_{max}. The effect of alanine on the partially purified PK is shown in figure 52. The inhibitor exerts its effects both on the enzyme incubated in the absence of $Fru_{16}BP$ and in the presence of $Fru_{16}BP$. The $S_{0.5}$ values for PEP vary from 45 µM with $Fru_{16}BP$ only, to 1.0 mM with alanine. Since the concentration of PEP in isolated hepatocytes found in the present study (0.15 µmoles g cells^{-1}) clearly lies within this range then it is evident that these changes in enzyme activity could obviously regulate flux through PK, but the high level of $Fru_{16}BP$ in the cell may prevent such a regulation. Thus it may be that the intracellular ATP concentration plays an important role in determining PK activity.

The stimulation of PK by Fru-1-P is also reversed by alanine (figure 53 & 54). The half maximal stimulation of the control enzyme was obtained at 0.34 mM Fru-1-P, whereas for the alanine treated enzyme the value was 3.3 mM. Once again the $S_{0.5}$ values for PEP vary from 0.24 mM with Fru-1-P only, to 1.0 mM with alanine (figure 54B).

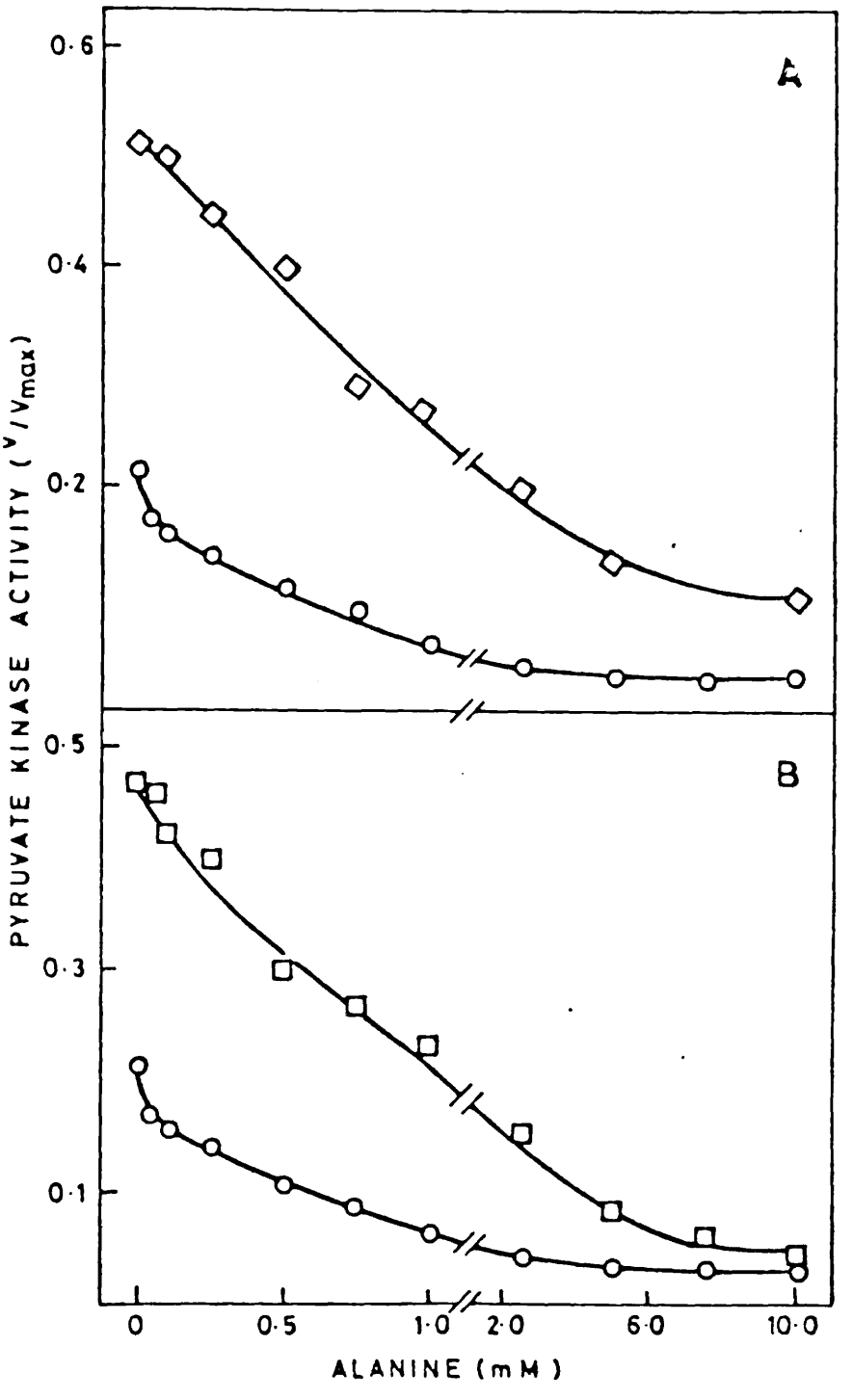


Figure 50. Effect of alanine concentrations on the activity of a partially purified pyruvate kinase preparation assayed in the presence of 1 μM $Fru_{16}BP$ (◇), 1 mM Fru-1-P (□) or no added stimulator (○).

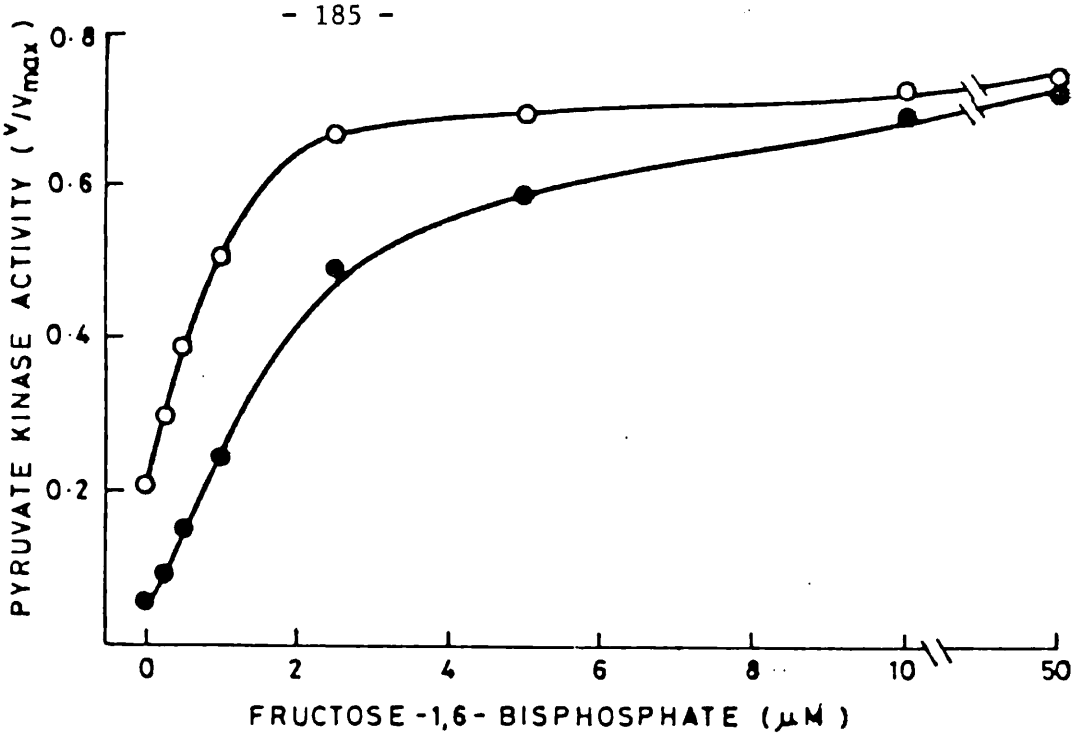


Figure 51. Effect of $Fru_{1,6}BP$ concentrations on the activity of a partially purified pyruvate kinase preparation assayed in the presence of 1 mM alanine (●) or with no added alanine (○).

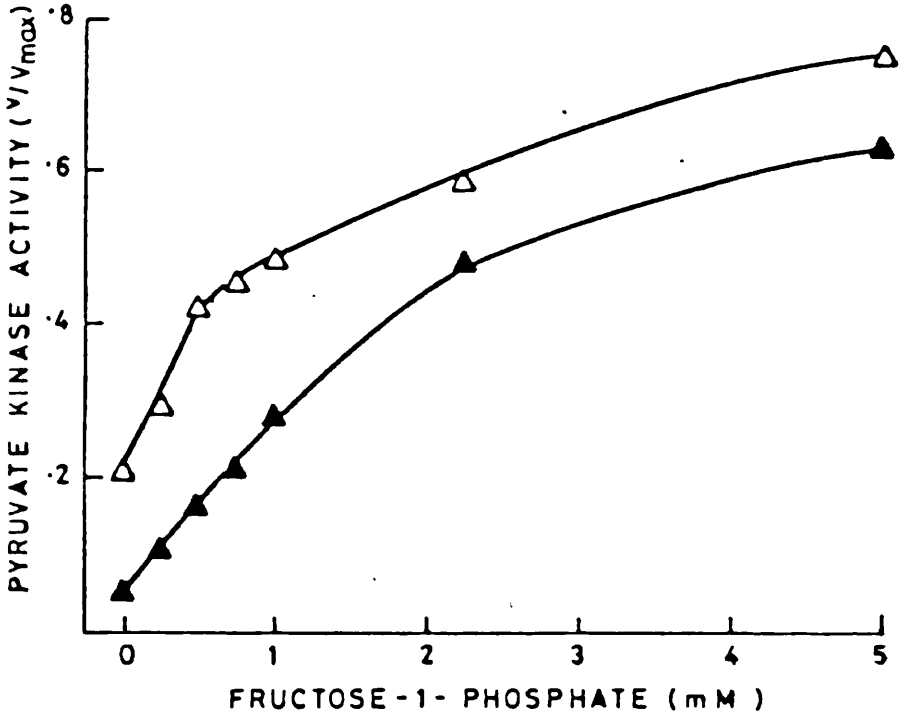


Figure 53. Effect of Fru-1-P concentrations on the activity of a partially purified pyruvate kinase preparation assayed in the presence (▲) or absence (△) of 1 mM alanine.

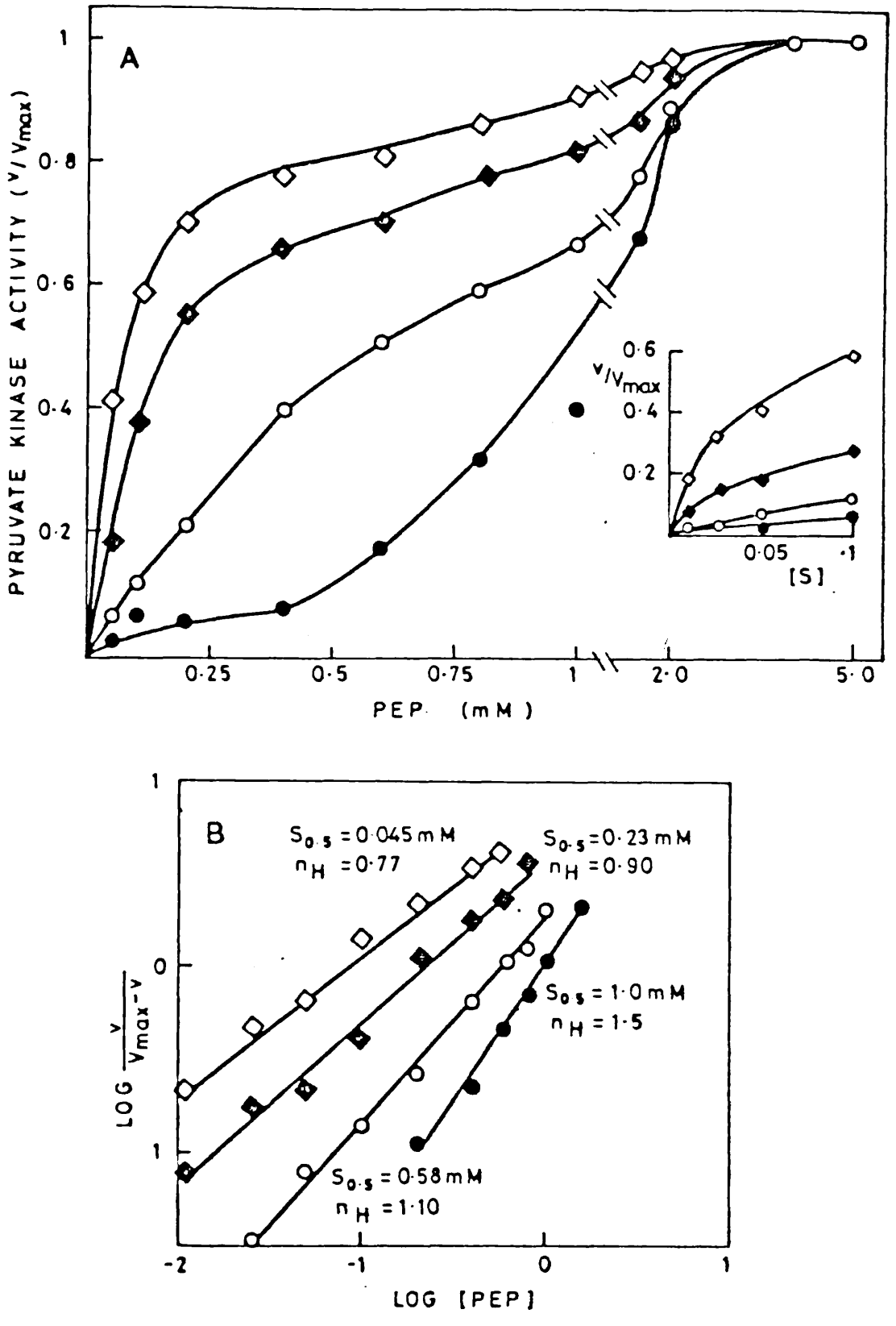


Figure 52. A, Effect of PEP concentrations on the activity of a partially purified pyruvate kinase preparation assayed in the presence of 50 µM $Fru_{16}BP$ (◇), 50 µM $Fru_{16}BP$ and 1 mM alanine (◆), 1 mM alanine (●) or no added effector(○). Insert shows the data obtained with low PEP concentrations.

B, Hill plots of data in figure 52A.

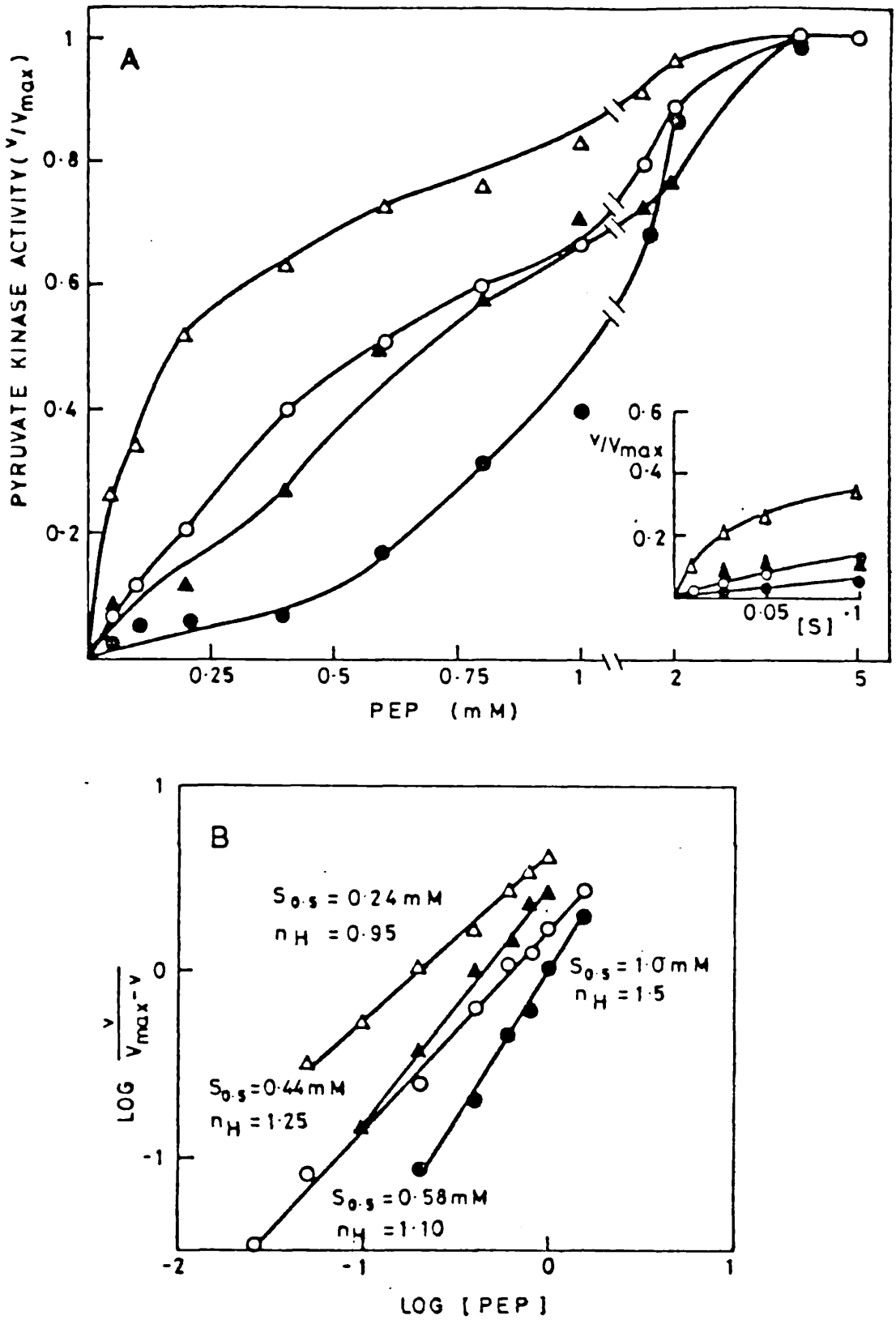


Figure 54. A, Effect of PEP concentrations on a partially purified pyruvate kinase preparation assayed in the presence of 1 mM Fru-1-P (△), 1 mM Fru-1-P and 1 mM alanine (▲), 1 mM alanine(●) or no added effector (○). Insert shows the data obtained with low PEP concentrations.
B, Hill plots of data in figure 54.A.

