STUDIES ON

HEPATIC FRUCTOSE METABOLISM

A Thesis submitted by

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in

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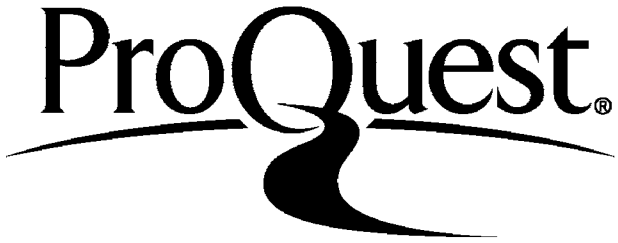

To my Grandparents with love.

ABSTRACT

The hepatic metabolism of fructose in fed rats was studied using perfused liver and isolated hepatocytes. Fru-1-P has been shown by many workers to be the major initial product of fructose metabolism. However, the present study shows the simultaneous accumulation of a higher molecular-weight compound. This product was found to predominate at low fructose concentrations, whereas Fru-1-P was the major product on incubation with more than 4mM-fructose. The unknown metabolite was isolated and purified by a variety of techniques. Gel-filtration studies indicated a molecular weight of 583 and on acid hydrolysis the compound was shown to contain carbohydrate, phosphate and amino acid positive material. The unknown was unstable to acid, alkaline phosphatase and barium acetate precipitation, yielding a variety of different products in each case.

Fructokinase, purified 100-fold from the livers of adult male rats, was shown to utilise GTP as well as ATP as nucleotide triphosphate. In both cases Fru-1-P was the sole labelled product of the fructokinase reaction with [U-^{14}C] fructose. The purified enzyme was found, with ATP as substrate, to have K_m values of 0.83mM for fructose and 1.43mM for MgATP. With GTP as substrate, constants of 0.56mM for fructose and 1.65mM for MgGTP were determined. Both reactions were inhibited by ADP and GDP and inhibition constants calculated. The utilisation of both nucleotide triphosphates following a fructose load is discussed.

Phosphofructokinase activity, assayed in crude extracts of isolated hepatocytes, was shown to be increased on incubation of the cells with low concentrations (1-8mM) of fructose, dihydroxyacetone or galactose or with high glucose (20mM) concentrations. The activation, proposed to be due to Fru-2,6-P_2 accumulation, was abolished by filtration of the extract down a Sephadex G-25 column. Glycerol (2-8mM) and glucose (1-8mM) were without significant effect and 20mM-fructose caused an inhibition of enzyme activity relative to controls. The latter effect is thought to be due to the accumulation of Fru-1-P, an inhibitor of hepatic fructokinase.

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ABBREVIATIONS

Enzymes

Fructose-1,6-bisphosphatase	Fru-1,6-P_2ase
Fructose-2,6-bisphosphatase	Fru-2,6-P_2ase
Phosphofructokinase	PFK
Phosphofructokinase-2	PFK-2
Dehydrogenase	DH

Substrates

Fructose-1-phosphate	Fru-1-P
Fructose-2-phosphate	Fru-2-P
Fructose-6-phosphate	Fru-6-P
Fructose-1,6-bisphosphate	Fru-1,6-P_2
Fructose-2,6-bisphosphate	Fru-2,6-P_2
Glucose-6-phosphate	Glc-6-P
Glucose-1,6-bisphosphate	Glc-1,6-P_2

Nucleotides

Adenosine 5'-mono-,di-, and triphosphate	AMP, ADP, ATP
Cyclic AMP	cAMP
Guanosine 5'-mono-, di-, and triphosphate	GMP, GDP, GTP
Inorganic phosphate	P_i
Inosine 5' - mono - and triphosphate	IMP, ITP
β-nicotinamide adenine dinucleotide and its reduced form	NAD^+, NADH
β-nicotinamide adenine dinucleotide phosphate and its reduced form	$NADP^+$, NADPH
Uridine 5'-triphosphate	UTP

Chelators and buffers

Ethylenediaminetetraacetic acid	EDTA
Ethyleneglycolbis(amino-ethylether)tetra-acetic acid	EGTA
2-amino-2-hydroxymethylpropane-1,3-diol	Tris
4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid	Hepes

Kinetic constants

Michaelis constant	K_m
Inhibition constant	K_i
Maximal velocity	V_{max}

Others

Chemical ionisation	c.i.
Electron impact	e.i.
Ionized molecule	M^+

The mass of an ion divided by its charge	m/z
The decomposing ion in any reaction	parent
The product of an ionic reaction	daughter
Trifluoroacetic acid	TFA
2,5-diphenyloxazole	PPO

I. INTRODUCTION

The increasing incidence of heart disease in Western countries has focused attention on the constituents of the daily diet. The consumption of D-fructose, a major dietary carbohydrate, has been shown to lead to several conditions which predispose the susceptible patient to heart disease. The major dietary sources of fructose are sucrose, fruits and honey. Sucrose is a disaccharide which is broken down by sucrase in the small intestine, to glucose and fructose. Fructose is the sweetest natural sugar and can therefore sweeten with fewer calories than glucose. Against this apparent dietary advantage must be weighed the proposed adverse effects of fructose in relation to heart disease.

Yudkin et al. (1969) have shown that serum insulin levels and platelet adhesiveness can be correlated with sucrose intake in patients with peripheral vascular disease. No such correlations were found in men without the disease or its predisposing factors. Sucrose-rich diets have been shown to increase serum cholesterol (Simko, 1980) and triglyceride levels (Sleder et al., 1980). Both glucose and fructose diets were reported to cause an increase in serum triglyceride and insulin levels, the effect being greater with fructose than with an isocaloric intake of glucose (Sleder et al., 1980). Hyperinsulinaemia only occurred in rats maintained on a 60% fructose diet for at least a week (Sleder et al., 1980). Hara and Saito (1981) reported that oral fructose inhibited the insulin response to oral glucose. No effect on the insulin response to glucose was observed when fructose was administered orally together with glucose

by intravenous infusion or when fructose was administered intravenously and glucose orally. It was therefore proposed that the inhibitory effect of fructose on insulin secretion was mediated through signals generated in the gastointestinal tract (Hara and Saito, 1981).

High fructose diets also appear to lead to increased serum uric acid and blood lactate levels (Solyst et al., 1980). No evidence has been found for a link between hyperuricaemia and coronary heart disease but good correlation does exist between the other predisposing factors (high serum triglycerides and cholesterol levels) and uric acid levels (Solyst et al., 1980).

A. Fructose metabolism in rat liver

The liver has been established as the main organ of fructose metabolism in most animal species (Heinz, 1972). Fructose can be phosphorylated by hexokinase to Fru-6-P, however, the enzyme has only low activity in the liver (0.25 U/g-Heinz et al., 1968) and a higher affinity for glucose, K_m 0.1mM (Heinz et al., 1968), than for fructose, K_m 3.4mM (Gumaa and Mclean, 1972; Grossband and Schimke, 1966). It therefore seems unlikely that this pathway plays a major role in fructose metabolism. The existence of a specific fructokinase for the utilisation of fructose was known as early as 1950 and its primary phosphorylation product was shown to be Fru-1-P (Cori et al., 1951; Hers, 1955). The metabolism of Fru-1-P involves the sequential operation of liver aldolase and triokinase resulting in the formation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate which are converted mainly to glucose and lactate in the short-term (Fig. 1).

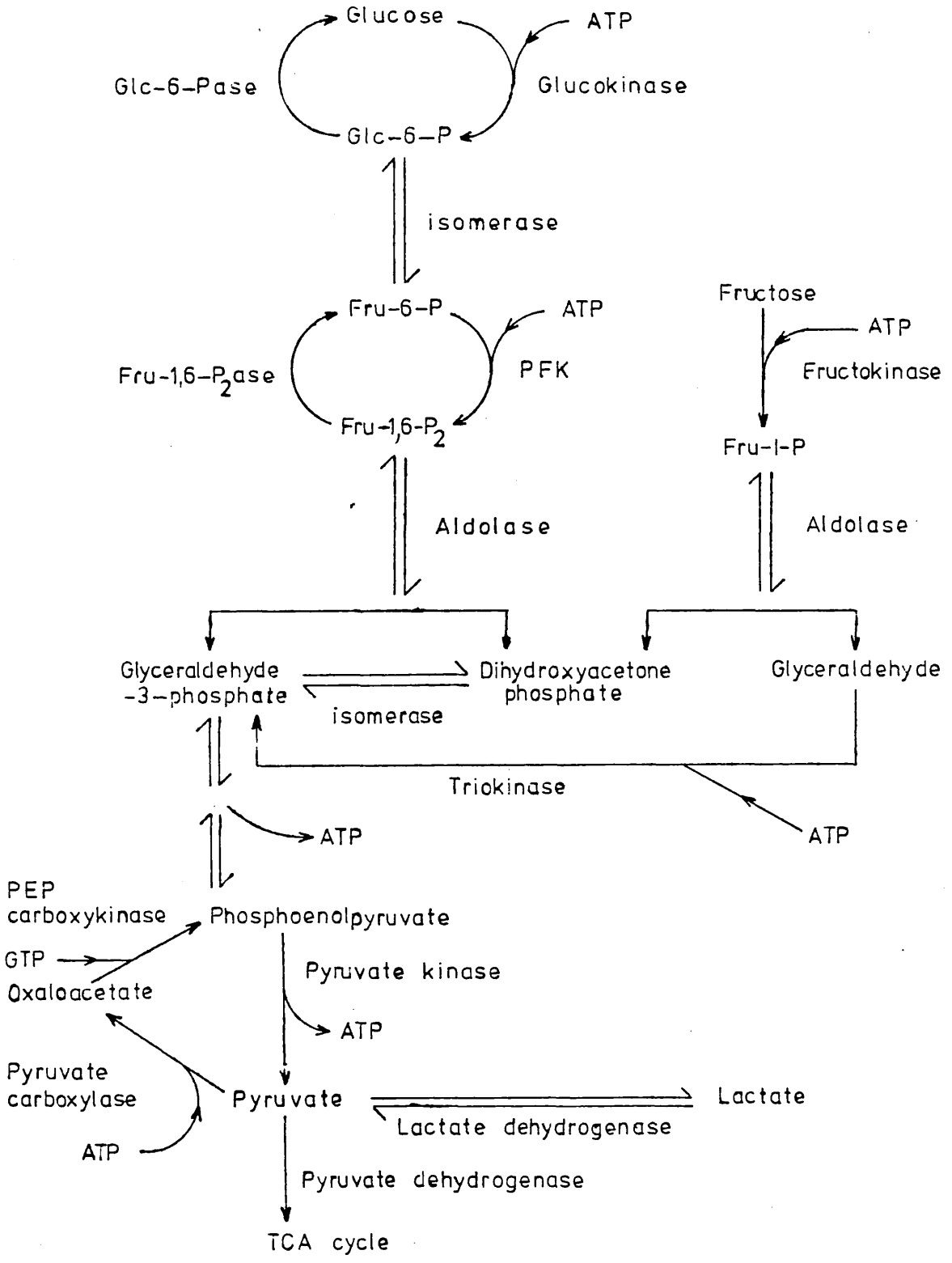


Fig. 1. Hepatic metabolism of glucose and fructose.

1. Transport of D-fructose

In 1958, Cahill et al. proposed that hexoses enter and leave liver cells by free diffusion across the plasma membrane. Ten years later Williams et al. (1968), using perfused liver, demonstrated that D-glucose was taken up more rapidly than L-glucose and that transport of either sugar was temperature dependent and inhibited by phlorizin. From this they concluded that a specific transport system exists for glucose and estimated the V_{max} to be 345 µmol/min/ml cell water and the K_m to be 17mM.