ATP is also known to be a potent inhibitor of PK (Seubert & Schoner, 1971). The effect of increasing ATP concentrations on the partially purified enzyme is shown in figure 55. In the absence of effectors, 5 mM ATP-Mg almost completely abolished the enzyme activity. The addition of Fru-1-P (1 mM) relieved the inhibition to a small extent, but a concentration of $Fru_{16}BP$ lower than physiological concentrations(see section III.B) was much more effective in relieving the inhibition caused by ATP-Mg. However, the inhibitory effect of ATP was noticeable even when a high concentration of $Fru_{16}BP$ was present (see also figure 56A). Thus, it is clear that the increase in $Fru_{16}BP$ and the decrease in ATP which occur together when the hepatocyte is treated with fructose (see figure 19 & 25) would result in the increased glycolytic flux observed (see figure 30). However, it is clear that the fall in ATP concentration appears to be more important in determining glycolytic flux since dihydroxyacetone raises $Fru_{16}BP$ but does not affect ATP levels to any great extent (see section III.B). These observations seem to suggest that Fru-1-P accumulation in the hepatocyte does not play an important role in the high rate of fructolysis. This is in agreement with the results in figure 57A, which shows that in the presence of 1 mM ATP-Mg, Fru-1-P exerts only a small stimulatory effect on the enzyme.

Earlier in the present study, it was found that allantoin was an inhibitor of PK activity measured at 0.2 mM PEP (Table 11). The effect of allantoin on the enzyme appears to be very similar to the effect of alanine and also appears to be equally potent (cf figure 52 & 58). Allantoin (1 mM) inhibited the control enzyme, the $S_{0.5}$ value for PEP in the absence of inhibitor was 580 µM and in the presence of allantoin the value was 930 µM. Inclusion of $Fru_{16}BP$ (50 µM) in the assay medium resulted in these values changing to 45 µM and 370 µM respectively. It was also found that allantoin (1 mM) causes inhibition of the enzyme at all concentrations of $Fru_{16}BP$ (figure 56A) and Fru-1-P (figure 57A) examined. Urate (0.5 mM) also exerted a similar effect on PK (results not shown), but since this metabolite

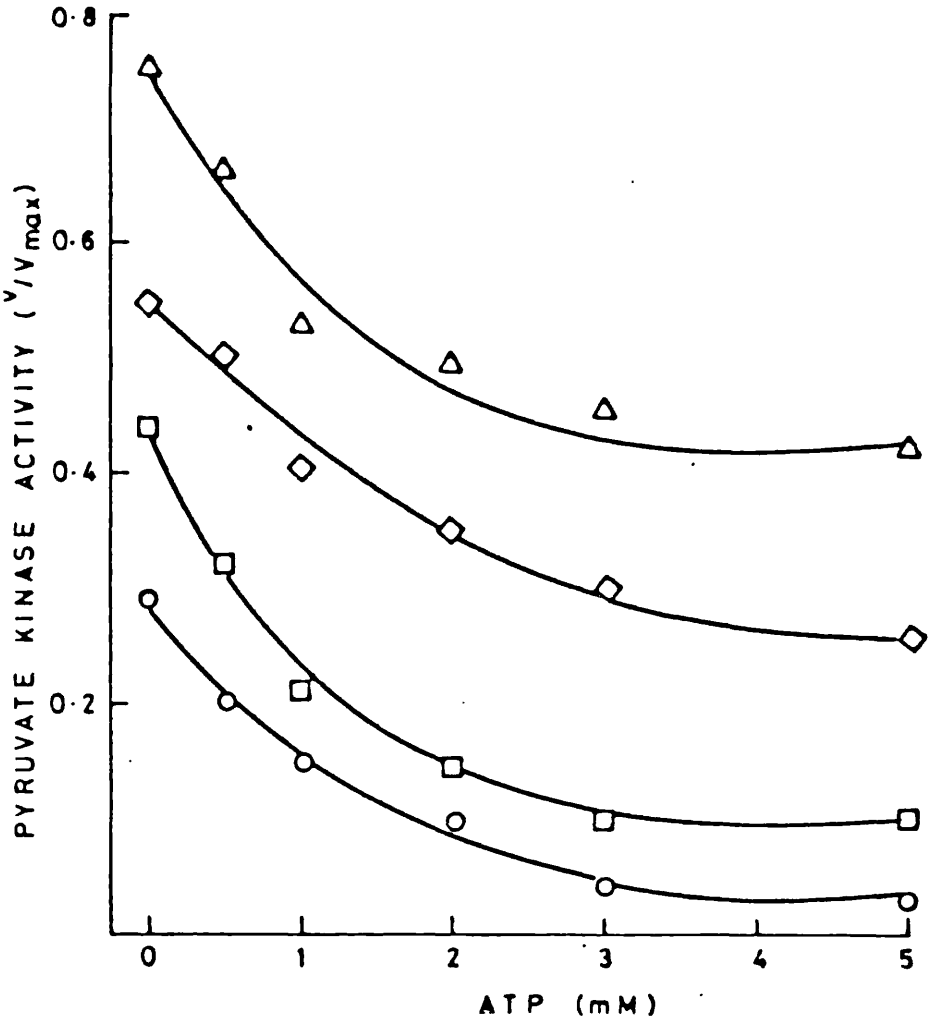


Figure 55. Effect of ATP concentrations on a partially purified pyruvate kinase preparation assayed with no added effector (○) and with 1 mM Fru-1-P (□), 1 μM $Fru_{16}BP$ (◇) or 50 μM $Fru_{16}BP$ (△).

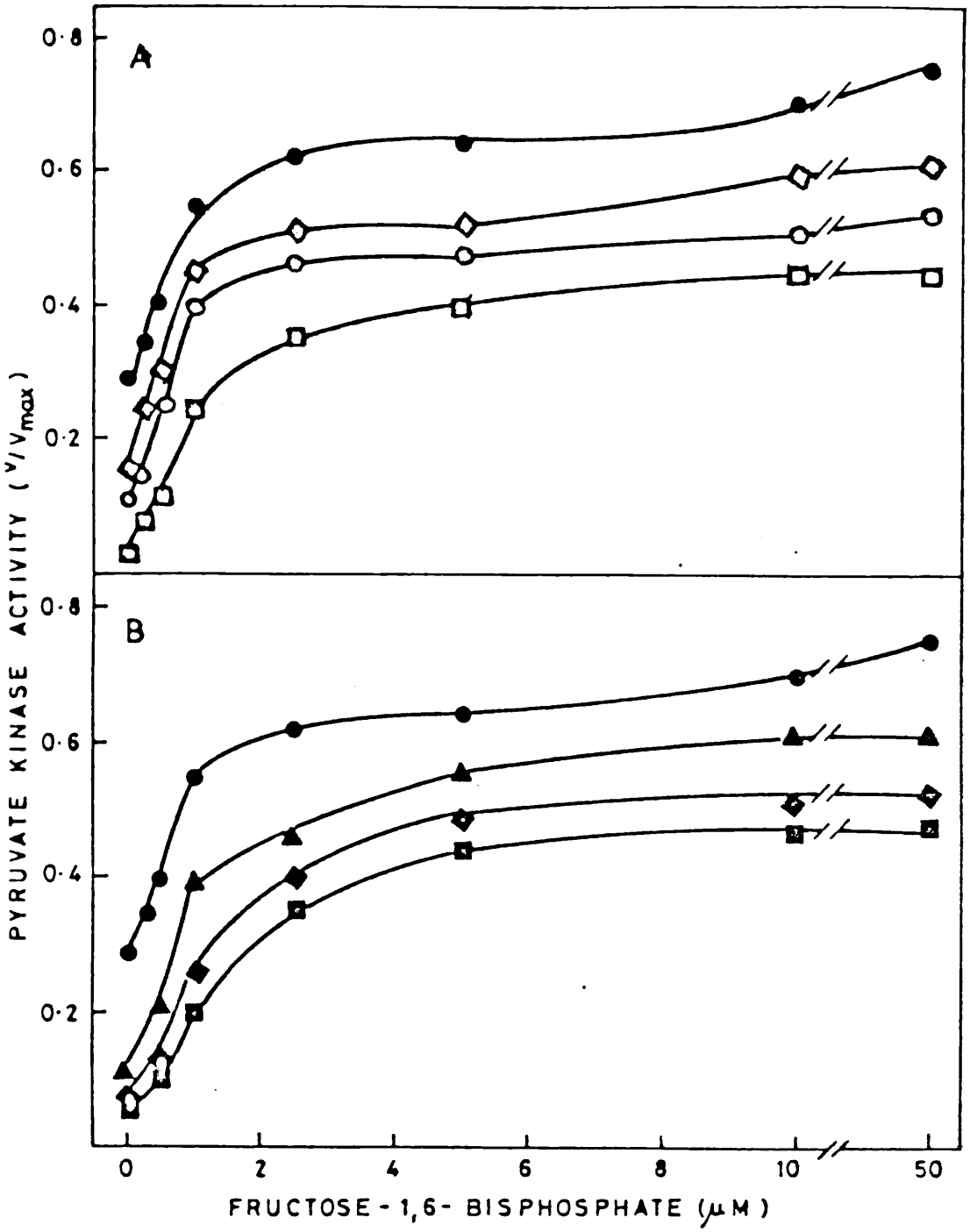


Figure 56. Effect of $Fru_{1,6}BP$ concentrations on a partially purified pyruvate kinase preparation assayed with no added effector (●) or in the presence of 1 mM allantoin (◇), 1 mM ATP (○), 3 mM ATP (□), 1 mM ATP and 1 mM alanine (▲), 3 mM ATP and 1 mM alanine (◆) or 1 mM ATP, 1 mM alanine and 1 mM allantoin (■).

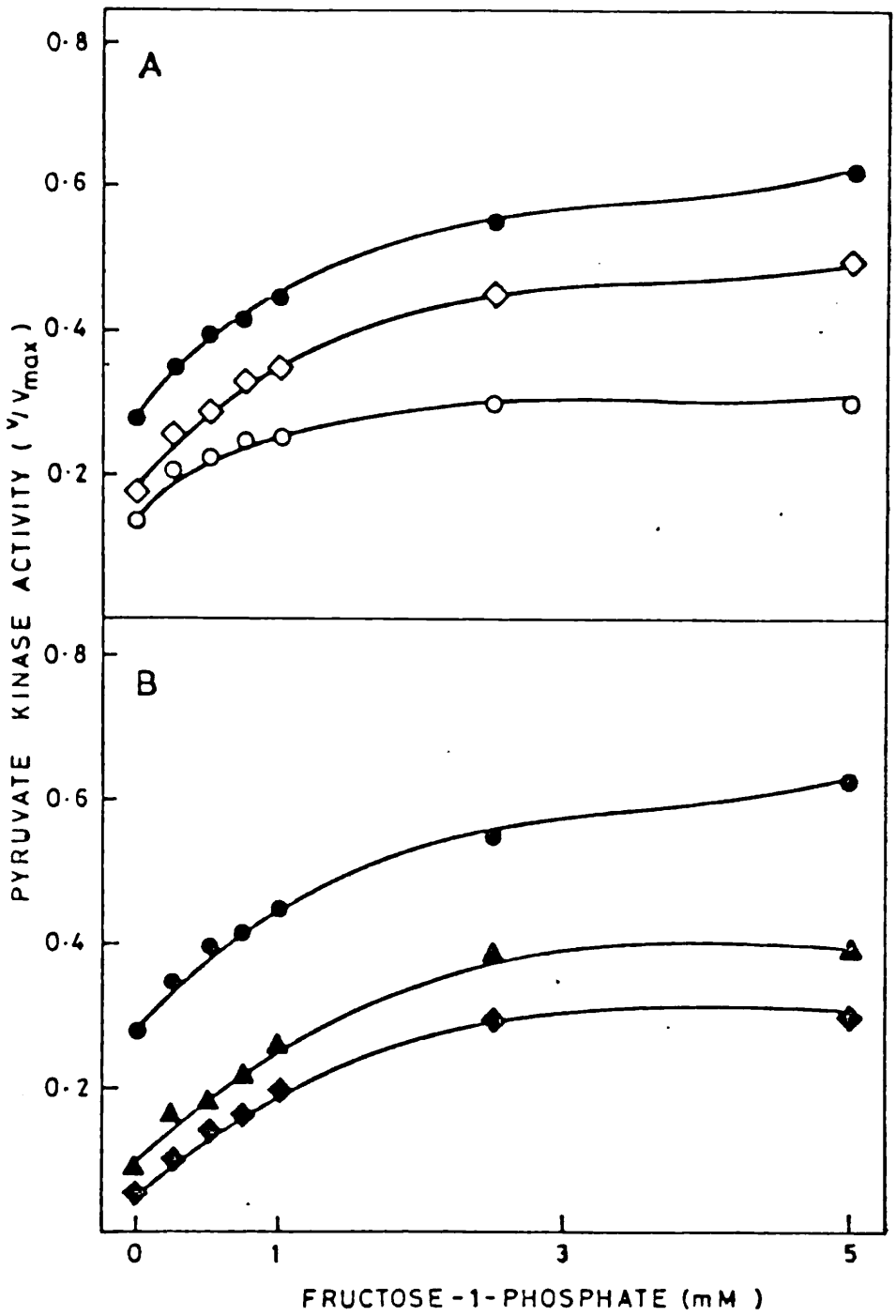


Figure 57. Effect of Fru-1-P concentrations on the activity of a partially purified pyruvate kinase assayed in the presence of 1 mM allantoin (◇), 1 mM ATP (○), 1 mM ATP and 1 mM alanine (▲), 1 mM ATP, 1 mM alanine and 1 mM allantoin (◆) or no added effector (●).

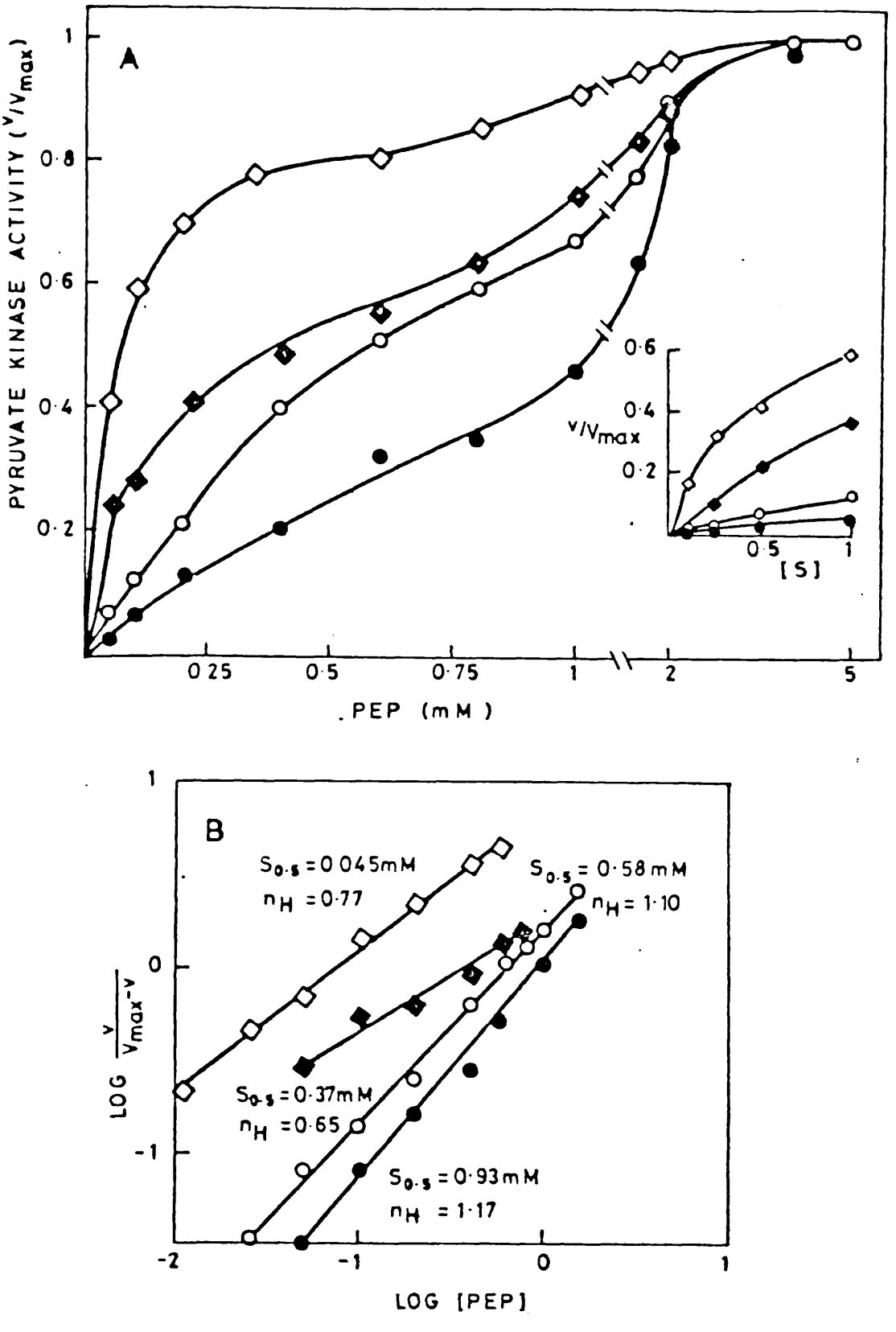


Figure 58. A, Effect of 50 µM $Fru_{16}BP$ (◇), 50 µM $Fru_{16}BP$ and 1 mM allantoin (◆), 1 mM allantoin (●) or no added effector (○) on the activity of a partially purified pyruvate kinase preparation assayed with various PEP concentrations. Insert shows data obtained with low PEP concentrations. B, Hill plots of data in figure 58A.

does not accumulate in isolated hepatocytes treated with fructose, it is clearly not involved in the inhibition of the enzyme observed in the present study. However, it is possible that allantoin accumulation observed in the presence of 10 mM fructose (figure 36) is sufficient to cause an inhibition of PK not by direct inhibition in the assay, since the dilution would be too great for the accumulated allantoin to exert an effect, but possibly by altering the conformation of the PK thus making it easier to remove the tightly bound $Fru_{16}BP$ which is carried over in the control extracts.

This was tested by incubating hepatocytes with 10 mM fructose for 20 min, removing the extracellular medium and then adding fresh KRB (plus Ca^{2+} and BSA), containing either allantoin or alanine or both effectors before freezing the samples as in the previous experiments. Table 19 shows that the inhibition of the fructose-treated PK occurs with the unwashed hepatocyte preparation and stimulation with the washed cell preparation. The stimulation was reversed by adding allantoin (1.0 mM) or alanine (1.0 mM) to the washed cells. A lower concentration of alanine (0.1 mM) partially reverses the stimulation. Concentrations of the metabolite comparable to those which occur in hepatocytes treated with fructose i.e. 1.0 mM allantoin and 0.1 mM alanine produced an inhibition comparable to that which occurs in unwashed cells. It is clear, therefore, that the 'inhibition' of PK which occurs is the result of the loss of bound $Fru_{16}BP$ from the PK during the freezing-thawing treatment, enzyme dilution and assay procedure. This is also apparent in the kinetic study of the enzyme from the fructose treated hepatocytes and control hepatocytes (figure 45 & 46). It is clear that the intracellular ATP does not interfere with the binding of $Fru_{16}BP$ to PK since the enzyme becomes activated after the washing step.

The physiological significance of the inhibitory effect of allantoin is somewhat doubtful since the normal plasma level of allantoin in rats is about 0.12 mM and that of urate is about 0.06 mM (Mdenpdd et al.,1968). However, on fructose infusion in vivo these values rise to 0.5 mM and 0.22 mM respectively (Mdenpdd et al.,1968).

Table 19. Pyruvate kinase activity in isolated hepatocytes treated with allantoin and/or alanine.

	Pyruvate Kinase Activity (v/V_{max})	
	No Substrate	Fructose (10 mM)
Unwashed Hepatocytes	0.44	0.36
Washed Hepatocytes	0.47	0.60
Washed Hepatocytes + 1.0 mM Allantoin	0.39	0.38
Washed Hepatocytes + 1.0 mM Alanine	0.38	0.38
Washed Hepatocytes + 0.1 mM Alanine	0.46	0.45
Washed Hepatocytes + 1.0 mM Allantoin + 1.0 mM Alanine	0.36	0.33
Washed Hepatocytes + 1.0 mM Allantoin + 0.1 mM Alanine	0.39	0.41

Isolated hepatocytes were incubated for 20 min in the presence and absence of 10 mM fructose. After the incubation period, an aliquot was frozen in liquid N_2 to stop the reaction, at the same time samples were removed and washed as described in methods (section IV.B.3). After the washing step, the cells were resuspended in KRB ($+Ca^{2+}$ and 1.5g% BSA) containing allantoin and/or alanine and then frozen in liquid N_2 for enzyme assay (section IV.B.3).

This suggests that under these conditions the effect of allantoin and urate on PK at least partially compensates for the fall in ATP levels which occurs following a fructose load.

The interaction of physiological concentrations of alanine, ATP-Mg and allantoin on partially purified PK activity was studied (figure 56B & 57B). The greatest inhibition was observed with 3 mM ATP-Mg at all $Fru_{16}BP$ concentrations examined (cf Figure 56, 57A & B). The inhibition observed with 1 mM ATP was considerably less than that observed with the higher concentration of ATP, but the addition of alanine (1 mM) to ATP-Mg (1 mM) appeared to have little further inhibitory effect over that observed with ATP-Mg (1 mM) on its own, whereas a combination of ATP, allantoin and alanine (concentration of all effectors 1 mM), produced an inhibition similar to that found with 3 mM ATP. Thus, under these conditions,found in the hepatocyte after a fructose load, i.e. low ATP, high alanine and allantoin, it is possible that the PK is in an inhibited form. However, since the latter inhibitors leave the cell freely while the ATP remains in the cytoplasm, it is evident that the inhibitory effect of the ATP remains the most important factor which determines PK activity.

E. GENERAL DISCUSSION AND CONCLUSIONS

It is clear from the data presented in section A - D that hepatic PK is subject to a number of diverse control mechanisms, involving changes in the concentrations of the allosteric effectors in addition to the control by phosphorylation - dephosphorylation observed by others (Engström, 1978). Fructose administration is well known to cause lactic acidosis in humans (Bergström et al., 1968), and it has also been shown that high rates of lactate output occur when perfused liver (Exton & Park, 1967) and isolated liver cells (Seglen, 1974) are subjected to high fructose concentrations. However, it is unlikely that liver cells are exposed to such high concentrations of the ketose in vivo since in animals ingesting fructose the concentration of the ketose in the hepatic portal vein has been shown not to exceed 2.5 mM (Topping & Mayes, 1971). In the present studies when the effects of fructose on hepatocyte metabolism were examined both at high fructose concentrations and physiological concentrations of the ketose considerable discrepancies were observed. Such differential effects of fructose have also been observed on other biochemical parameters. For example, fructose concentrations (up to 5 mM) stimulate glycogen deposition by isolated hepatocytes whereas higher concentrations cause increased glycogen mobilization (Wood et al., 1981). Lipogenesis from ^3H_2O is stimulated by low concentrations of fructose but inhibited at high concentrations (Clark D.G. et al., 1974). Hepatic pyruvate dehydrogenase activity is not significantly affected by low fructose concentrations whereas higher concentration of the ketose increases the enzyme activity (Topping & Mayes, 1977). Gluconeogenesis from fructose is stimulated by glucagon at low substrate concentrations but the hormone has no effect when the ketose concentration is increased (see section I.D.3).

The effect of fructose on PK activity measured in vitro in the present study appeared to fit in with the pattern of the differential effects of fructose. However, a closer examination revealed that the effect observed did not reflect the in vivo changes in enzyme activity,

but rather was an artefact of the hepatocyte extraction and PK assay procedure. This procedure has been employed successfully to show the effects of insulin and glucagon (Felíu et al., 1976) and dihydroxyacetone on PK activity in isolated hepatocytes (Claus et al., 1979).

From the present study, it is clear that a number of factors could alter the v/V_{max} value observed with this assay technique. The results indicate that the value observed is not a reflection of the phosphorylation state of the enzyme but that it is affected by the carry-over of $Fru_{16}BP$ and/or Fru-1-P associated with PK during the extraction and assay procedure. This carry-over of $Fru_{16}BP$ and/or Fru-1-P is dependent on a number of factors. Firstly, the level of the hexose phosphate in the hepatocytes after incubation is clearly important since dihydroxyacetone, fructose and D-glyceraldehyde all increase the level of one or both these intermediates and PK activity. Secondly, the accumulation of the inhibitors, alanine and allantoin, causes the dissociation of the stimulator - enzyme complex resulting in a lowering of the v/V_{max}. Thus the dissociation probably occurs during the freeze-thaw procedure for the extraction of the PK, since addition of alanine or allantoin to the washed hepatocytes incubated with fructose also results in a lowering of the enzyme activity. A kinetic analysis shows that the concentrations of alanine and allantoin in the assay medium are unlikely to substantially inhibit PK directly. Further evidence for the dissociation of the $Fru_{16}BP$ - PK complex comes from comparing the kinetics of the $(NH_4)_2SO_4$ treated enzyme with the enzyme from the washed and unwashed fructose treated hepatocytes. The effect of fructose in the unwashed cells is to decrease the $S_{0.5}$ for PEP to a similar value as that observed in the $(NH_4)_2SO_4$ treated enzyme.

Therefore, the use of a spectrophotometric assay for examining the effects of substrates on PK activity is associated with a number of problems such as the dilution of the enzyme preparations. Thus, the activity of the enzyme found by this method does not necessarily

reflect the activity of the enzyme in the cytosol of the hepatocyte. Perhaps a better indicator of the latter parameter is the measurement of lactate and pyruvate output which is a measure of the net glycolytic flux. This assay, however, ignores the potential contribution by pyruvate to amino acid synthesis, gluconeogenesis and mitochondrial pyruvate metabolism. Thus some care must be taken in the interpretation of the results.

The effect of fructose at all concentrations is to increase the lactate and pyruvate output by isolated hepatocytes suggesting that the apparent inhibition of PK by high fructose does not occur in the cell. The large accumulation of Fru-1-P even at low fructose concentrations indicates that Ketohexokinase is operating at its maximum velocity under these conditions. If it is assumed that the aldolytic cleavage of Fru-1-P to DHAP and D-glyceraldehyde is not affected by changes in fructose concentration, then the output of lactate and pyruvate must reflect the state of activity of the only regulatory enzyme (i.e. PK) in the glycolysis of fructose. Thus the increased glycolytic flux in response to increasing concentrations of fructose must be related to the intracellular accumulation of Fru-1-P and $Fru_{16}BP$, both allosteric activators of PK. That PK is rate limiting under these conditions is indicated by the accumulation of PEP at low fructose concentrations. At high concentrations of the ketose, a further factor must be taken into account, that is the depletion of ATP which results in a further increase in the glycolytic flux with a concomitant decrease in PEP levels.

The relative importance of the ATP-depleting effect and the increase in $Fru_{16}BP$ is apparent when the glycolytic flux with dihydroxyacetone is examined. Here again an increase of lactate output, reflected an increased concentration of $Fru_{16}BP$ in the cell, but in this case PK appeared to be rate limiting since PEP accumulated at all concentrations of dihydroxyacetone examined. This substrate does not substantially deplete ATP which could account for the lower rate of glycolysis from the triose. The physiological significance of

this observation is that,normally changes in PK would probably be mediated via changes in $Fru_{16}BP$, but under conditions where ATP is depleted, then glycolytic flux is enhanced.

Thus it may be postulated that glycerol could have a similar effect to fructose since ATP is depleted by this substrate. However, an examination of the glycolytic flux in this case indicates that there was an inhibition of endogenous glycolysis from glycogen in hepatocytes from fed rats. The explanation for this phenomenon is probably related to the redox changes induced by the metabolism of glycerol. Therefore, in the presence of glycerol, the deficiency of NAD^+ probably limits glycolysis. This was borne out by the inhibition of lactate and pyruvate output by xylitol which has a similar effect on the $(NADH)/(NAD^+)$ ratio in the cell.

The effects of sorbitol, another reduced substrate, were more complex. Initially, the sugar alcohol caused little or no effect on the glycolytic flux, whereas at a later time there was a stimulation of flux. Sorbitol has a number of different effects which could possibly account for this apparent contradiction.The metabolism of sorbitol is via fructose (see figure 3), therefore it shares with fructose a number of effects (for example, increases in $Fru_{16}BP$ and Fru-1-P levels and , probably depletion of ATP).Thus the stimulatory effect of sorbitol on glycolytic flux is probably due to these factors which overcome the effect of the NAD^+ depletion caused by this substrate. The time lag in the stimulatory effect of sorbitol probably reflects the slower rate of Fru-1-P accumulation. The lack of inhibition of glycolysis by sorbitol may be the result of reoxidation of NADH by pyruvate accumulating as the result of the stimulation of PK. Thus the increased metabolism of the triose phosphates in this case is the result of a 'pull' mechanism which does not involve any net change in NAD^+ levels. Thus the increased $Fru_{16}BP$ and Fru-1-P levels caused by sorbitol, but not by glycerol, stimulate glycolysis irrespective of the depletion of ATP by the latter substrate.

It is well documented that the effect of fasting is to increase phosphorylation of L-type PK by a cAMP-dependent protein kinase which results in the inactivation of the enzyme (see section II.C.6). This was confirmed in the present study and the effect of this inactivation on the glycolytic rate of the hepatocytes in the presence of fructose and dihydroxyacetone examined. A high glycolytic rate was observed in hepatocytes incubated with fructose and this was comparable to that by cells from fed animals. Dihydroxyacetone stimulated lactate and pyruvate output but to a much lesser extent than fructose despite the fact that $Fru_{16}BP$ concentrations are raised to a much greater extent by the triose.

A comparison of the PK activity assayed *in vitro* shows that dihydroxyacetone stimulates PK at least as efficiently as fructose. However, it is clear from the low glycolytic rate observed, in the presence of the triose that PK must be inhibited in the cytoplasm of the isolated hepatocyte. The explanation for this discrepancy must be related to the phosphorylated state of the enzyme and the ATP concentration in the cytoplasm. The enzyme is mainly phosphorylated in the fasted state and thus the effect of $Fru_{16}BP$ is not so marked and that of ATP is enhanced, hence the low rate of glycolysis from dihydroxyacetone. Fructose however, lowers ATP levels and allows glycolysis to proceed. Thus a similar, but more marked, effect of fructose compared to dihydroxyacetone is observed with hepatocytes from fasted rats compared to cells from fed animals. Again the discrepancy between the activation of PK observed *in vitro* and the low rate of glycolysis from dihydroxyacetone emphasises the problems of extrapolating the results of enzyme assays on crude extracts to explain the changes which occur *in vivo*.

Another approach to the problem of determining enzyme activity *in vivo* is to examine the levels of substrates and effectors under various conditions and to attempt to examine the regulation of the purified enzyme under conditions which are as close to physiological

situation as possible. Thus at the levels of ATP, alanine and PEP found under physiological conditions; $Fru_{16}BP$ is a far better stimulator of PK activity than Fru-1-P if one compares the effects of the levels of the hexose phosphates which accumulate in the hepatocytes. At lower ATP levels, Fru-1-P and $Fru_{16}BP$ stimulate the enzyme to a greater extent, suggesting a possible synergistic effect of ATP depletion and $Fru_{16}BP$ and/or Fru-1-P accumulation resulting in an increased glycolytic rate.

One other consequence of increased glycolysis and decreased ATP levels is the accumulation of alanine as a result of increased synthesis of the amino acid by the transamination of glutamate and pyruvate. This transamination is stimulated by NH_4^+ formed during the catabolism of AMP. The increased level of alanine which occurs in the hepatocytes might be expected to impose an inhibitory effect on glycolysis via its effect on PK. This would be expected to counteract the decreased inhibitory effect due to the depletion of ATP. However, there is little evidence in the present study that the increased alanine content of the hepatocytes substantially counteracts the activating effect of ATP depletion on PK.