Craik and Elliott (1980), using isolated heptocytes, showed that a specific carrier system exists for fructose and they proposed that galactose and 3-O-methyl-D-glucose are transported by the same system, since all three sugars competitively inhibit each other's transport. The system exhibits typical Michaelis-Menten kinetics with V_{max} and K_m values of 291µmol/min/ml cell water and 212mM respectively for fructose (Craik and Elliott, 1980). This K_m value is considerably higher than the K_m of 70mM reported by Sestoft (1974) from clearance studies in the perfused liver. However, the latter method does not accurately differentiate between transport and metabolism. These kinetic studies together with the low K_m for fructose (0.46mM) of fructokinase (Sánchez et al., 1971a) suggest that the rate-limiting step in fructose metabolism is not the phosphorylation of fructose but its rate of entry into the liver cell. Perfusion studies with fructose have supported this proposal, indicating a steep gradient between extra- and intracellular fructose concentrations (Woods et al., 1970; Sestoft, 1974).

2. Fructokinase (EC 2.7.1.3)

Fructokinase activity is located in the cytoplasm (Adelman et al., 1967a) of mammalian liver (Heinz et al., 1968; Heinz and Weiner, 1969), kidney (Heinz and Lamprecht, 1967) and intestinal mucosa (Grand et al., 1974 Bode et al., 1980). Rat liver fructokinase activity is 2.5 - 3.0U/g at 25°C and the enzyme has been partially purified by several groups of workers (Parks et al., 1957; Adelman et al., 1967a; Sánchez et al., 1971a).

ATP-Mg has been shown to be the true substrate for fructokinase, since an excess of either ATP or Mg^{2+} causes inhibition of enzyme activity (Parks et al., 1957; Sánchez et al., 1971b). 2'-dATP and 3'-dATP are reported to be the only alternative phosphate donors for the phosphorylation of fructose (Adelman et al., 1967a). The purified enzyme has an absolute requirement for K^+, which also stimulates enzyme activity (Parks et al., 1957; Sánchez et al., 1971b). K_m values of 0.46mM and 1.56mM have been reported for fructose and MgATP respectively at 0.4M-KCl(Sánchez et al., 1971a).

In addition to D-fructose, fructokinase also catalyses the phosphorylation of D-xylulose, L-sorbose, L-galactoheptulose (Adelman et al., 1967a) and D-tagatose (Sánchez et al., 1971a) to the corresponding 1-phosphates. The substrate and anomeric specificities of fructokinase have been studied,using purified beef liver enzyme and a variety of anhydroalditol derivatives,by Raushel and Cleland (1973; 1977a). From this the enzyme appears to be specific for a tetrahydrofuran ring, with the hydroxymethyl group which is phosphorylated having the β-D (or α-L)configuration and the hydroxyls on C-3 and C-4

having preferred configurations of L and D respectively. The anomeric hydroxyl group on C-2 contributes to enzyme binding and a wide range of groups are possible at C-5 (Fig. 2). The affinity of the enzyme was shown to be considerably reduced for all substrates other than D-fructose and L-sorbose.

A pathway for the metabolism of D-xylitol to glycolaldehyde (a known oxalate precursor) has recently been proposed (Fig. 3) involving the sequential operation of fructokinase and liver aldolase (James et al., 1982; Barngrover and Dills, 1983). Fructokinase, at 0.1M-KCl, has a K_m of 6mM for D-xylulose (James et al., 1982) compared to 0.8mM for D-fructose (Sánchez et al., 1971a; James et al., 1982). In 1982, James et al. demonstrated that dihydroxyacetone phosphate and glycolaldehyde were formed by the incubation of purified human liver fructokinase and aldolase with D-xylulose. Barngrover and Dills (1983) have since shown accumulation of xylulose-1-phosphate and glycolaldehyde in isolated hepatocytes incubated with 2 to 20mM-D-xylitol, thereby providing further evidence for the proposed pathway for glycolaldehyde production. These workers do not dispute the existence of an alternative pathway of D-xylitol metabolism, via xylulose-5-phosphate and the pentose phosphate pathway, although attempts to link this to glycolaldehyde production through a transketolase reaction have not proved conclusive.

Rognstad (1982b) showed that the distribution of [^{14}C] in glucose, produced from [3-^{14}C] D-fructose or [3-^{14}C] D-tagatose by isolated hepatocytes, was identical indicating that D-tagatose is metabolised by the same pathway as D-fructose.