The evidence described above suggests that the rate of glycolysis from the triose phosphates is normally limited by L-type PK activity in the liver cell from both fed and fasted animals. This is despite a concentration of $Fru_{16}BP$ which would normally be expected to fully stimulate the enzyme. The current belief is that much of the $Fru_{16}BP$ in the cell is protein-bound and thus not available to stimulate PK. However, the possibility remains that there is as yet another undiscovered regulator of PK which further inhibits the enzyme. The inhibition by allantoin and urate may provide part of the explanation, but the concentration of these catabolic products *in vivo* would not impose a physiologically important inhibition on PK under normal conditions. Another possibility is that the levels of $Fru_{16}BP$ are over-estimated by the enzymatic assay mechanisms normally used.

From a physiological viewpoint it is important that the regulation of PK by fructose is not considered in isolation. For example, fructose is generally regarded as a lipogenic precursor presumably because of the ease of its conversion to lactate (also regarded as a good precursor for fatty acids) (Hopkirk & Bloxham, 1979). Thus the regulation of PK may be important in determining the flux of carbon from carbohydrate to fatty acids. This allied to the observation that pyruvate dehydrogenase is activated at high fructose concentrations (Topping & Mayes, 1977) should result in an increased provision of acetyl CoA for lipogenesis. However, lipogenesis is inhibited at high fructose concentration (Clark, D.G. et al., 1979) suggesting that there is an inhibition of the lipogenesis at a point subsequent to the provision of intramitochondrial acetyl CoA. However, fructose does stimulate lipogenesis from [^{14}C] acetate and [^{14}C] lactate at physiological concentrations of the substrate (Davies & Daneshmand, unpublished results).

Another aspect of the present study which may be important is that the depletion of ATP caused by fructose may cause activation of PK. In this context it has been reported that the hypoglycemia caused by administrating fructose in patients with hereditary fructose intolerance is caused by inhibition of gluconeogenesis in addition to inhibition of phosphorylase 'a' by Fru-1-P (Van den Berghe, 1978). This decreased gluconeogenesis may be the result of a stimulation of glycolysis via PK and, possibly, by the relieving of ATP inhibition of PFK 1; which is also inhibited at physiological concentrations of ATP (Van Shaftingen et al., 1981b).

In conclusion it is evident from the work presented in this thesis and from a considerable amount of the published data that, the effects of fructose on hepatic metabolism are many and varied. It is clear that the effects of the ketose are concentration dependent and thus a clear distinction must be made between the effects of physiological levels of fructose, such as those found in animals given fructose in the diet (Topping & Mayes, 1971), and the toxic effects

of the high levels of fructose which are sometimes administered intraveneously in certain clinical conditions (Van den Berghe, 1978).

It is apparent that low concentrations of the sugar stimulate glycolysis and flux through PK. This is probably related to the elevation of Fru-1-P and $Fru_{16}BP$. The depletion of ATP by higher concentrations of fructose causes a further increase of the glycolytic rate. The ATP depletion is followed by a number of changes in the levels of metabolites, e.g. alanine and allantoin, which would also influence PK activity.

This study also shows that the *in vitro* assay of an enzyme in a crude extract does not necessarily represent the activity of the enzyme *in vivo* and that artefacts can arise as the result of effects which are apparently unrelated. However, it should be emphasised that, only changes which involve a covalent modification are seen when purification of the enzyme is performed and thus there is a valid reason for using crude extracts to examine the effects of various substrates and hormones on enzyme activity. Therefore, a careful and critical analysis of the data from such studies is important.

MATERIALS AND METHODS

IV. MATERIALS AND METHODS

A. MATERIALS

1. Animals and diets

All rats used for this project were mature male rats (200 - 300 g) of the Wistar strain bred in this laboratory. Unless otherwise stated all animals were fed ad libitum on a normal laboratory chow diet (PRD). The animals were maintained on a 12 h light and 12 h dark cycle and all experiments were commenced 2 h after the start of the light cycle. Fasted animals were deprived of food 24 h before the start of the experiment.

2. Reagents

All enzymes and substrates were obtained from Sigma Chemical Company or Boehringer Mannheim. All phosphorylated substrates used were in the form of their sodium salts. Inorganic chemicals and solvents were of the Analar grade from BDH. All solutions and buffers were prepared using glass distilled, deionized water.

B. METHODS

1. Isolation of hepatocytes

Isolated hepatocytes were prepared from adult male rats by the method described by Berry & Friend (1969); with some modifications (Krebs et al., 1974; Wagle & Ingebertson, 1975). Mature rats were anaesthetized with nembutal (60 mg of pentabarbitone/ml; Abbot, UK, 0.1 ml/100 g rat) and placed on a dissecting tray. The peritoneal cavity was opened by making incisions starting at the midline of the lower abdomen and proceeding upwards until the diaphram was reached but not penetrated. The muscle and skin was then lifted to expose the peritoneal cavity where the portal vein and the inferior vena cava

can be located. Two ligatures were loosely placed around the portal vein and a third ligature was placed loosely around the inferior vena cava just anterior to the branch point of the right renal vena cava.

A sterile luer cannula (Braunula sterile luer; Armour Pharmaceutical Co.Ltd.) was used to cannulate the portal vein. The two ligatures around the portal vein were then tied to secure the cannula. Krebs-Henseleit bicarbonate medium (KRB) (Krebs & Henseleit, 1932) minus Ca^{2+} (preincubated at 37°C, gassed with 95% O_2 and 5% CO_2 and already circulating in the perfusion cabinet) was connected to the cannula to allow a continuous flow into the liver. Immediately following this the inferior vena cava was severed to prevent the liver from swelling. When the blood had been cleared from the liver, the rib cage was cut exposing the thoracic cavity. A fourth ligature was placed loosely around the inferior vena cava, and then a cannula was placed via the right atrium of the heart into the inferior vena cava. The ligature around the cannula was then tied. To ensure that the KRB ($-Ca^{2+}$) was flowing from the portal vein through the liver to the inferior vena cava, the ligature adjacent to the kidney was tied. The liver was perfused with KRB ($-Ca^{2+}$) at a rate of approximaterly 25 ml/min until the perfusate was free of blood cells.

The liver was then perfused with 30 mg crude collagenase in 100 ml KRB ($+Ca^{2+}$). The buffer was maintained at 37°C and was gassed continuously with O_2 + CO_2 (95:5 v/v). The perfusion was continued for about 15-20 min and any leaky perfusion fluid appearing in the abdominal cavity was collected and returned to the reservoir. The rate of digestion of the tissue was reflected by the degree of leakage of the medium and when this was rapid, the liver was removed and placed in a beaker containing KRB ($-Ca^{2+}$).

The liver was then finely cut with a scissors and gently gassed with O_2 + CO_2 (95:5 v/v). A plastic funnel with a nylon mesh (Nybolt No. 10; 132 micron, J.Staniar & Co., Manchester) placed over the top

of centrifuge tubes (MSE 50 ml) was used to filter the cells. The samples were centrifuged at 50 g for 60 s in a bench centrifuge and the cells resuspended in KRB ($+Ca^{2+}$), containing 1.5 g% of essentially fatty acid free-bovine serum albumin (BSA) (fraction V, Sigma Chemical Co.).

The cell suspension was examined microscopically for trypan blue exclusion; 85-95% of hepatocytes isolated in this way excluded trypan blue indicating that under these conditions a high proportion of the cells were viable.

2. Incubation procedure

All flasks used for incubating hepatocytes were siliconized to give them water repelling properties. This was done by rinsing the flasks thoroughly with dimethyldichlorosilane solution (about 2% v/v in 1, 1, 1-trichloroethane) and leaving them to dry in an oven overnight. Cells suspended in KRB($+Ca^{2+}$) with 1.5g% BSA (8-10 ml) were placed in the siliconized conical (25 ml) flasks and preincubated in a shaking water bath (100 strokes/min) at 37°C with continuous gassing with O_2 + CO_2 (95:5 v/v). After a period of 10 min the cells were used for various metabolic studies.

Aliquots of the cell suspension were then transferred to a 25 ml conical flask containing an equal volume of substrate dissolved in KRB ($+Ca^{2+}$) containing 1.5g% BSA. The final cell concentration was between 6 and 8 x 10^6 cells/ml (48-64 mg wet wt/ml). The cells were incubated in a shaking water bath at 37°C (gassing with O_2 + CO_2 (95:5 v/v). After the desired time interval, aliquots of the incubation mixture were removed and stored appropriately before being used for metabolite and enzyme assays. Duplicate incubations were carried out for every experiment. Unless otherwise stated all experiments were done on at least three different hepatocyte preparations.

3. Sampling procedure for pyruvate kinase assay

Following incubation, an aliquot of cell suspension was taken and the reaction stopped by freezing in liquid N_2, and these samples

were used as a source of 'unwashed' cells. The washing of the hepatocytes was done by the following procedure: the cell extracts were taken at the appropriate time and rapidly sedimented through ice cold KRB + Ca^{2+} containing BSA (1.5g%). After spinning the suspension for 60 s (at 50g), the supernatant was discarded and the pellet resuspended in KRB + Ca^{2+} containing 1.5g% BSA and then frozen in liquid N_2 and stored at -70°C before use for PK assay.

4. Partial purification of pyruvate kinase from isolated hepatocytes

One vol. of the cell suspension was frozen in liquid N_2 and thawed by adding 4 volumes of ice cold suspending medium (containing 0.1 M KF, 15 mM ethylene glycol bis(β-aminoethyl ether) -N, N, N', N'-tetraacetic acid (EGTA) and 50 mM glycylglycine, pH 7.4) PK was precipitated by adding one vol. of extract to one vol. of saturated ammonium sulphate (Carbonell et al., 1973; Felíu et al., 1977). The mixture was kept for 30 min in the cold and the precipitate was collected by centrifugation at 17,000 g for 30 min. The pellet was dissolved in one vol. of suspending medium and aliquots stored at -70°C. When required the samples were thawed by the addition of five vol. of suspending medium and PK was assayed.

5. Measurement of pyruvate kinase activity

PK was measured using a coupled reaction system (Felíu et al., 1976). The incubation mixture (total volume 1 ml) contained 50 mM glycyglycine, pH 7.4, 0.1 M KCl, 10 mM $MgCl_2$, 1.3 mM ADP, 0.15 mM NADH and 2 units of LDH, 50 µl of homogenate was added to the mixture and preincubated at 25°C for 10 min. The reaction was started by the addition of PEP and the PK activity was measured by following the oxidation of NADH at 340 nm. In all cases the rate of reaction was followed for 5 min. The enzyme activity was calculated using 6.22 x 10^3 $L.mole^{-1}cm^{-1}$ as the molar extinction coefficient of NADH at 340 nm.

6. Metabolite assays

For the measurement of metabolite levels in hepatocytes, ice cold perchloric acid (0.05 vol.of 4.2M $HClO_4$)was used to terminate

the reaction at the appropriate time intervals. The resulting extract was blended on a Vortex mixer and the protein precipitate removed by centrifugation at 3,000g for 10 min. The supernatant fraction was neutralized with K_2CO_3 and any precipitate of $KClO_4$ formed was removed by centrifugation at 3,000g for 10 min. The metabolite levels in the neutralized supernatant were assayed as described below.

a) Adenosine triphosphate

ATP levels were measured using a U.V. method. The principle of the test was the conversion of glycerate-3-phosphate to glycerol-1-phosphate in a sequence of reactions catalysed by glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase with the utilisation of ATP and NADH oxidation. The incubation mixture (total vol. 1.2 ml) contained 0.41 M triethanolamine, pH 7.6, 3.3 mM $MgSO_4$, 5 mM glycerate-3-phosphate, 0.21 mM NADH and the deproteinized supernatant. The decrease in absorbance at 340 nm was followed after the addition of an enzyme mixture (10 µl) containing glyceraldehyde -3- phosphate dehydrogenase (350 U/ml; phosphoglycerate kinase 450 U/ml; triose phosphate isomerase 800 U/ml; glycerol-3-phosphate dehydrogenase 60 U/ml.

b) Alanine

The method of Williamson, (1974) was used to measure L-alanine levels. Hydrazine-tris buffer (prepared fresh daily) containing 12.9 mM tris, 0.2 mM hydrazine, 0.45 mM EDTA, pH 10.0, was placed in a cuvette containing 0.8 mM NAD^+ and the deproteinized sample (final vol 1.55 ml). The reduction of NAD^+ was recorded spectrophotometrically at 340 nm after addition of 5 µl dialysed L-alanine dehydrogenase. This enzyme preparation was dialysed against 20 mM phosphate, pH 7.4 to remove NH_4^+ ions. The enzyme was diluted with the same buffer to give an activity of 15 U/ml before use.

c) Allantoin

The allantoin in the perchloric acid extracts was first subjected to alkaline-acid hydrolysis. 1 ml of sample was mixed with water (2 ml)

and 0.5 M NaOH (1 ml) and heated for 8 min at 100°C. After cooling, 0.65 M HCl (1 ml) was added and the mixture was heated for 4 min at 100°C. The mixture was cooled once more and 0.4 M phosphate buffer, pH 7.0 (1 ml) and 1 ml phenylhydrazine (3.3 mg /ml) were added and the mixture allowed to stand at room temperature for at least 5 min. After this hydrolysis procedure the standard method for assaying glyoxylate derivatives was used (Vogels & Van der Drift, 1970).

The tubes containing the hydrolysed samples were cooled to 0°C in an ice-water bath and concentrated HCl (5 ml) precooled to 0°C as well as 1 ml of ferricyanide solution (16.5 mg/ml) were added. The tubes were then kept at room temperature for 15 min and the absorbance was measured spectrophotometrically at 535 nm. A standard curve using commercial allantoin was prepared.

d) Dihydroxyacetone phosphate and fructose-1, 6-bisphosphate

These two metabolites can be measured in the same reaction mixture using a method described by Michal & Beutler, (1974). The reaction mixture (total vol. 1.5 ml) containing 200 mM triethanolamine, pH 7.6, 17 µM NADH and 0.75 ml of the deproteinized sample were placed in a cuvette. The decrease in absorbance at 340 nm was followed using a recording spectrophotometer after the addition of various enzymes (5 µl of each enzyme). Glycerol-3-phosphate dehydrogenase (1 mg protein/ml) was added initially and the decrease in absorbance was quantitatively related to the amount of DHAP present in the extract. A further decrease in absorbance was observed when triose phosphate isomerase (50 µg protein/ml) was added and this was related to the glyceraldehyde-3-phosphate levels in the extract. Muscle aldolase (1.5 mg protein/ml) caused a further decrease in the absorbance which is equivalent to the amount of $Fru_{1,6}BP$ in the sample. Contamination of the enzymes with LDH and with each other should be minimal to achieve accurate results with this method. Enzyme preparations from Boehringer Mannheim were found to be sufficiently pure.

e) Fructose-1-phosphate

The method described by Eggleston (1974), was used to determine Fru-1-P levels in the neutralized supernatants. In this method liver aldolase is used to convert Fru-1-P to DHAP and D-glyceraldehyde. The DHAP produced can be reduced to G3P by glycerol-3-phosphate dehydrogenase coupled with the oxidation of NADH to NAD^+.

Liver aldolase was prepared from 3-5 rats killed by cervical fracture. After insertion of a hypodermic syringe needle into the hepatic portal vein, the inferior vena cava was severed and the liver was slowly perfused with 0.15 M KCl at 2°C to wash out the blood. The combined livers were homogenized with 4 volumes of KCl at 2°C, and centrifuged at 30,000g for 20 min. $(NH_4)_2SO_4$ (27.7g/100 ml of supernatant) was added to the supernatant with stirring and the final pH was adjusted to 7.8 with 2 M NaOH. This mixture was left in an ice-bath with stirring for 60-90 min then it was centrifuged at 30,000 g for 20 min. A further portion of $(NH_4)_2SO_4$ (6.5g/100 ml of supernatant) was added with stirring. After 90 min. in an ice-bath the mixture was centrifuged at 30,000 g for 20 min and the resulting precipitate dissolved in a small volume of water (0.3 ml for every g of original liver). This solution was dialysed against 200 volumes of distilled water at 2°C, changing the water every h, for 4 hours to remove the $(NH_4)_2SO_4$ as completely as possible. The cloudy protein solution was cleared by centrifugation at 30,000 g for 20 min and 0.1 M EDTA, pH 7.4 added to give a final concentration of 2.5 mM EDTA. The mixture was allowed to stand at room temperature for 1 h to completely inactivate any polyol dehydrogenase present.