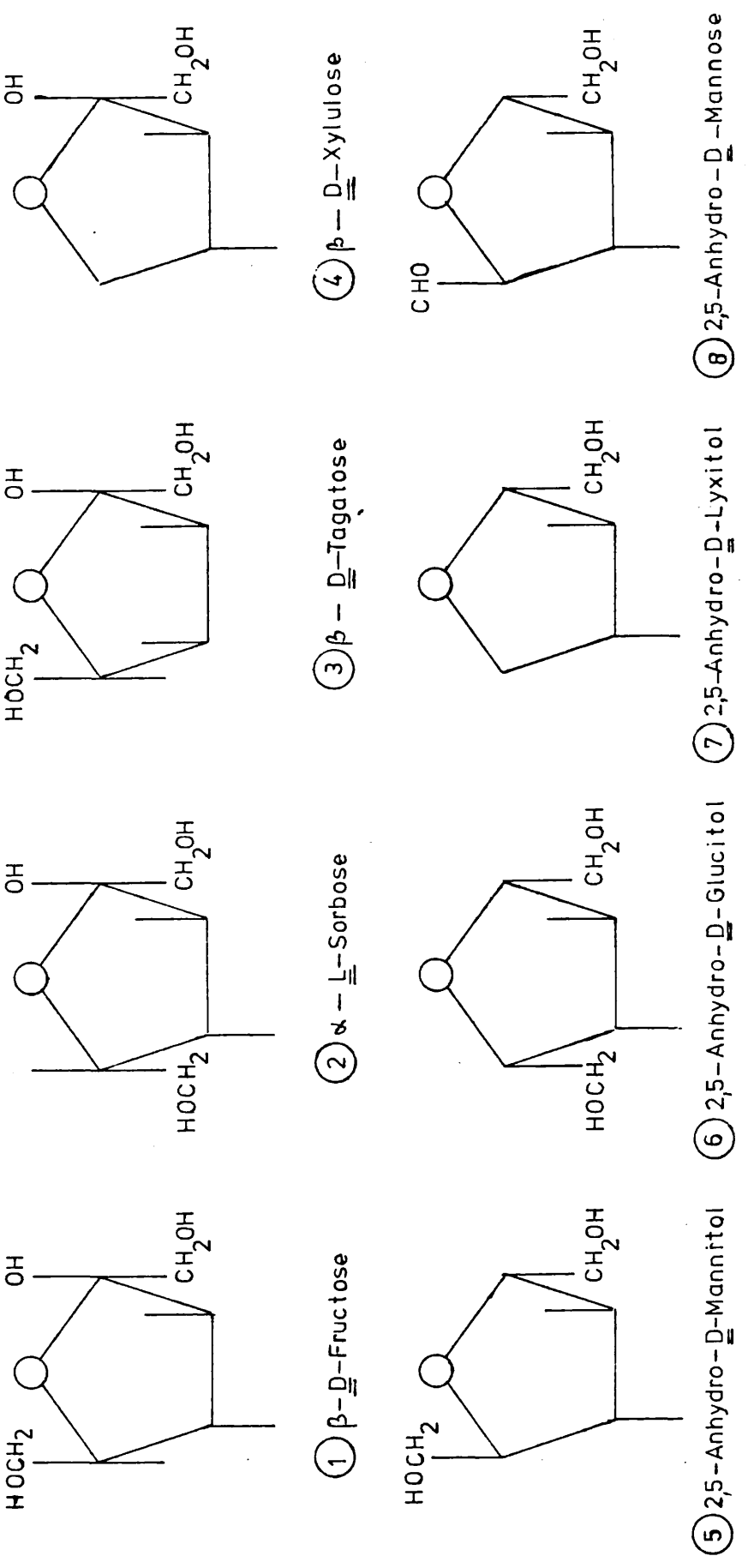


Fig. 2. Substrates for fructokinase (Raushel and Cleland, 1973).

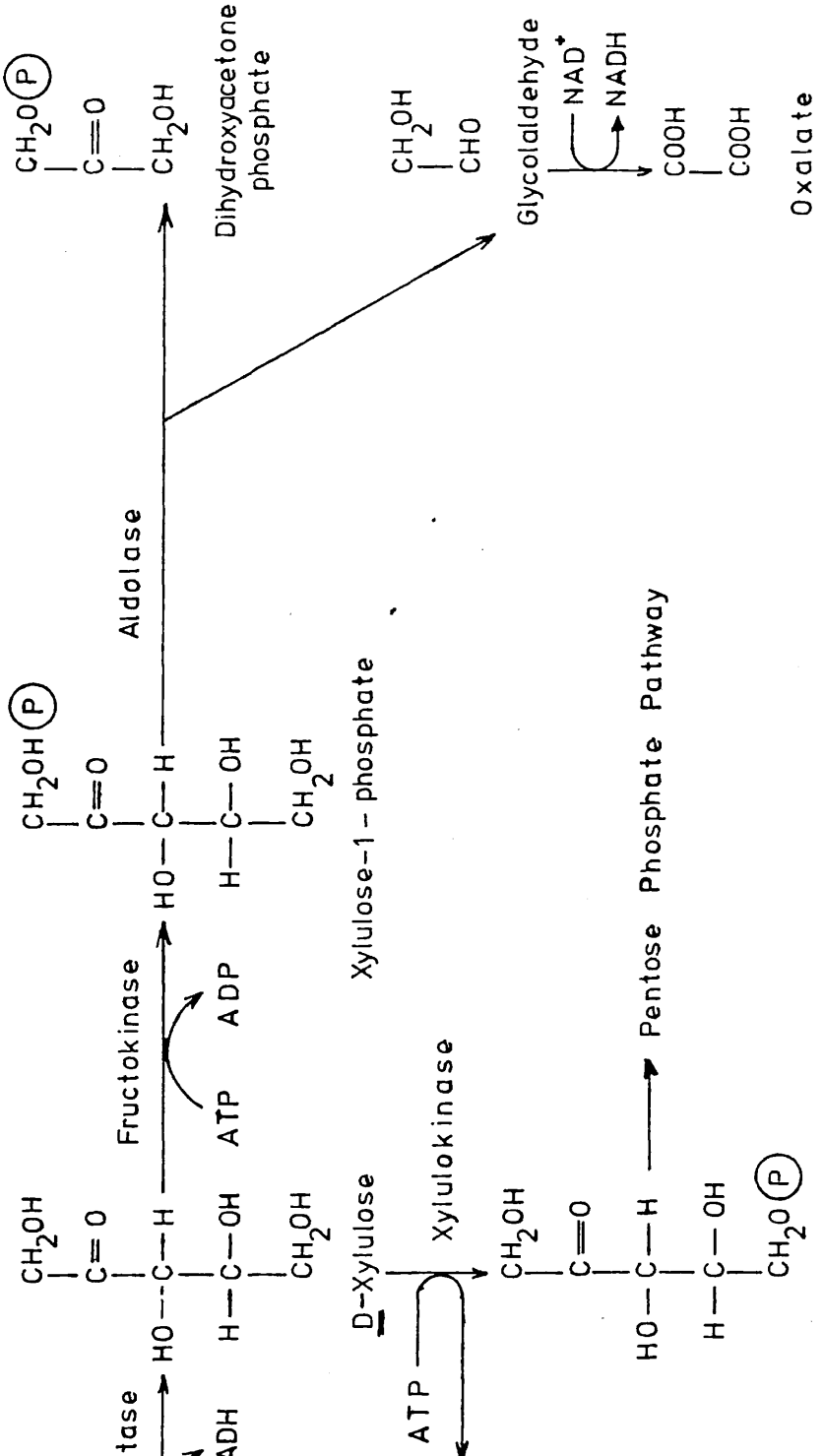


Fig. 3. Pathways for D-xylitol metabolism (James et al., 1982).