To assay Fru-1-P, the decrease in absorbance was measured at 340 nm and this is proportional to the amount of Fru-1-P present. The deproteinized cell extracts contain $Fru_{1,6}BP$ and triose phosphates which can be removed using muscle aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase. The assay mixture

(total volume 1.4 ml) contained 50 mM Tris, pH 7.4, 70 µM NADH, 50 µl of the enzyme mixture (i.e. LDH 15 units; aldolase 0.42 units; triose phosphate isomerase 4.2 units; glycerol-3-phosphate dehydrogenase 0.3 units) and the sample (0.575 ml). The enzyme mixture was first dialysed against 200 volumes of water at 2°C to completely remove NH_4^+ ions before use. Fru-1-P was measured from the change in absorbance observed after adding 0.1 ml of liver aldolase to the reaction mixture. There was a slow rate of NADH oxidation observed even in the reaction mixture with no sample. This was overcome by subtracting the change in absorbance observed in blank cuvettes from the sample values.

f) Glycerol-3-phosphate

The formation of NADH as measured by the increase in optical density at 340nm was used as a measure of G3P present (Michal & Lang, 1974). The reaction mixture (total volume 1.05 ml) contained hydrazine-glycine buffer pH 9.5 (i.e. 0.189 M hydrazine, 0.47 M glycine and 2.7 mM EDTA); 2.31 mM NAD^+ and the deproteinized sample (0.5 ml). The reaction was started by adding 10 µl of glycerol-3-phosphate dehydrogenase (10 mg/ml) and the formation of NADH was followed using a recording spectrophotometer.

g) Glucose

Glucose levels were determined using a colorimetric method involving glucose oxidase and peroxidase (Bergmeyer & Bernt, 1974). A sample of deproteinized cell extract was placed in the reaction tube and the volume adjusted to 1 ml using distilled water. To each sample, 2 ml of glucose oxidase reagent (i.e. 75 mg glucose oxidase, 7.5 mg peroxidase and 25 mg O-dianisidine HCl in 250 ml Tris-glycerol buffer pH 7.0) was added. After shaking thoroughly, the tubes were incubated at 37°C for 1 hour in a water bath. At the end of this period, 9 M H_2SO_4 (2 ml) was added to stop the reaction.A standard curve was simultaneously prepared using 0-50 µg glucose. The optical density was read at 540 nm.

h) Lactate

This metabolite was measured using a method described by Gutmann & Wahlefeld,(1974). The enzyme used is LDH which catalyses the conversion of lactate to pyruvate. The reaction product (i.e. pyruvate) is removed by trapping with hydrazine. The assay mixture (total volume 1.0 ml) contained hydrazine-glycine buffer pH 9.5 (i.e.0.178 M hydrazine,0.45 M glycine, 2.55 mM EDTA); 2.47 mM NAD^+ and the neutralized supernatant (0.5 ml). The reaction was started by adding 10 µl of . Lactate dehydrogenase (10 mg protein/ml) and the oxidation of NAD^+ followed at 340 nm. Lactate levels in the cell extracts are proportional to the NADH produced. At the end of the assay a standard amount of lactate was added to check the assay method.

i) Phosphoenolpyruvate and pyruvate

PEP and pyruvate levels were determined by the method described by Czok & Lamprecht,(1974). In this assay system, pyruvate and PEP can be assayed sequentially, in the same cuvette. The reaction mixture (total volume 1.10 ml) contained 45 mM triethanolamine, pH 7.6; 0.7 mM $MgSO_4$; 67 mM KCl; 0.2 mM ADP; 89 µM NADH and the sample (0.75 ml). The decrease in absorbance was measured spectrophotometrically after the addition of the enzymes. Initially 10 µl of LDH (130 units) was added and the decrease in absorbance was quantitatively related to the pyruvate present in the extract. A further decrease in absorbance was observed when 10 µl of PK (60 units) was added and this was related to the PEP levels present.

j) Uric Acid

Uric acid was assayed using both the colorimetric and U.V. method (Scheibe et al., 1974). The test principle for the U.V.method is the conversion of uric acid (which absorbs at 293 nm) to allantoin and H_2O_2 which do not absorb at this wavelength. Borate buffer (0.1 M;

pH 9.5) was used to dilute the sample. Then 3.5 ml of the diluted sample was placed in a quartz cuvette containing 45 mU uricase (20µl) and the change in absorbance at 293 nm measured against a blank. The change in optical density is due to the degradation of uric acid.

The colorimetric method was carried out using uricase and catalase. The reaction product of uricase (i.e. H_2O_2) oxidizes methanol to formaldehyde. The latter reacts with acetylacetone and ammonia to give a yellow product 3,5-diacetyl-1, 4-dihydrolutridine, which has an absorption maximum at 410 nm (Kageyama, 1971). 5 ml of uric acid reagent (0.57 M ammonium phosphate buffer, pH 7.0; 1.7M methanol; 20 mM acetylacetone; 670 U/ml catalase) was placed in a test tube containing 0.5 ml of sample. After mixing thoroughly, 2.5 ml of the mixture was put in a cuvette containing 5 U/ml uricase (20 µl). The sample and sample blanks were incubated for at least 60 min at 37°C and the absorbance of the sample was measured against a sample blank at 410 nm.

7. Expression of results

The enzyme activity is expressed as v/V_{max}, where v is the enzyme activity at 0.2 mM substrate (PEP) concentration and V_{max} is the enzyme activity at 4.0 mM PEP. The use of this activity ratio eliminates any slight variations of v and V_{max} which are due to non-uniform cell distribution during sampling. In cases where the enzyme activity is not expressed as v/V_{max}, the activity is given in µmoles min^{-1} g $cells^{-1}$. Metabolite levels are expressed as µmole (or n moles) per g cells. The weight of hepatocytes was calculated from the total number of cells counted (Krebs et al., 1974).

8. Statistical Analysis

Unless otherwise stated all data presented is the mean ± standard error from at least three different cell preparations. The standard error of mean was calculated from the standard deviation.

$$\text{i.e.} \quad SEM = \frac{SD}{\sqrt{n}}$$

where n = number of observations

SD = standard deviation

SEM = standard error of the mean

The standard deviation was calculated using the formula

$$SD = \pm \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$$

where Σ = sum of

X = observation or observed value

$\bar{X}$ = mean of the sample (i.e. $\frac{\Sigma x}{n}$)

A t-test for small population was used to test the significance of the results. The formula used was

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\hat{\sigma}w}$$

degrees of freedom n_1+n_2-2

where

t = test of significance

$\bar{X}_1;\bar{X}_2$ = mean of sample

$$\hat{\sigma}w = \hat{\sigma}\sqrt{\frac{n_1+n_2}{n_1 n_2}}$$

$$\hat{\sigma}^2 = \frac{n_1 s_1^{\,2} + n_2 s_2^{\,2}}{n_1+n_2-2}$$

n - number of observation

s_1 - standard error of mean

(Ballentine, 1974).

BIBLIOGRAPHY

BIBLIOGRAPHY

Adam, P.A.J. and Haynes, R.C.Jr. (1969), J.Biol.Chem. 244, 6444-6450.

Adelman, R.C., Ballard, F.J. and Weinhouse, S. (1967), J.Biol. Chem., 242, 3360-3365.

Arinze, I.J. and Rowley, D.L. (1975), Biochem.J., 152, 393-399.

Arion, W.J., Wallin, B.K., Carlson, P.W. and Lange, A.J. (1972), J.Biol.Chem. 247, 2558-2565.

Assimacopoulos-Jeannet,F.D., Exton, J.H and Jeanrenoud,B., (1973), Am.J.Physiol. 225, 25-32.

Assimacopoulos-Jeannet,F.D., Blackmore,P.F. and Exton,J.H., (1977), J.Biol.Chem., 252, 2662-2669.

Bachelard,H.S., (1970), in "Handbook of Neurochemistry" (Leytha A.ed.), vol.4, pp.1-12, Plenum, New York.

Baer,H.P., Drummond,G.I. and Duncan,E.L., (1966), Mol.Pharmacol., 2, 67-76.

Bailey,E., Stripe,F. and Taylor,C.B., (1968a), Biochem.J., 108, 427-436.

Bailey,E., Taylor,C.B. and Bartley,W., (1968b), Nature (London) 217, 471-472.

Ballard,F.J., (1970), Biochem.J., 120, 809-814.

Ballentine,R.B., (1974), in "Experimental Techniques in Biochemistry" (Brewer J.M., Pescoe A.J. and Ashworth R.B., eds). pp 10-31, Prentice-Hall, New Jersey.

Bar-on,H. and Stein,Y. (1968), J.Nutr., 94, 95-105.

Barngrover,D.A., Stevens,H.C. and Dills,W.L. Jr., (1981), Biochem. Biophys.Res.Commun., 102, 75-80.

Barritt,G.J., Zander,G.L. and Utter,M.F., (1976), in "Gluconeogenesis: Its Regulation in Mammalian Species", (Hanson R.W. and Mehlman M.A., eds.), pp 3-46, J.Wiley & Sons, New York.

Baur,H. and Heldt,H.W., (1976), in "Use of Isolated Liver cells and Kidney Tubules in Metabolic Studies", (Tager J.M., Soling H.D. and Williamson J.R., eds.), pp 357-362, North-Holland Publishing Company, Amsterdam.

Beaufay,H. and de Duve,C., (1954), Bull.Soc.Chim.Biol., 36, 1525-1537.

Bender,A.E. and Damji,K.B., (1971), in "Sugar:Chemical Biological and Nutritional Aspects of Sucrose", (Yudkin J., Edelman J. and Hough L., eds.,) pp 172-182, Butterworths, London.

Berglund,L., Ljungström,O. and Engström,L., (1977), J.Biol.Chem., 252, 613-619.

Bergmeyer,H.U. and Bernt,E., (1974) in "Methods in Enzymatic Analysis", (Bergmeyer,H., ed.), Vol.3, pp 1205-1212, Academic Press, New York.

Bergström,G., Ekman,P., Humble,E. and Engström,L., (1978), Biochim. Biophys. Acta., 532, 259-267.

Bergström,J., Hultman,E. and Roch-Norlund,A.E., (1968), Acta Med. Scand., 184, 359-364.

Berry,M.N., (1980), FEBS Lett., 117, K106-K120.

Berry,M.N. and Friend,D.S., (1969), J.of Cell Biol., 43, 506-520.

Berry,M.N. and Werner,H.V., (1973), Biochem.Soc.Trans., 1, 190-193.

Berry, M.N., Kun, E. and Werner, H.V., (1973), Eur.J.Biochem., 33, 407-417.

Betheil,J.J., Feigelson,M. and Feigelson,P., (1965), Biochim. Biophys. Acta, 104, 92-97.

Bischofberger,A., Hess,B., Röschlau,P., Wieker,H.J. and Zimmarmann-Teschow,H., (1970), Hoppe-Seyler's Z.Physiol.Chem., 351, 401-408.

Blackmore,P.F., Brumley,F.T., Marks,J.L. and Exton,J.H., (1978), J. Biol.Chem., 253, 4851-4858.

Blackmore,P.F., Dehaye,J-P., Strickland,W.G. and Exton,J.H., (1979), FEBS Lett., 100, 117-120.

Blackshear,P.J., Holloway,P.A.H. and Alberti,K.G.M.M., (1975), Biochem.J., 150, 379-389.

Blair,J.B., Cook,D.E. and Lardy,H.A. (1973), J.Biol.Chem., 248, 3601-3607.

Blair,J.B., Cimbala,M.A., Foster,J.L. and Morgan,R.A. (1976), J.Biol. Chem., 251, 3756-3762.

Blair,J.B., James,M.E. and Foster,J.L., (1979a), J.Biol.Chem., 254, 7579-7584.

Blair,J.B., James,M.E. and Foster,J.L., (1979b), J.Biol.Chem., 254, 7585-7590.

Bloxham,D.P. and Lardy,H.A. (1973), in "The Enzymes", (Boyer P.B.ed.), 3rd Edition, vol. 8, pp 239-278, Academic Press, New York.

Bode,J.C., Zelder,O., Rumpelt,H.J. and Wittkamp,U., (1973), Eur.J. Clin.Invest., 3, 436-441.

Bode,J.C., Bode,C., Rumpelt,H.J. and Zelder,O., (1974), in "Regulation of Hepatic Metabolism" (Lundquist F. and Tygstrup N.,eds)., pp 267-281, Munksgaard, Copenhagen.

Bold,A.M. and Wilding,P., (1975), in "Conversion scales for SI Units with normal values in Clinical Chemistry", Blackwell Scientific Publication, Oxford.

Bollman,J.L. and Mann,F.C. (1931), Am.J.Physiol., 96, 683-695.

Bonney,R.J., Walker,P.R. and Potter,V.R., (1973), Biochem.J., 136, 947-954.

Brand,I. and Söling,H.D., (1974), J.Biol.Chem., 249, 7824-7831.

Brand,I. and Söling,H.D., (1975), FEBS Lett., 57, 163-168.

Brand,I., Muller,M.K., Unger,C. and Söling,H.D., (1976), FEBS Lett., 68, 271-274.

Brocks,D.G., Siess,E.A. and Wieland,O., (1980), Eur.J.Biochem., 113, 39-43.

Brosnan,J.T., Krebs,H.A. and Williamson,D.H., (1970), Biochem.J., 117, 91-96.

Brown,S.S., Forrest,J.A.H. and Roscoe,P., (1972), Lancet, 2, 898-900.

Buc,H.A., Demaugre,F. and Leroux,J-P., (1978), Biochem.Biophys. Res. Commun., 85, 774-779.

Buc,H.A., Demaugre,F., Moncion,A. and Leroux,J-P., (1981), Biochimie, 63, 595-605.

Bücher,Th., Krejci,K., Rüssmann,W., Schnitger,H. and Wessemann W., (1964), in "Rapid Mixing and Sampling Techniques in Biochemistry", (Chance,B., Eisenhardt,R.H., Gibson,Q.H. and Lonberg-Holm,K.K., eds.), Academic Press, New York.

Bücher,Th., Brauser,B., Conze,A., Klein,F., Langguth,O. and Siess,H., (1972), Eur.J.Biochem., 27, 301-317.

Burch,H.B., Max,P.Jr., Chyu,K. and Lowry,O.H., (1969), Biochem.Biophys. Res. Commun., 34, 619-626.

Burch,H.B., Lowry,O.H., Meinhardt,L., Max,P.Jr. and Chyu,K.J., (1970), J.Biol.Chem., 245, 2092-2102.

Burke,V., (1971), in "Sugar: Chemical, Biological and Nutritional Aspects of Sucrose", (Yudkin,J., Edelman,J. and Hough,L., eds.), pp 203-220, Butterworths, London.

Cahill,G.F.Jr., Ashmore,J., Earle,A.S. and Zottu,S., (1958a), Am.J. Physiol., 192, 491-496.

Cahill, G.F.Jr., Hastings, A.E., Ashmore, J. and Zottu, S., (1958B), J.Biol.Chem., 230, 125-135.

Carbonell, J., Felíu, J.E., Marco, R., and Sols, A., (1973), Eur.J. Biochem., 37, 148-156.

Cardenas,J.M. and Dyson,R.D., (1973), J.Biol.Chem., 248, 6938-6944.

Carlson,C.W., Baxter,R.C., Ulm,E.F. and Pogell,B.M., (1973), J.Biol. Chem., 248, 5555-5561.

Carminatti,H., Jiménez De Asúa,L., Recondo,E., Passeron,S. and Rozengurt,E., (1968), J.Biol.Chem., 243, 3051-3056.

Castaña,I.K., Nieto,A. and Feliu,D.E., (1979), J.Biol.Chem., 254, 5575-5579.

Cederbaum,A.I. and Dicker,E., (1979), Arch.Biochem.Biophys., 197, 415-423.

Cederbaum,A.I., Lieber,C.S., Beattie,D.S. and Rubin,E., (1973), Arch. Biochem.Biophys., 158, 763-781.

Chan,T.M. and Exton,J.H., (1978), J.Biol.Chem., 253, 6393-6400.

Chen,J.L.J., Babcock,D.F. and Lardy,H.A., (1978), Proc.Natl.Acad. Sci., USA., 75, 2234-2238.

Cherrington,A.D., Assimacopoulos,F.D., Harper,S.C., Corbin,J.D., Park,C.R. and Exton,J.H., (1976), J.Biol.Chem., 251, 5209-5218.

Clark,D.G., Rognstad,R. and Katz,J., (1973), Biochem.Biophys.Res. Commun., 54, 1141-1148.

Clark,D.G., Filsell,O.H. and Topping,D.L., (1979), Biochem.J., 184, 501-507.

Clark,M.G., Bloxham,D.P., Holland,P.C. and Lardy,H.A., (1973), Biochem.J., 134, 589-597.

Clark,M.G., Kneer,N.M., Bosch,A.Z. and Lardy,H.A., (1974), J.Biol. Chem., 249, 5695-5703.

Claus,T.H. and Pilkis,S.J., (1976), Biochim. Biophys.Acta., 421, 246-262.

Claus,T.H., El-Maghrabi,M.R. and Pilkis,S.J., (1979), J.Biol.Chem., 254, 7855-7864.

Claus,T.H., Schlumpf,J., Pilkis,J., Johnson,R.A. and Pilkis,S.J., (1980), Biochem.Biophys.Res.Commun., 98, 359-366.

Cohen,R.D. and Iles,R.A., (1975), CRC Crit.Rev.Clin.Lab.Sci., 6, 101-143.