England et al. (1972) reported that while 5-keto-D-fructose is phosphorylated by fructokinase, the product, 5-keto-D-Fru-1-P, competitively inhibits the cleavage of Fru-1-P by liver aldolase. The presence of the carbonyl group on C-5 appears to prevent the cleavage of aldolase between C-3 and C-4.

ADP has been shown to be an inhibitor of the fructokinase reaction, with a K_i of 1.3 - 1.7mM (Parks et al., 1957; Sánchez et al., 1971b). Sánchez et al. (1971b) showed that K^+ partially reversed ADP inhibition and proposed two binding sites on the enzyme, one for the phosphate donor (ATP-Mg), the other for K^+ and the phosphate acceptor (fructose).

Isotope exchange studies, on the beef liver enzyme, have shown fructokinase to have a random kinetic reaction mechanism in which the release of fructose is slower than the catalytic reaction (Raushel and Cleland, 1977b). Enzyme activity appeared, from pH profiles, to require a group (possibly carboxyl) to be ionised and another group (possibly lysine) to be protonated. A reaction mechanism was therefore proposed, in which a carboxyl group on the enzyme accepts a proton from the 1-hydroxyl of fructose (Raushel and Cleland, 1977b).

Grand et al. (1974) were able to demonstrate fructokinase activity in foetal liver four days before birth. The incorporation of [U-^{14}C] fructose into liver glycogen in the developing liver parallels the increase in fructokinase and triokinase activities, which approach adult levels within 25 days of birth (Sillero et al., 1970; Ballard and Oliver, 1964).

Several workers have shown that fructokinase activity is affected by diet and hormones (Grand et al., 1974; Adelman et al., 1966). Feeding adult rats with sucrose or fructose, after a 48-72h carbohydrate fast, increases enzyme activity while actinomycin D treatment, before or during sucrose feeding, inhibits the increase in fructokinase activity (Grand et al., 1974). Thus, fructokinase was proposed to be induced by a high fructose diet, with regulation at the level of transcription. Recent work by Bode et al. (1980) has shown that feeding a 60% fructose diet to fed rats increases intestinal enzyme levels, but has little or no effect on liver activity. Earlier Adelman et al. (1966) had shown that enzyme activity was only affected by fructose feeding if the total liver activity, as opposed to specific activity (expressed in terms of liver weight), was considered. Bode et al. (1980) confirmed this result, although they only measured the latter activity, whereas Grand et al. (1974) showed an effect in terms of specific activity expressed per mg protein. Whether pre-starving the rats before a fructose load is essential to show any fructose-induced effects remains unclear from the results so far obtained.

Essential fructosuria is a rare, symptomless condition characterised by high blood fructose levels and the excretion of fructose in urine after ingestion of fructose (Hers and Joassin, 1961). It is proposed to be due to a lack of fructokinase and is inherited as an autosomal recessive trait (Van den Berghe, 1978).

3. Aldolase IB (EC 4.1.2.7)

Depending on their properties aldolases are described as

Class I (resembling mammalian muscle aldolase) or Class II (resembling yeast enzyme activity). In addition to different cofactor requirements and molecular and catalytic properties the two classes are proposed to have different reaction mechanisms. Class I aldolases, in the presence of sodium borohydride, form a stable covalent bond with dihydroxyacetone phosphate *in vitro*. The resulting stable enzyme-substrate complex is catalytically inactive. A similar loss of aldolase activity is observed on incubation of aldolase with Fru-1,6-P_2 and sodium borohydride (Rutter, 1964). It has been proposed that the carbonyl group of dihydroxyacetone phosphate forms a Schiff's base with the ε-amino group of a lysine residue at the active site of the enzyme. As shown in Fig. 4 cleavage is then achieved by proton loss, gain and exchange yielding dihydroxyacetone phosphate and glyceraldehyde from Fru-1-P.

Class I aldolase activity in mammals has been found to be due to three distinct isoenzymes; A, B and C, with different specificities towards Fru-1,6-P_2 and Fru-1-P. Aldolase B has been found in the liver, kidney cortex and small intestine of mammals and of some vertebrates (Heinz, 1972). The properties of the enzyme from rat liver (Woods *et al.*, 1970), kidney (Heinz and Lamprecht, 1967) and intestinal mucosa (Bode *et al.*, 1980) have been studied. It cleaves Fru-1-P to dihydroxyacetone phosphate and D-glyceraldehyde and is also able to cleave Fru-1,6-P_2 at a similar rate, to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Rutter, 1964; Adelman *et al.*, 1967b; Sillero *et al.*, 1969). Aldolase A predominates in the foetal liver and adult muscle and utilises Fru-1,6-P_2 at least 50 times faster than Fru-1-P (Rutter, 1964; Adelman *et al.*, 1967b). The major enzyme form in brain is aldolase C which