Cohen,R.D., Iles,R.A., Barnett,D., Howell,M.E.O. and Strunim,J., (1971), Clin.Sci., 41, 159-170.

Cook,G.A., Nielsen,R.C., Hawkins,R.A., Mehlman,M.A., Lakshmanan,M.R. and Veech,R.L., (1977), J.Biol.Chem., 252, 4421-4424.

Cori,C.G., Garland,R.C. and Chang,H.W., (1973), Biochemistry, 12, 3126-3130.

Cori,G.T., Ochoa,S., Slein,M.W. and Cori,C.G., (1951), Biochim. Biophys.Acta., 1, 304-317.

Craig,J.W., Rall,T.W. and Larner,J., (1969), Biochim.Biophys.Acta, 177, 213-219.

Crane,R.K., (1960), Physiological Review, 40, 789-825.

Crane,R.K., (1968), in "Handbook of the Physiology of the Alimentary Canal" (Code C.F.,ed)., Am.Physiol.Soc., Washington, III, Section 6, pp.1323-1352.

Crisp,D.M. and Pogson,C.I., (1972), Biochem.J., 126, 1009-1023.

Czok,R. and Lamprecht,W., (1974), in "Methods in Enzymatic Analysis", (Bergmeyer,H. ed.), vol.3, 1446-1451, Academic Press, New York.

Dahlqvist,A., (1974), in "Sugars in Nutrition", (Sipple,H.L. and McNutt,K.W., eds.), pp 187-214, Academic Press, New York.

Datta,A.G., Abrams,B., Sasaki,T. and Van den Berg,T.W., (1974), Arch.Biochem.Biophys., 165, 641-645.

Dieter,H., Koberstein,R. and Sund,H., (1981), Eur.J.Biochem., 115, 217-226.

Di Pietro,D.L and Weinhouse S., (1960), J.Biol.Chem., 235, 2542-2545.

Dudman,N.P.B., de Maine,M.M. and Benkovic,S.J., (1978), J.Biol.Chem., 253, 5712-5718.

Dunaway,G.A.Jr. and Weber,G., (1974), Arch.Biochem.Biophys., 162, 620-628.

Edlund,B., Andersson,J., Titanji,V., Dahlqvist,U., Ekman,P., Zetterqvist, O. and Engström,L., (1975), Biochem.Biphys.Res.Commun., 67, 1516-1521.

Edmondson,J.W., Lumeng,L. and Li,T-K., (1977), Biochem.Biophys.Res. Commun., 76, 751-757.

Edmondson,J.W., Lumeng,L. and Li,T-K., (1979), J.Biol.Chem., 254, 1653-1658.

Eggleston,L.V. (1974) in "Methods in Enzymatic Analysis" (Bergmeyer,H., ed), vol.3, pp 1308-1313, Academic Press, New York.

Eggleston,L.V. and Woods,H.F., (1970), FEBS Lett., 6, 43-45.

Eigenbrodt,E. and Schoner,W., (1975), Hoppe-Seyler's Z.Physiol.Chem., 356, 227-228.

Eigenbrodt,E. and Schoner,W., (1977a), Hoppe-Seyler's Z.Physiol.Chem., 358, 1047-1054.

Eigenbrodt,E. and Schoner,W., (1977b), Hoppe-Seyler's Z.Physiol.Chem., 358, 1057-1067.

Ekman,P., Dahlqvist,U., Humble,E. and Engström,L., (1976), Biochim. Biophys.Acta, 429, 374-382.

Elion,G.B., Kovensky,A., Hitchings,G.H., Metz,E. and Rundles,R.W., (1966), Biochem.Pharmacol., 15, 863-889.

El-Maghrabi,M.R., Haston,S.H., Flockhart,D.A., Claus,T.H. and Pilkis, S.J., (1980), J.Biol.Chem., 255, 668-675.

Emmerson,B.T., (1974), Ann.Rheum.Dis., 33, 276-280.

Engström,L., (1978), Curr.Top.Cell.Reg., 13, 29-51.

Exton,J.H., (1979), Biochem.Pharmacol., 28, 2237-2240.

Exton,J.H. and Harper,S.C. (1975), Adv.Cyclic.Nucleotide Res., 5, 519-532.

Exton,J.H. and Park,C.R., (1966), Pharmacol.Rev., 18, 181-188.

Exton,J.H. and Park, C.R., (1967), J.Biol.Chem., 242, 2622-2636.

Exton,J.H. and Park,C.R., (1969), J.Biol.Chem., 244, 1424-1433.

Exton,J.H., Mallette,L.E., Jefferson,L.S., Wong,E.H.A., Friedman,N., Miller,T.B.Jr. and Park,C.R., (1970), Recent Prog.Horm.Res., 26, 411-461.

Exton,J.H., Lewis,S.B., Ho,R.J., Robison,G.A. and Park,C.R., (1971), Ann.N.Y.Acad.Sci., 185, 85-100.

Exton,J.H., Harper,S.C., Tucker,A.L. and Ho,R.J., (1973), Biochim. Biophys.Acta., 329, 23-40.

Fain,J.N. and Shepherd,R.E., (1977), J.Biol.Chem., 252, 8066-8070.

Faupel,R.P., Seitz,H.G. and Tarnowski,W., (1972), Arch.Biochem. Biophys., 148, 509-522.

Felig,P., (1973), Metabolism, 22, 179-207.

Felíu,J.E., Hue,L. and Hers,H-G., (1976), Proc.Nat.Acad.Sci. USA, 73, 2762-2766.

Felíu,J.E., Hue,L. and Hers,H-G., (1977), Eur.J.Biochem., 81, 609-617.

Flanders,L.E., Bamburg,J.R. and Sallach,H.J., (1971), Biochim.Biophys. Acta, 242, 566-579.

Forsander,O.A., Räihä,N., Salaspuro,M. and Mäenpää,P., (1965), Biochem.J., 94, 259-265.

Förster,H., (1974), in "Sugars in Nutrition" (Sipple H.L. and McNutt K.W., eds.), pp 259-280, Academic press, New York.

Foster,J.L. and Blair,J.B., (1976), Fed.Proc., Fed.Am.Soc.Exp.Biol., 35, 1428.

Foster,J.L. and Blair,J.B., (1978), Arch.Biochem.Biophys., 189, 263-276.

Frandsen,E.K. and Grunnet,N., (1971), Eur.J.Biochem., 23, 588-592.

Friedmann,G.,Goodman,E.H. and Weinhouse,S., (1965), J.Biol.Chem., 240, 3729-3733.

Friedmann,B.,Goodman,E.H. and Weinhouse,S., (1970), Endocrinology, 86, 1264-1271.

Friedman,N., Miller,T.B.Jr. and Park,C.R., (1971), Rec.Prog.Horm.Res., 26, 411-461.

Friedrichs,D., (1976), in "Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies", (Tager,T.M., Soling,H.D. and Williamson,J.R., eds.), pp 444-447, North-Holland Publishers, Amsterdam.

Froesch,E.R. and Jakob,A., (1974), in "Sugars in Nutrition", (Sipple, H.L. and McNutt,K.W., eds.), pp 241-258, Academic Press, New York.

Furfine,C.S. and Velick,S.F., (1965), J.Biol.Chem., 240, 844-855.

Furuya, E. and Uyeda, K., (1980), Proc.Natl.Acad.Sci. USA, 77, 5861-5864.

Furuya,E. and Uyeda,K., (1981), J.Biol.Chem., 256, 7109-7112.

Gabrielli,F. and Baldi,S., (1972), Eur.J.Biochem. 31, 209-214.

Gale,M.M. and Crawford,M.A., (1969), Metabolism, 18, 1021-1023.

Garber,A.J., Karl,I.E. and Kipnis,D.M., (1976), J.Biol.Chem., 251, 826-835.

Garrison,J.C. and Borland,M.K., (1978), J.Biol.Chem., 253, 7091-7100.

Garrison,J.C. and Haynes,R.C., (1973), J.Biol.Chem., 248, 5333-5343.

Garrison,J.C. and Haynes,R.C. Jr., (1975), J.Biol.Chem., 250, 2769-2777.

Gessner,P.K., Parke,D.V. and Williams,R.T., (1961), Biochem.J., 79, 482-489.

Gibson,D.M., Davisson,E.O., Bachhawart,B.K., Ray,B.R. and Vestling,C.S., (1953), J.Biol.Chem., 203, 397-409.

Gimpel,J.A., de Haart,E.J. and Tager,J.M., (1973), Biochim.Biophys. Acta., 292, 582-591.

Gracey,M., Burke,V. and Oshin,A., (1972), Biochim.Biophys.Acta., 266, 397-406.

Gracia,A., Williamson,J.R. and Cahill,G.F.Jr., (1964), Fed.Proc., Fed.Am.Soc.Exp.Biol., 23, 520.

Grande,F., (1967), Am.J.Clin.Nutr., 20, 176-184.

Green,D.E., (1936), Biochem.J., 30, 629-644.

Greene,H.L., Taunton,O.D., Stifel,F.B. and Herman,R.H., (1974), J.Clin. Invest., 53, 44-51.

Gregory,R.B. and Ainsworth,S., (1981), Biochem.J., 195, 745-751.

Günther,M.A., Sillero,A. and Sols,A., (1967), Enzymol.Biol.Clin., 8, 341-352.

Gutmann,I. and Wahlefeld,A.W., (1974), in "Methods in Enzymatic Analysis" (Bergmeyer,H.U. ed.), vol.3, pp 1464-1468. Academic Press, New York.

Guy,M.J. and Deren,J.J., (1971), Am.J.Physiol., 221, 1051-1056.

Hall,E.R., McCully,V. and Cottam,G.L., (1979), Arch.Biochem. Biophys. 195, 315-324.

Harris, R.A., (1975), Arch.Biochem.Biophys., 169, 168-180.

Haslam,J.M. and Krebs,H.A., (1968), Biochem.J., 107, 659-667.

Haynes,R.C.Jr., (1972) in "Energy Metabolism and the Regulation of Metabolic Processes in Mitochondria" (Mehlman,M.A. and Hanson,R.W., eds.), pp 239-252, Academic Press, New York.

Heinz,F. and Junghänel,J., (1969), Hoppe-Seyler's Z.Physiol.Chem., 350, 859-866.

Heinz,F. and Lamprecht,W., (1967), Hoppe-Seyler's, Z.Physiol.Chem., 348, 855-863.

Hems,R., Lund,P. and Krebs,H.A., (1975), Biochem.J., 150, 47-50.

Herman,R.H. (1974), in "Sugars in Nutrition" (Sipple,H.L. and McNutt, K.W., eds.), pp 145-172, Academic Press, New York.

Hers,G.H., (1952a), Biochim.Biophys.Acta, 8, 416-423.

Hers,G.H., (1952b), Biochim.Biophys.Acta., 8, 424-430.

Hers,G.H., (1976), Ann.Rev.Biochem., 45, 167-189.

Hers,G.H. and Kusaka,T., (1953), Biochim.Biophys.Acta., 11, 427-437.

Hers,G.H., Berthert,J., Berthert,L. and de Duve,C., (1951), Biochem. Soc.Trans., 2, 1051-1055.

Hers,G.H., Stalmans,W., de Wulf,H., Laloux,M. and Hue,L., (1973), in "Regulation of Hepatic Metabolism" (Lundquist,F. and Tygstrup,N., eds.), Alfred Benzon Symposium Vol.6, pp 237-253, Munksgaard, Copenhagen.

Hess,B., Haeckel,R. and Brand,K., (1966), Biochem.Biophys.Res.Commun., 24, 824-831.

Hjelmquist,G., Anderson,J., Edlund,B. and Engström,L., (1974), Biochem.Biophys.Res. Commun., 61, 559-563.

Ho,L.Y. and Pace,N., (1958), Arch.Biochem.Biophys., 75, 125-128.

Hodgkinson,A., (1977), in "Oxalic Acid in Biology and Medicine", pp 179-192, Academic Press, New York.

Hofmann,E., (1976), Rev.Physiol.Biochem.Pharmacol., 75, 1-68.

Hohorst,H.J., Kreutz,F.H. and Bucher,Th., (1959), Biochem.Z., 332, 18-46.

Hopkirk,T.J. and Bloxham,D.P., (1979), Biochem.J., 182, 383-397.

Hopper,S. and Segal,H.L., (1962), J.Biol.Chem., 237, 3189-3195.

Horecker,B.L., Melloni,E. and Pontremoli,S., (1975), Adv.Enzymol., 42, 193-226.

Humble,E., (1980), Biochim.Biophys.Acta., 626, 179-187.

Humble,E., Berglund,L., Titanji,V., Ljungström,O., Edlund,B., Zetterquist,O. and Engström,L., (1975), Biochem.Biophys.Res. Commun., 66, 614-621.

Hue,L., (1981), Personal Communication.

Hue, L. and Feliu,J.E., (1978), Biochem.Soc.Trans., 6, 29-33.

Hue,L. and Hers,H.G., (1974), Biochem.Biophys.Res.Commun., 58, 540-548.

Ibsen,K., (1977), Cancer Research, 37, 341-351.

Ibsen,K. and Trippet,P., (1972), Biochemistry, 11, 4442-4450.

Iles,R.A., Griffiths,J.R., Stevens,A.N., Gadian,D.G. and Porteous,R., (1980), Biochem.J., 192, 191-202.

Imamura,K. and Tanaka,T., (1972), J.Biochem. (Tokyo), 71, 1043-1051.

Imamura,K., Taniuchi,K. and Tanaka,T., (1972), J.Biochem.(Tokyo), 72, 1001-1015.

Irving,M.G. and Williams,J.F., (1973), Biochem.J., 131, 287-301.

Ishibashi,H. and Cottam,G.L., (1978), J.Biol.Chem., 253, 8767-8771.

Ishikawa,E., (1976), Adv.Enz.Reg., 14, 117-136.

Ishikawa,E., Aikawa,T. and Matsutaka,H., (1972), J.Biochem.(Tokyo), 71, 1093-1095.

Jakob,A., (1976), Mol.Cell.Endorcrinol., 6, 47-56.

Jakob,A., Williamson,J.R. and Asakura,T.,(1971), J.Biol.Chem., 246, 7623-7531.

Jiménez De Asúa,L., Rozengurt,E. and Carminatti,H., (1970), J.Biol. Chem., 245, 3901-3905.

Jiménez De Asúa,L., Rozengurt,E., Devalle,J.J. and Carminatti,H., (1971), Biochim.Biophys.Acta, 235, 326-334.

John,R.A., Charteris,A. and Fowler,L.J. (1978), Biochem.J., 171, 771-779.

Jomain-Baum,M. and Schramm,V.L. (1978), J.Biol.Chem., 253, 3648-3659.

Jomain-Baum,M., Schramm,V.L. and Hanson,R.W., (1976), J.Biol.Chem., 251, 37-44.

Kageyama,N., (1971), Clin.Chim.Acta., 31, 421-425.

Kagimoto,T. and Uyeda,K., (1979), J.Biol.Chem., 254, 5584-4487.

Kagimoto,T. and Uyeda,K., (1980), Arch.Biochem.Biophys., 203, 792-799.

Kahn,A., Garreau,M.J. and Sprengers,E.D., (1978) Biochim.Biophys. Acta, 523, 59-74.

Kato,K. and Bishop,J.S. (1972), J.Biol.Chem., 247, 7420-7429.

Keech,D.B. and Utter,M.F., (1963), J.Biol.Chem., 238, 2609-2614.

Kekomäki,M., Louhimo,I., Rahiala,E-L. and Suutarinen,T., (1972), Acta. Clin.Scand., 138, 239-243.

Kemp,B.E. and Clark,M.G., (1978), J.Biol.Chem., 253, 5147-5154.

Keppens,S., Van den Heede,J.R. and de Wulf,H., (1977), Biochim.Biophys. Acta., 496, 448-457.

Kjerulf-Jensen,K., (1942), Acta.Physiol.Scand., 4, 249-258.

Klingenberg,M. and Bucher,T., (1960), Ann.Rev.Biochem., 29, 669-710.

Kneer,N.M., Bosch,A.L., Clark,M.G. and Lardy,H.A., (1974), Proc.Natl. Acad.Sci.USA, 71, 4523-4527.

Kneer,N.M., Wagner,M.J. and Lardy,H.A., (1979), J.Biol.Chem., 254, 12160-12168.

Kohl,E.A. and Cottam,G.L., (1976), Arch.Biochem.Biophys., 176, 671-682.

Kohl,E.A. and Cottam,G.L., (1977), Biochim.Biophys.Acta., 484, 49-58.

Koshland,D.E., (1970), in "The Enzymes" (Boyer P.D.ed.), vol.1, pp 305-344, Academic Press, New York.

Koshland,D.E. and Neet,K.W., (1968), Ann.Rev.Biochem., 37, 359-410

Koster,J.F. and Hulsmann,W.C., (1970), Arch.Biochem.Biophys., 141, 98-101.

Koster,J.F., Slee,R.G., Staal,G.E.J. and Van Berkel,T.J.C., (1972), Biochem.Biophys.Acta., 258, 763-768.

Krebs,H.A., (1968), Adv.Enz.Reg., 6, 467-479.

Krebs,H.A. and Eggleston,L.V. (1965), Biochem.J., 94, 3c-7c.

Krebs,H.A. and Henseleit,K., (1932), Hoppe-Seyler's Z.Physiol.Chem. 210, 33-66.

Krebs,H.A., Freedland,R.A., Hems,R.and Stubbs,M., (1969), Biochem.J., 112, 117-124.

Krebs,H.A., Cornell,N.W., Lund,P. and Hems,P., (1974), in "Regulation of Hepatic Metabolism" (Lundquist,F. and Tygstrup,N., eds.), vol. 6, 726-753, Munksgaard, Copenhagen.

Kuo,J.F. and Greengard,P., (1969), J.Biol.Chem., 244, 3417-3419.

Kutzbach,C. and Hess,B., (1970), Hoppe-Seyler's Z., Physiol.Chem., 351, 272-273.

Kutzbach,C., Bischofberger,H., Hess,B. and Zimmermann-Telschow,H., (1973), Hoppe-Seyler's Z.Physiol.Chem., 354, 1473-1489.

Kwan,C-Y. and Davis,R.C., (1981), Can.J.Biochem., 59, 92-99.

Lardy,H.A., Paetkau,V. and Walter,P., (1965), Proc.Natl.Acad.Sci. USA, 53, 1410-1415.

Lawson,J.W.R., Guynn,R.W., Cornell,N. and Veech,R.L., (1976), in "Gluconeogenesis: Its regulation in Mannalian Species" (Hanson, R.W. and Mehlamn,M.A., eds.), pp 481-512, J.Wiley & Sons, New York.

Lea,M.A. and Weber,G., (1968), J.Biol.Chem., 243, 1096-1102.

LeCam,A. and Freychet,P., (1977), J.Biol.Chem., 252, 148-156.

Lehninger,A.L., (1951), J.Biol.Chem., 190, 334-337.

Leissing,N. and McGuinness,E.T. (1978), Biochim.Biophys.Acta., 524, 254-261.

Leiter,A.B., Weinberg,M., Isohashi.F., Utter,M.F. and Linn,T.C., (1978), J.Biol.Chem., 253, 2716-2723.

Leuthart,F. and Wolf,H.P. (1954), Helv.Chim.Acta., 37, 1732.

Levin,B., Oberholzer, V.G., Snodgrass,G.J.A.I., Stimmler,L. and Wilmers,M.J. (1963), Arch.Dis.Chil., 38, 220-230.

Lieber,C.S.and De Carti,L.M., (1968), Science, 162, 917-918.

Lincoln,D.R., Black,J.A. and Rittenberg,M.B., (1975), Biochim. Biophys.Acta., 410, 279-284.

Lindros,K.O., Vihma,R. and Forsander,O.A., (1972), Biochem.J., 126, 945-952.

Ljungström,O. and Ekman,P., (1977), Biochem.Biophys.Res.Commun., 78, 1147-1155.

Ljungström,O., Hjelmquist,G. and Engström,L., (1974), Biochim.Biophys. Acta., 358, 289-298.

Ljungström,O.,Berglund,L. and Engström,L., (1976), Eur.J.Biochem., 68, 497-506.

Llorente,P., Marco,R. and Sols,A., (1970), Eur.J.Biochem., 13, 45-54.

Lloyd,M.H., Iles,R.A., Simpson,B.R., Strunim,J.M., Layton,J.M. and Cohen,R.D., (1973), Clin.Sci.Mol.Med., 45, 543-549.

Lowenstein,L.M., Simone,R., Boulter,P. and Nathan,P., (1970), J.Am. Med.Assoc., 213, 1899-1901.

Lundquist,F., Damgaard,S.E. and Sestoft,L., (1974), in "Alcohol and Aldehyde Metabolising Systems" (Thurman,R.G. ed.), pp 405-416, Academic Press, New York.

Macdonald,I., (1971), in "Sugar: Chemical, Biological and Nutritional Aspects of Sucrose" (Yudkin,J., Edelman,J. and Hough,L., eds.), pp 192-199, Butterworths, London.

Madison,L.L., Lochmer,A. and Wulff,J., (1967), Diabetes, 16, 252-258.

Mäenpää,P.H., Raivio,K.O. and Kekomaki,M.P., (1968), Science, 161, 1253-1254.

Mahmoud,S.A.F. (1979), Ph.D. Thesis, London University.

Mallette,L.E., Exton,J.H. and Park,C.R., (1969a), J.Biol.Chem., 244, 5713-5723.

Mallette,L.E., Exton,J.H. and Park,C.R., (1969b), J.Biol.Chem., 244, 5724-5728.

Mansour,T.E., (1972), Curr.Top.Cell.Reg., 5, 1-46.

Marie,J., Kahn,A. and Boivin,P., (1976), Biochim.Biophys.Acta., 438, 393-406.

Massey,V., Komai,H., Palmer,G. and Elion,G., (1970a), J.Biol.Chem., 245, 2837-2844.

Massey,V., Komai,H., Palmer,G. and Elion,G., (1970b), Vit.Horm., 28, 505-531.

McClurke,W.R. and Lardy,H.A., (1971), J.Biol.Chem., 246, 3591-3596.

Mendeloff,A.I. and Weichselbaum,T.E., (1953), Metab., Clin.Exp., 2, 450-458.

Mendicino,J. and Vasarhely,F. (1963), J.Biol.Chem., 238, 3528-3534.

Mendicino,J., Leibach,F. and Reddy,S., (1978), Biochemistry, 17, 4662-4669.

Michal,G. and Beutler,H.O., (1974), in "Methods in Enzymatic Analysis" (Bergmeyer,H., ed.), vol.3, pp 1314-1319, Academic Press, New York.

Michal,G. and Lang,F., (1974), in "Methods in Enzymatic Analysis" (Bergmeyer,H., ed.), vol.3, pp 1417-1418, Academic Press, New York.

Miller,T.B.Jr., (1978), Biochim.Biophys.Acta, 540, 151-161.

Monod,J., Wyman,J. and Changeux,J.P., (1965), J.Mol.Biol., 12, 88-118.

Muirhead,H., Grant,J.P., Lawton,M.A., Midwinter,C.A., Nocton,J.C. and Stuart,D.I., (1981), Biochem.Soc.Trans., 9, 212-213.

Naismith,D.J. (1971), in "Sugar: Chemical, Biological and Nutritional Aspects of Sucrose" (Yudkin,J., Edelman,J. and Hough,L., eds.), pp 183-191, Butterworths, London.

Nakashima,K., (1974), Clin.Chim.Acta, 55, 245-254.

Newsholme,E.A., (1978), Biochem.Soc.Symp., 43, 183-205.

Newsholme,E.A. and Crabtree,B., (1976), Biochem.Soc. Symp., 41, 61-109.

Newsholme,E.A. and Start,C., (1976), in "Regulation in Metabolism", A.Wiley-Interscience Publication.

Niemeyer,R., Ureta,T. and Clar-Turn,L., (1975), Mol.Cell.Biochem., 6, 100-126.

Nífhaoláin,I. and Coughlan,M.P., (1978), FEBS Lett., 90, 305-308.

Nikiforuk,G. and Colowick,S.P., (1956), J.Biol.Chem., 219, 119-129.

Nikkilä,E.A., (1969), Adv.Lipid.Res., 1, 63-134.

Nikkilä,E.A., (1974), in "Sugars in Nutrition", (Sipple,H.L. and McNutt,K.W., eds.), pp 439-448, Academic Press, New York.

Nimmo,H.G. and Tipton,K.F., (1975), Biochem.J., 145, 323-334.

Nordlie,R.C. and Lardy,H.A., (1963), J.Biol.Chem., 238, 2259-2263.

Nyfeler,F., Fasel,P. and Walter,P., (1981), Biochem.Biophys.Acta., 675, 17-23.

Odessey,R., Khairallah,E. and Goldberg,A.L., (1974) J.Biol.Chem., 249, 7623-7629

Otto,K., (1965), Hoppe-Seyler's Z.Physiol.Chem., 341, 99-104.

Otto,M., Jacobasch,G. and Rapoport,S., (1976), Eur.J.Biochem., 65, 201-206.

Pande,S.V. and Mead,J.F., (1968), J.Biol.Chem., 243, 6180-6185.

Papenberg,J., Von Wartburg,J.P. and Aebi,N., (1970), Enz.Biol.Clin., 11, 237-250.

Pardridge,W.M. and Oldendorf,W.J. (1977), J.Neurochem., 28, 5-12.

Parks,R.E.Jr., Ben-Gershom,E. and Lardy,H.A., (1957), J.Biol.Chem., 227, 231-242.

Passeron,S., Jiménez De Asúa,L. and Carminatti,H., (1967), Biochem. Biophys.Res.Commun., 27, 33-38.

Pawan,G.L.S., (1968), Nature (London), 220, 374-376.

Penhoet,E., Rajkumar,T. and Rutter,W.J., (1966), Proc.Natl.Acad.Sci. USA., 56, 1275-1282.

Perheentupa,J. and Raivio,K., (1967), Lancet, 2, 528-531.

Periera,J.N. and Jangaard,N.O., (1971), Metabolism, 20, 392-400.

Pilkis,S.J., (1970), Biochim.Biophys.Acta, 215, 461-476.

Pilkis,S.J., Claus,T.H., Johnson,R.A. and Park,C.R., (1975), J.Biol. Chem., 250, 6328-6336.

Pilkis,S.J., Riou,J.P. and Claus,T.H., (1976a), J.Biol.Chem., 251, 7841-7852.

Pilkis,S.J., Claus,T.H., Riou,J.P. and Park,C.R., (1976b), Metab. Clin.Expt., 25, Supplement 1, 1341-1355.

Pilkis,S.J., Park,C.R. and Claus,T.H., (1978a), Vit.Horm., 36, 383-460.

Pilkis,S.J., Pilkis,J. and Claus,T.H., (1978b), Biochem.Biophys.Res. Commun., 81, 139-146.

Pilkis,S.J., Claus,T.H., Riou,J.P., Cherrington,A.D., Chaisson,J.E., Liljenquist,J.E., Lacy,W.W. and Park,C.R., (1978c), FEBS Symp., 42, 13-29.

Pilkis,S.J., El-Maghrabi,M.R., Coven,B., Claus,T.H., Tager,H.S., Steiner, D.F., Kein,P.S. and Heinrikson,R.L., (1980), J.Biol.Chem., 255, 2770-2775.

Pilkis,S.J., El-Maghrabi,M.R., Pilkis,J., Claus,T.H. and Cumming,D.A., (1981), J.Biol.Chem., 256, 3171-3174.

Pitot,H.C. and Yatvin,M.B. (1973), Physiol.Rev., 53, 228-328.

Pogell,B.M., Tanaka,A. and Siddons,R.C., (1968), J.Biol.Chem., 243, 1356-1367.

Pozefsky,T. and Tancredi,R.G., (1972), J.Clin.Invest., 51, 2359-2369.

Purich,D.L., Fromm,H.J. and Rudolf,F.B., (1973), Adv.Enzymol., 39, 249-326.

Raivio,K.O., Kekomäki,M.P. and Mäenpää,P.H., (1968), Biochem. Pharmacol., 18, 2615-2624.

Raivio,K.O., Becker,M.A., Meyer,L.J., Greene,M.L., Nuki,G. and Seegmiller,J.E., (1975), Metabolism, 24, 861-869.

Ramaiah,A., (1974), Curr.Top.Cell.Reg., 8, 297-350.

Raushel, F.M. and Cleland,W.W., (1977), Biochemistry, 16, 2169-2175.

Reinecke,R.M. (1944), Am.J.Physiol., 141, 669-676.

Renold,A.E., Hastings,A.B. and Nesbett,F.B., (1954), J.Biol.Chem., 209, 687-691.

Richards,C.F. and Uyeda,K., (1980), Biochem.Biophys.Res.Commun., 97, 1535-1540

Riou,J.P., Claus,T.H. and Pilkis,S.J., (1976), Biochem.Biophys.Res. Commun., 73, 591-599.

Riou,J.P., Claus,T.H., Flockhart,D., Corbin,J. and Pilkis,S.J., (1977), Proc.Natl.Acad.Sci.USA, 74, 4615-4619.

Riou,J.P., Claus,T.H. and Pilkis,S.J., (1978), J.Biol.Chem., 253, 656-659.

Robinson,J. and Newsholme,E.A., (1969), Biochem.J., 112, 455-464.

Robison, G.A., Butcher,R.W. and Sutherland,E.W., (1971), in "Cyclic AMP", Academic Press, New York.

Rofe,A.M., Thomas,D.W., Edwards,R.G. and Edwards,J.B., (1977), Biochem.Med., 18, 440-451.

Rognstad,R. and Clark,D.G., (1974), Arch.Biochem.Biophys., 161, 638-646.

Rognstad,R. and Katz,J., (1972), J.Biol.Chem., 247, 6047-6054.

Rognstad,R. and Katz,J., (1976), Arch.Biochem.Biophys., 117, 337-345.

Rosenberg,J.S., Tishima,Y. and Horecker,B.L., (1973), Arch.Biochem. Biophys., 154, 283-293.

Ross,B.D., Hems,R. and Krebs,H.A., (1967a), Biochem.J., 102, 942-951.

Ross,B.D., Hems,R. and Krebs,H.A., (1967b), Biochem.J., 105, 869-875.

Rozengurt,E., Jiménez De Asúa,L. and Carminatti,H., (1969),J.Biol. Chem., 244, 3142-3147.

Rozengurt,E., Jiménez De Asúa,L. and Carminatti,H., (1970), FEBS Lett., 11, 284-286.

Rozengurt,E., Jiménez De Asúa,L. and Carminatti,H., (1973), FEBS Lett., 37, 225-227.

Ruderman,N.B. and Berger,M., (1974), J.Biol.Chem., 249, 5500-5506.

Ruderman,N.B. and Lund,P., (1972), Isr.J.Med.Sci., 8, 295-302.

Rufo,G.A.Jr., Yorek,M.A. and Ray,R.D., (1981), Biochim.Biophys. Acta., 674, 297-305.

Sainsbury,G.M., (1980), Biochim.Biophys.Acta., 631, 305-316.

Salas,M., Vinela,E. and Sols,A., (1963), J.Biol.Chem., 238, 3535-3538.

Sanchez,J.J., Gonzolez,N.S. and Pontis,H.G., (1971), Biochim. Biophys.Acta., 227, 67-68.

Scheibe,P., Bert,E. and Bergmeyer,H.U., (1974) in "Methods in Enzymatic Analysis" (Bergmeyer,H.U., ed.), vol.4, pp 1951-1957, Academic Press, New York.

Schimassek,H. and Mitzkat,H.J., (1963), Biochem.Z., 337, 510-518.

Scholz,R. and Nohl,H., (1976), Eur.J.Biochem., 63, 449-458.

Schoner,W., Haag,U. and Seubert,W., (1970), Hoppe-Seyler's Z.Physiol. Chem., 351, 1071-1088.

Scrutton,M.C. and Utter,M.F., (1968), Ann.Rev.Biochem., 37, 249-302.

Scrutton,M.C. and White,M.D., (1974), J.Biol.Chem., 249, 5405-5415.

Seglen,P.O., (1974), Biochim.Biophys.Acta., 338, 317-336.

Sestoft,L., (1974), Biochim.Biophys.Acta., 343, 1-16.

Sestoft,L. and Fleron,P., (1974), Biochim.Biophys.Acta., 345, 27-38.

Sestoft,L., Tønnesen,K., Hansen,F.V. and Damgarrd,S.E., (1972), Eur. J.Biochem., 30, 542-552.

Seubert,W. and Schoner,W., (1971), Curr.Top.Cell.Reg., 3, 237-267.

Seubert,W., Henning,H.V., Schoner,W. and L'age,M., (1968), Adv. Enz.Reg., 6, 153-187.

Sharma,C., Manjeshwar,R. and Weinhouse,S., (1963), J.Biol.Chem., 238, 3840-3845.

Sherline,P., Lynch,A. and Glinsmann,W.H., (1972), Endocrinology, 91, 680-690.

Siess,E.A. and Wieland,O.H., (1972), Eur.J.Biochem., 26, 96-105.

Siess,E.A., Brocks,D.G., Lattke,H.K. and Wieland,O.H., (1977), Biochem. J., 166, 225-235.

Sigrist-Nelson,K. and Hopfer,U., (1974), Biochim.Biophys.Acta., 364, 247-254.

Sillero,M.A.G., Sillero,A. and Sols,A., (1969), Eur.J.Biochem., 10, 345-351.

Simkin,P.A., (1972), Metab.Clin.Exp., 21, 1029-1036.