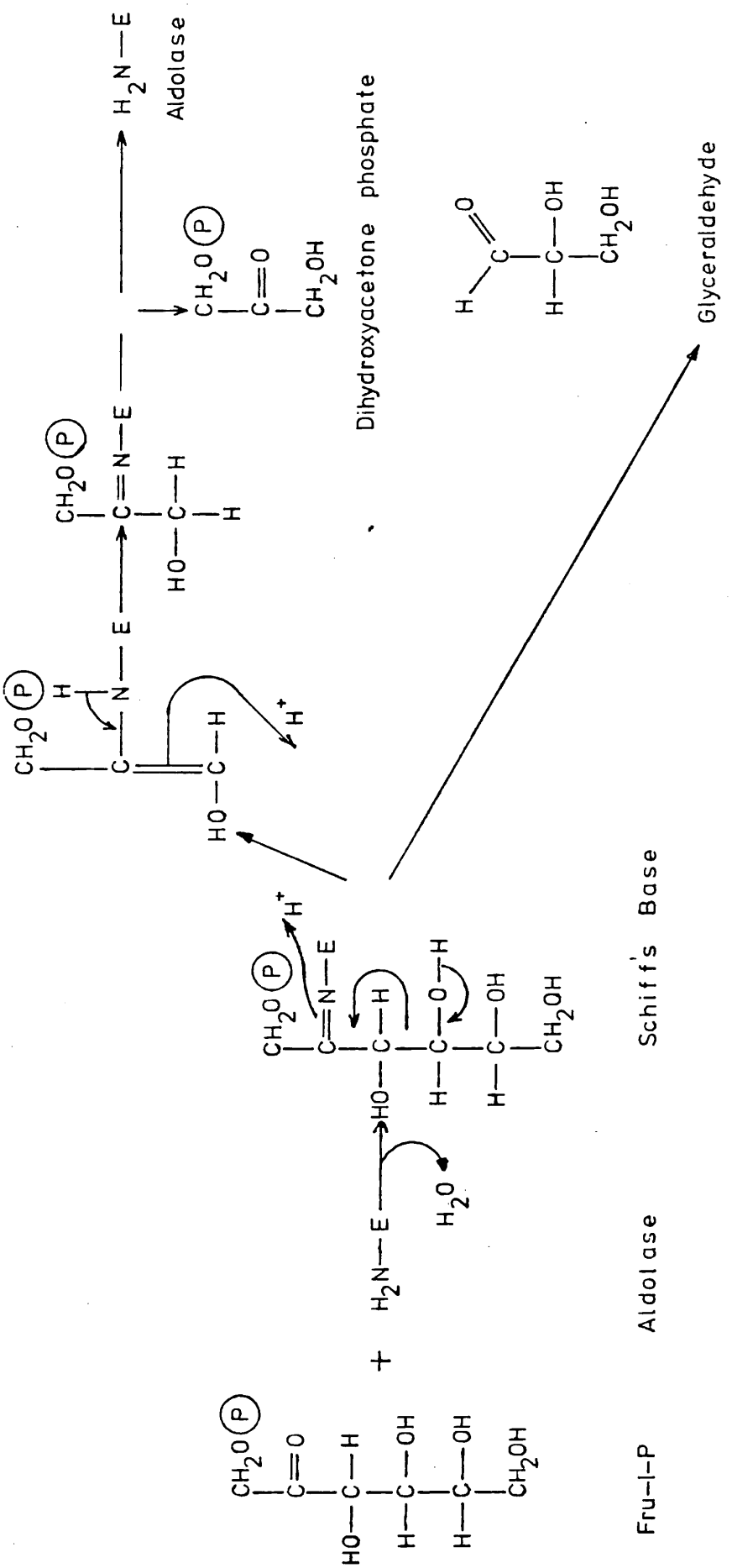


Fig. 4. Proposed mechanism for the cleavage of Fru-1-P by aldolase 1B.

has a Fru-1,6-P_2: Fru-1-P activity ratio of 10 (Van den Berghe, 1978).

In liver aldolase B is the predominant isoenzyme, accounting for 98% of the aldolase protein (Gitzelmann et al., 1982). Whilst this profile is maintained in well-differentiated hepatic tumours, aldolase A predominates in poorly-differentiated tumours (Adelman et al., 1967b). The isoenzymes have been shown to differ in their kinetic properties. Aldolase A is competitively inhibited by ATP, ADP and AMP, with the ATP inhibition being overcome by Mg^{2+}. Aldolase B is not affected by ATP but is inhibited by AMP and ADP (Adelman, 1972). Rat liver aldolase B has a K_m for Fru-1-P of 0.35mM (Woods et al., 1970). The rabbit liver enzyme is a tetramer with a molecular weight of 154 000 and reported K_m values of 0.8mM for Fru-1-P and 2μM (Rutter, 1964) or 10μM (Heinz, 1973) for Fru-1,6-P_2.

Rat liver aldolase B activity, at 25°C, has been reported to be 1.63U/g (Heinz et al., 1968), 3.4U/g (Adelman et al., 1966; Woods et al., 1970) and 4.5U/g (Sillero et al., 1970). The effect of diet on enzyme activity over a period of days has been studied by several groups of workers. Adelman et al. (1966) claimed a 50% decrease in total liver aldolase activity on fasting (48-72h), normal activity being restored in 24h by fructose or glucose feeding. These workers observed no change in specific enzyme activity and their initial observation can probably be attributed to the normal decrease in liver weight which occurs on fasting. They were also unable to show any significant change in enzyme activity per gram of liver on fructose feeding but did report a small increase in total

activity per liver. Other workers have shown that rats fed a 60% fructose diet for at least 3 days show no significant change in aldolase activity per gram of liver (Sillero et al., 1969; Bode et al., 1980) but a 3-fold increase in intestinal mucosa enzyme activity has been reported (Bode et al., 1980). Aldolase B activity in the liver of newborn rats has been shown to be similar to adult levels, in common with many other carbohydrate metabolising enzymes (Sillero et al., 1970).

Hereditary fructose intolerance is a disorder of fructose metabolism in which the symptoms are induced by the intake of food containing fructose or sucrose (Harris, 1975). The clinical manifestations resulting include vomiting, hypoglycemia, shock, and liver failure and can eventually lead to death if the intake of fructose continues. Laboratory investigations have revealed liver failure, proximal tubular dysfunction and a derangement of intermediary metabolism (Gitzelmann et al., 1982). Treatment involves the removal from the diet of all foods containing fructose or sucrose. The inheritance is autosomal recessive and the enzyme defect is that of aldolase B (Gitzelmann et al., 1982). The disease is therefore characterised by a decrease in aldolase activity in liver, but not in muscle, and an accumulation of hepatic Fru-1-P (Hers and Joassin, 1961). The decrease in aldolase activity in liver is greater with Fru-1-P as substrate than with Fru-1,6-P_2. The Fru-1,6-P_2: Fru-1-P aldolase activity ratios for sufferers of the disease are 6:1 compared to 1:1 in normal livers (Hers and Joassin, 1961). It is not clear whether this is due to an increase in the activity ratio of aldolase B or to the persistence of foetal aldolase A activity and the presence of

aldolase C in the liver. There is sufficient residual aldolase activity for normal glycolysis and gluconeogenesis, the residual activity being only rate-limiting following a fructose load (Gitzelmann et al., 1982).

The fructose-induced hypoglycemia is proposed to arise as a result of inhibition of glucose release from the liver. Since dihydroxyacetone administration cannot correct glucose levels a block of gluconeogenesis, through inhibition of Glc-6-P isomerase, by Fru-1-P, (Zalitis and Oliver, 1967) and aldolase activity, towards condensation of triose phosphates to Fru-1,6-P_2, is proposed (Gitzelmann et al., 1982). Neither glucagon or dibutyryl cAMP administration restore blood glucose concentrations and hence inhibition of glycogenolysis is also suspected (Van den Berghe et al., 1973). Since galactose infusion can overcome the hypoglycemia, phosphoglucomutase activity must be unaffected by fructose loading (Zalitis and Oliver, 1967). Inhibition of phosphorylase activity (Section I.B.3.a) and an inhibition of gluconeogenesis below Glc-6-P are proposed to account for the hypoglycemia arising in patients with hereditary fructose intolerance consuming fructose.

4. Triokinase (EC.2.7.1.28)

The D-glyceraldehyde, formed as a result of the aldolase IB reaction, can be further metabolised to:

(a) glyceraldehyde-3-phosphate by triokinase,

(b) 2-phosphoglycerate by aldehyde dehydrogenase and glycerate kinase,

(c) dihydroxyacetone phosphate by NAD^+- or $NADP^+$-dependent alcohol dehydrogenase, glycerol kinase and glycerol-3-phosphate dehydrogenase,

as shown in Fig. 5. The pathways operating during fructose metabolism have been exhaustively studied. In 1961, Kattermann et al. showed an increase in glycerate concentration following intraperitoneal injection of fructose, thereby providing evidence for the operation of the oxidative pathway. However, Sillero et al. (1969) have shown that at physiological pH triokinase would have the greatest affinity for glyceraldehyde, the contribution from aldehyde dehydrogenase would be minimal and the aldehyde dehydrogenase pathway would only be expected to operate when glyceraldehyde concentrations were high (Table 1).

Table 1. Activities of enzymes metabolising D-glyceraldehyde in rat liver (Sillero et al., 1969).

Enzyme	V_{max} (μmol/min/g liver)	Km (mM)
Triokinase	1.0	0.01
Aldehyde dehydrogenase	0.4	0.30
Alcohol dehydrogenase		
NAD^+- dependent	4.0	11.00
$NADP^+$- dependent	0.5	0.60

Support for this came from studies involving perfusion of rat liver with 10mM-fructose, glycerol-3-phosphate levels increased 8-fold, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate increased 4-fold. The concentration of 2- and 3-phosphoglycerate initially fell and then showed only a slight increase (Woods et al., 1970). Isotope labelling experiments,