Sips,H.J., Van Amelsuoort,J.M.M. and Van Dam,K., (1980a), Eur.J. Biochem., 105, 217-224.

Sips,H.J., Groen,A.K. and Tager,J.M., (1980b), FEBS Lett., 119, 217-274.

Smith,C.M., Ravamo,L.M., KekomÄki,M.P. and Raivio,K.O., (1977a), Can.J.Biochem., 55, 1134-1139.

Smith,C.M., Ravamo,L.M. and Raivio,K.O., (1977b), Can.J.Biochem., 55, 1237-1240.

Smith,M.G., (1962), Biochem.J., 83, 135-144.

Smith,S.B. and Freedland,R.A., (1979), J.Biol.Chem., 254, 10644-10648.

Smith,S.B. and Freedland,R.A., (1981), Am.J.Physiol., 240, 279-285.

Snell,K., (1976), Biochem.Soc.Trans., 4, 287-288.

Snell,K., (1979), TIBS, 4, 124-128.

Snell,K. and Duff,D.A., (1977), Biochem.J., 162, 399-403.

Sobell,S., Scholz,R., Freisl,M., Elbers,R. and Heldt,H.W., (1976), in "Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies" (Tager,J.M., Söling,H.D. and Williamson,J.R., eds.), pp 22-40, North-Holland Publishing Company, Amsterdam .

Sokoloff,L., Fitzgerald,G.G. and Kaufman,E.E., (1977), in "Nutrition and the Brain", (Wurtman,P.J. and Wurtman,J.J., eds.), vol.1, pp 87-139, Raven, New York.

Söling,H.D. and Kleineke,J., (1976), in "Gluconeogenesis: Its Regulation in Mammalian Species", (Hanson,R.W. and Mehlman,M.A., eds.),pp 369-462, J.Wiley & Sons, New York.

Söling,H.D., Williams,B. and Janson,G., (1970), FEBS Lett., 11, 324-327.

Söling,H.D., Kuduz,J. and Brand,I.A., (1981), FEBS Lett., 130, 309-313.

Sols,A. and Marco,R., (1970), Curr.Top.Cell.Reg., 2, 227-273.

Soterakis,J. and Iber,F.L., (1975), Am.J.Clin.Nutr., 28, 254-257.

Spector,T. and Johns,D.G., (1970), J.Biol.Chem., 254, 5079-5085.

Spiro,R., (1959), J.Biol.Chem., 233, 546-550.

Spiro,R., Ashmore,J. and Hastings,A.B., (1958), J.Biol.Chem., 230, 761-771.

Stadman,E.R. (1966), Adv.Enzymol., 28, 41-154.

Stalmans,W., (1976), Curr.Top.Cell.Reg., 11, 51-97.

Stalmans,W. and Hers,H.G., (1975), Eur.J.Biochem., 54, 341-350.

Stifel,F.B., Taunton,O.D., Greene,H.L. and Herman,R.H., (1974), J.Biol.Chem., 249, 7240-7244.

Stripe,F., Della Corte,F., Bonetti,E., Abbondanza,A., Abbati,A. and De Stefano,F., (1970), Lancet, 2, 1310-1311.

Struck,E., Ashmore,J., Wieland,O., (1965), Biochem.Z., 343, 107-110.

Stubbs,M., Kirk,C.J. and Hems,D.A., (1976), FEBS Lett., 69, 199-202.

Susor,W.A. and Rutter,W.J., (1968), Biochem.Biophys.Res.Commun., 30, 14-20.

Sutherland,E.W., (1972), Science, 177, 401-408.

Taketa,K. and Pogell,B.M., (1963), Biochem.Biophys.Res.Commun., 12, 229-235.

Taketa,K. and Pogell,B.M., (1965), J.Biol.Chem., 240, 651-662.

Tanaka,T., Harano,Y., Morimura,H. and Mori,R., (1965), Biochem. Biophys.Res.Commun., 21, 55-60.

Tanaka,T., Sue,F. and Morimura,H., (1967a), Biochem.Biophys.Res. Commun., 29, 444-449.

Tanaka,T., Harano,Y., Sue,F. and Morimura,H., (1967b), J.Biochem. (Tokyo), 62, 71-91.

Taunton,O.D.,Stifel,F.B., Greene,H.L. and Herman,R.H., (1972), Biochem. Biophys.Res.Commun., 48, 1663-1676.

Taunton,O.D., Stifel,F.B., Greene,H. and Herman,R.H., (1974), J.Biol. Chem., 249, 7228-7239.

Taylor,C.B. and Bailey,E., (1967), Biochem.J., 102, 32c-33c.

Tejwani,G.A., Pedrosa,F.O., Pentrenoli,S. and Horecker,B.L., (1976), Arch.Biochem.Biophys., 177, 253-264.

Thieden,H.I.D. and Lundquist,F., (1967), Biochem.J., 102, 177-180.

Thieden,H.I.D., Grunnet,N., Damgaard,S.E. and Sestoft,L., (1972), Eur.J.Biochem., 30, 250-261.

Thurston,J.H., Jones,E.M. and Hauhart,R.E., (1974), Diabetes, 23, 597-604.

Tilghman,S.M., (1976), in "Gluconeogenesis: Its Regulation in Mammalian Species" (Hanson,R.W. and Mehlman,M.A.,eds.), pp 47-91, Academic Press, New York.

Tischler,M.E., Friedrichs,D., Coll,K. and Williamson,J.R., (1977), Arch.Biochem.Biophys., 184, 222-236.

Titanji,V.P.K., (1977), Biochim.Biophys.Acta., 481, 140-151.

Titanji,V.P.K., Zetterqvist,O. and Engström,L., (1976), Biochim. Biophys.Acta., 422, 98-108.

Titheradge,M.A. and Coore,H.G., (1976a), FEBS Lett., 63, 45-50.

Titheradge,M.A. and Coore,H.G., (1976b), FEBS Lett., 71, 73-78.

Tolbert,M.E.M. and Fain,J.N., (1974), J.Biol.Chem., 249, 1162-1166.

Tolbert,M.E.M., Butcher,F.R. and Fain,J.N., (1973), J.Biol.Chem., 248, 5686-5692.

Topping,D.L. and Mayes,P.A., (1971), Nutr.Metab., 13, 331-338.

Topping,D.L. and Mayes,P.A., (1977), Biochem.Soc. Trans., 5, 1001-1002.

Tygstrup,N., Winkler,K. and Lundquist,F., (1965), J.Clin.Invest., 44, 817-830.

Ui M., Claus,T.H., Exton,J.H. and Park,C.R., (1973a), J.Biol.Chem., 248, 5344-5349.

Ui,M., Exton,J.H. and Park,C.R., (1973b), J.Biol.Chem., 348, 5350-5359.

Underwood,A.H. and Newsholme,E.A.,(1965), Biochem.J., 95, 767-774.

Utter,M.F. and Keech,D.B. (1960), J.Biol.Chem., 235, PC17-PC18.

Utter,M.F. and Keech,D.B. (L963), J.Biol.Chem., 238, 2603-2608.

Uyeda,K., (1979), Adv.Enzymol., 48, 193-244.

Uyeda,K., Furuya,E. and Luby,L.J., (1981a), J.Biol.Chem., 256, 8394-8399.

Uyeda,K., Furuya,E. and Dean Sherry,A., (1981b), J.Biol.Chem., 256, 8679-8684.

Van Amelsvoort,J.M.M., Sips,H.J. and Van Dam,K., (1978), Biochem.J., 174, 1083-1086.

Van Amelsvoort,J.M.M., Sips,H.J., Apitule,M.E.A. and Van Dam,K., (1980), Biochim.Biophys.Acta., 600, 950-960.

Van Berkel,T.J.C., Koster,J.F. and Hulsmann,W.C., (1972), Biochim. Biophys.Acta., 276, 425-429.

Van Berkel,T.J.C., Koster,J.F., Kruijt,J.K. and Hulsmann,W.C., (1974), Biochim.Biophys.Acta., 370, 450-458.

Van Berkel,T.J.C., Kruijt,J.K. and Koster,J.F., (1975), FEBS Lett., 52, 312-316.

Van Berkel,T.J.C., Kruijt,J.K., Koster,J.F. and Hulsmann,W.C., (1976), Biochem.Biophys.Res.Commun., 72, 917-925.

Van Berkel,T.J.C., Kruijt,J.K. and Koster,J.F., (1977a), Eur.J. Biochem., 81, 423-432.

Van Berkel,T.J.C., Kruijt,J. and Koster,J.F., (1977b), Biochim.Biophys. Acta., 500, 267-276.

Van Berkel, T.J.C., Kruijt,J., Van den Berg,G.B. and Koster,J.F., (1978), Eur.J.Biochem., 92, 553-561.

Van den Berghe,G., (1978), Curr.Top.Cell.Reg., 13, 97-135.

Van den Berghe,G., Hue,L. and Hers,H.G., (1973), Biochem.J., 134, 637-645.

Van den Berghe,G.,Bronfman,M., Vannesle,R. and Hers,H.G., (1977a), Biochem.J., 162, 601-609.

Van den Berghe,G., Van Pottelsberghe,C. and Hers,H.G., (1977b) Biochem. J., 162, 611-616.

Van den Berghe,G., Bontemps,F. and Hers,H.G., (1980), Biochem.J., 188, 913-920.

Van de Werve,G. and Hers,H.G., (1979), Biochem.J., 178, 119-126.

Van de Werve,G., Hue,L. and Hers,H.G., (1977), Biochem.J., 162, 135-142.

Van Schaftingen,E. and Hers,H.G., (1980), Biochem.Biophys.Res. Commun., 96, 1524-1531.

Van Schaftingen,E. and Hers,H.G., (1981a), Proc.Natl.Acad.Sci.USA, 78, 2861-2863.

Van Schaftingen,E. and Hers,H.G., (1981b), Biochem.Biophys.Res. Commun., 101, 1078-1084.

Van Schaftingen,E., Hue,L. and Hers,H.G., (1980a), Biochem.J., 192, 263-261.

Van Schaftingen,E., Hue,L. and Hers,H.G. (1980b), Biochem.J., 192, 887-895.

Van Schaftingen,E., Hue,L. and Hers,H.G., (1980c), Biochem.J.,192, 897-901.

Van Schaftingen,E., Davies, D.R. and Hers, H-G., (1981a),Biochem.Biophys. Res.Commun., 103, 362-368.

Van Schaftingen,E., Jett,M-F., Hue,L. and Hers,H.G., (1981b), Proc.Natl. Acad.Sci.,USA, 78, 3483-3486.

Van Veelen,C.W.M., Rijksen,G., Vlug,A.M.C. and Staal,G.E.J., (1981), Clinica.Chimica Acta, 110, 113-120.

Veloso,D., Guynn,R.W., Oskarsson,M. and Veech,R.L., (1973), J.Biol. Chem., 248, 4811-4819.

Veneziale,C.M., (1971), Biochemistry, 10, 3443-3447.

Veneziale,C.M., (1972a), Biochemistry, 11, 3286-3289.

Veneziale,C.M., (1972b), Eur.J.Biochem., 31, 59-62.

Veneziale,C.M. (1976), in "Gluconeogenesis: Its Regulation in Mammalian Species" (Hanson,R.W. and Mehlman,M.A., eds.), pp 463-480, Academic Press, New York.

Veneziale,C.M. and Lohmar,P.H., (1973), J.Biol.Chem., 248, 7786-7791.

Vernon,R.G., Eaton,S.W. and Walker,D.G., (1968), Biochem.J., 110, 725-731.

Vessal,M., Choun,M.O., Bissel,M.J. and Bissell,D.M., (1980), Biochim. Biophys.Acta, 633, 201-210.

Videla,L. and Irael,Y., (1970), Biochem.J., 118, 275-281.

Vinuela,E., Salas,M., Sols,A., (1963), J.Biol.Chem., 238, PC1175-PC1177.

Vogels,G.D. and Van den Drift,C., (1970), Analytical Biochemistry, 33, 143-157.

Wagle,S.R. and Ingebreston,W.R.Jr., (1975), Methods in Enzymology, XXXV, 579-607.

Walker,D.G., (1963), Biochim.Biophys.Acta, 77, 209-226.

Walker,D.G. and Rao,S., (1964), Biochem.J., 90, 360-368.

Walker,P.R. and Potter,R., (1973), J.Biol.Chem., 248, 4610-4616.

Walli,A.K., Birkmann,L. and Schimassek,H., (1975), Biochem.Soc.Trans., 3, 1037-1042.

Walli,R.A., (1978), Biochim.Biophys.Acta, 539, 62-80.

Walsh,D.A. and Chen,L-J., (1971), Biochem.Biophys.Res.Commun., 45, 669-675.

Walsh,D.A., Ashley,C.D., Gonzalez,C., Calkins,D., Fisher,E.H. and Krebs,E.G., (1971), J.Biol.Chem., 246, 1977-1985.

Walter,P., (1976), in "Gluconeogenesis: Its Regulation in Mammalian Species" (Hanson,R.W. and Mehlman,M.A., eds.), pp 239-265, J.Wiley & Sons, New York.

Weber,G., Singhal,R.L. and Srivastava,S.K., (1965a), Adv.Enz.Reg., 3, 43-75.

Weber,G., Stamm,N.B. and Fisher,E.A., (1965b), Science, 149, 65-67.

Weber,G., Lea,M.A., Convery,H.J.H. and Stamm,N.B., (1968a), Adv. Enz.Reg., 5, 257-298.

Weber,G., Lea,M.A. and Stamm,N.B., (1968b), Adv.Enz.Reg., 6, 101-123.

Weinhouse,S., (1976), Curr.Top.Cell.Reg., 11, 1-45.

Widdas,W.F., (1971), in "Sugar: Chemical, Biological and Nutritional Aspects of Sucrose" (Yudkin,J., Edelman,J. and Hough,L., eds.), pp 155-171, Butterworths, London.

Williams,J.F., Layde,M.M. and Utter,M.F., (1969), Proc.Aust.Biochem. Soc., 2, 68-79.

Williams,T.F., Exton,J.H., Park,C.R. and Regen,D.M., (1968), Am.J. Physiol., 215, 1200-1209.

Williamson,D.H., (1974), in "Methods in Enzymatic Analysis", (Bergmeyer,H.U., ed.), vol.4, pp 1679-1685, Academic Press, New York.

Williamson,D.H. and Brosnan,J.T., (1974), in "Methods in Enzymatic Analysis" (Bergmeyer,H.U. ed.), vol.4, 2266-2302, Academic Press, New York.

Williamson,D.H., Lund,P. and Krebs,H.A., (1976a), Biochem.J., 103, 514-527.

Williamson,D.H., Lopes-Vieira,O. and Walker,B., (1967b), Biochem.J., 104, 497-502.

Williamson,J.R., Wright,P.H., Malaisse,W.J. and Ashmore,J., (1966a), Biochem.Biophys.Res.Commun., 24, 765-770.

Williamson,J.R., Browning,E.T. and Olson,M.S., (1968), Adv.Enz. Reg., 6, 67-100.

Williamson,J.R., Jakob,A. and Refine,C., (1971), J.Biol.Chem., 246, 7632-7641.

Wilson,J.E., (1980), Curr.Top.Cell.Reg.,16, 2-53.

Wolfe,B.M., Ahuja,S.P. and Marliss,E.B., (1975), J.Clin.Invest., 56, 970-977.

Wood,C.L., Babcock,C.J. and Blum,J.J., (1981), Arch.Biochem.Biophys., 212, 43-53.

Woods, H-F. and Krebs,H.A., (1973), Biochem.J., 132, 55-60.

Woods,H-F., Eggleston,L.V. and Krebs,H.A., (1970), Biochem.J., 119, 501-510.

Yip, B.P. and Lardy, H.A., (1981), Arch.Biochem.Biophys., 212, 370-377.

Ylikahri,R.H., Kähönen,M.T. and Hassinen,I., (1972), Acta.Med.Scand. Suppl., 542, 141-150.

Yorek,M.A., Rufo,G.A.Jr. and Ray,R.D., (1980), Biochim.Biophys.Acta, 632, 517-526.

Yorek,M.A., Rufo,G.A.Jr., Blair,J.B. and Ray,R.D., (1981), Biochim. Biophys.Acta, 675, 309-315.

Yudkin,J., (1971), in "Sugar:Chemical, Biological and Nutritional Aspects of Sucrose", (Yudkin,J., Edelman,J. and Hough,L., eds.), pp 231-242, Butterworths, London.

Zakin,D., Pardini,R.S., Herman,R.H. and Sauberlich,H.E., (1967), Biochim.Biophys.Acta, 144, 242-251.

Zahner,J., Loy,E., Müllhofer,G. and Bücher,T., (1973), Eur.J. Biochem., 34, 248-255.

Zuurendonk,P.F., Akerboom,T.B.F. and Jager,J.M., (1976), in "Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies", (Tager,J.M., Söling,H.D. and Williamson,J.R., eds.), pp 17-27, North-Holland Publishing Company, Amsterdam.