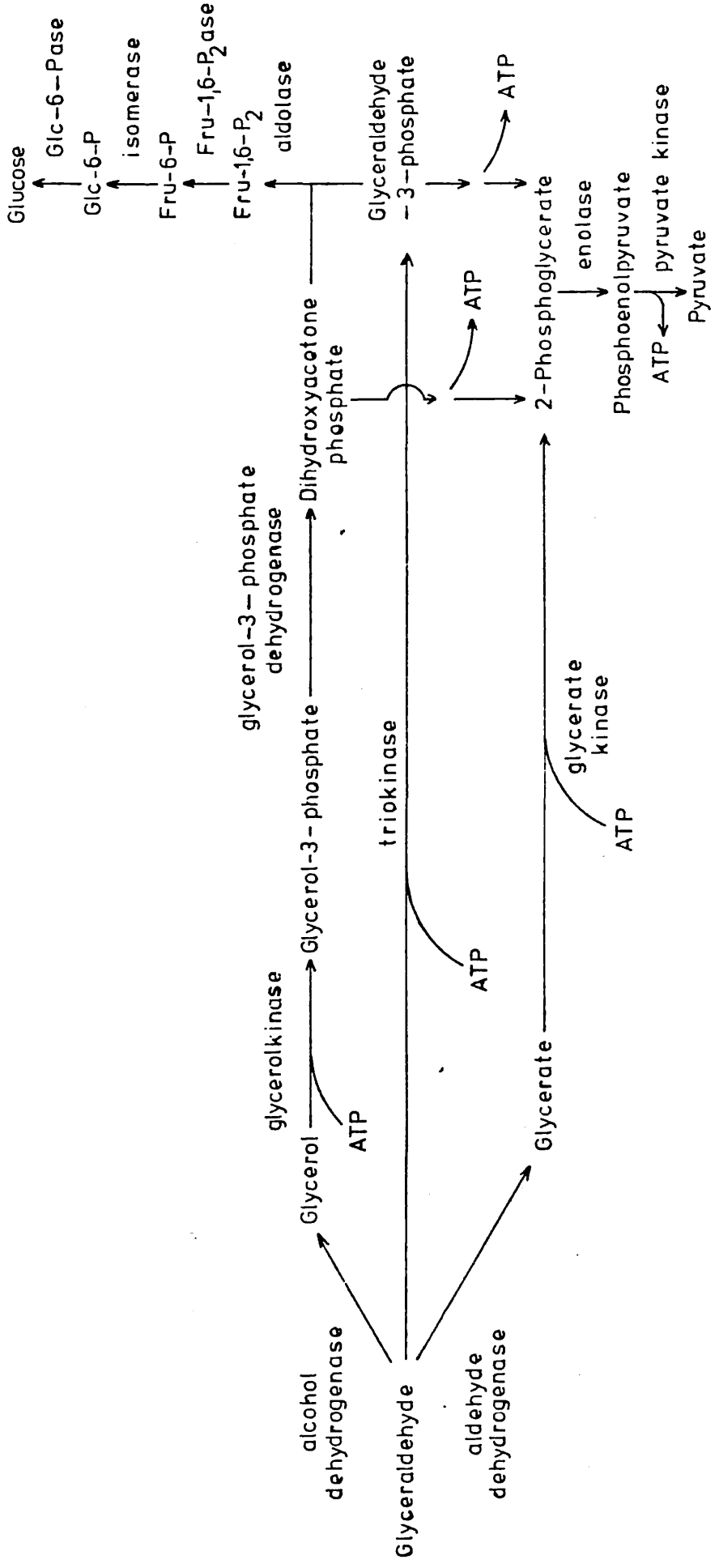


Fig. 5. Alternative pathways of D-glyceraldehyde metabolism.

with [6-^{14}C] fructose (Landau and Merlevede, 1963) and [4-^{3}H, 6-^{14}C] fructose (Hue and Hers, 1972) have shown randomisation of C-6 and C-1 in liver glycogen compatible with fructose being metabolised through conversion of glyceraldehyde to glyceraldehyde-3-phosphate. In the latter experiment, with [4-^{3}H,6-^{14}C] fructose, the loss of tritium to fructose metabolism was approximately equal to the C-6 - C-1 randomisation at 29%. Furthermore, the ^{3}H : ^{14}C ratio in glyceraldehyde-3-phosphate was the same as in fructose, if metabolism had been via glycerate a complete loss of tritium would have been expected (Hue and Hers, 1972).

Triokinase has been isolated and purified from the livers of guinea pigs (Hers and Kusaka, 1953), cattle (Heinz and Lamprecht, 1961) and rats (Frandsen and Grunnet, 1971). Triokinase activity in rat liver is 1.65 U/g at 37^{o}C (Heinz *et al.*, 1968). The enzyme was proposed to be ATP-specific by Heinz and Lamprecht (1961), although Hers and Kusaka (1953) had earlier shown that the beef liver enzyme can also utilise ITP as a phosphate donor. More recently, Frandsen and Grunnet (1971) have reported that GTP or ITP can act as alternative phosphate donors. However, the reaction with either of these nucleotides is only 10% of the rate obtained with ATP as substrate. ATP-Mg is the true substrate for the reaction with a reported K_m, which is independent of glyceraldehyde concentration, of 0.8mM (Frandsen and Grunnet, 1971; Thieden *et al.*, 1972). ADP (K_i 1.1mM) and free ATP (K_i 0.38mM) have been shown to be competitive inhibitors with respect to glyceraldehyde. Hill plots of the data with ATP and ADP suggest the existence of more than one binding site for ATP and at least one binding site for ADP (Frandsen and Grunnet, 1971). Free Mg^{2+} is a

competitive activator with respect to MgATP since it decreases the K_m for MgATP but has no effect on maximal enzyme activity (Frandsen and Grunnet, 1971). Whether Mg^{2+} acts as an allosteric activator, removes an inhibitor from the enzyme or is involved in aiding the binding of ATP to the enzyme is not clear from the data obtained. The results do, however, suggest three binding sites on the enzyme, two for the substrates, ATP-Mg and glyceraldehyde, and one for the effectors, ATP, Mg^{2+} and ADP.

The K_m for D-glyceraldehyde has been reported to be 10μM (Sillero et al., 1969) and between 14 and 17μM (Thieden et al, 1972), in the presence of 6mM-MgATP. Frandsen and Grunnet (1971) proposed that the K_m for glyceraldehyde was dependent on the MgATP concentration and reported K_m values of 8-35μM with 1-3mM-MgATP. Triokinase can also phosphorylate dihydroxyacetone with a K_m of 6μM and a V_{max} 2.5-times higher than with glyceraldehyde (Frandsen and Grunnet, 1971).

Liver, kidney and intestinal mucosa triokinase activities have been shown to increase 2-3 fold when rats are fed a fructose-enriched diet (Heinz, 1968; Bode et al., 1980). Fasting leads to a fall in hepatic enzyme activity which is fully restored by fructose feeding while glucose causes only a partial restoration of activity (Adelman et al., 1966). Fed adrenalectomised or hypophysectomised rats have normal triokinase activity which falls rapidly on fasting. However, normal levels are not restored either by fructose or glucose feeding (Adelman et al., 1966).

Since fructokinase and triokinase have a parallel course

of appearance in the developing rat (Sillero et al., 1970) and show a fall in activity in well-differentiated tumours and a total loss of activity in poorly-differentiated tumours (Adelman et al., 1967b), it has been proposed that the two enzymes are under co-ordinated gene control (Sillero et al., 1970).

B. Consequences of a fructose load

The characteristic features of the effect of intravenous injection, liver perfusion or hepatocyte incubation with fructose are a depletion of ATP and the total adenine nucleotide pool and a rapid increase in Fru-1-P levels. These metabolite changes can be at least partially explained in terms of adenine nucleotide degradation.

1. Effect of fructose on adenine nucleotide catabolism.

a. Degradation of adenine nucleotides

The adenine nucleotide pool is maintained in close equilibrium by myokinase in the cell. The hepatic catabolism of adenine nucleotides proceeds from AMP via two possible pathways to allantoin as shown in Fig. 6. Van den Berghe et al (1977a,b) have studied the kinetics of AMP deaminase and 5'-nucleotidase at physiological concentrations of substrate, AMP (0.2mM) and effectors, P_i (5mM), ATP (3mM) and GTP (0.5mM).

AMP deaminase has a K_m for AMP of 9mM however, the positive effector, ATP, increases the K_m to 0.25mM and stimulates enzyme activity 200-fold. ATP can completely overcome the 90% inhibition caused by P_i but has no effect on the inhibition resulting from GTP (60%) or from 1mM-IMP (25%). The presence

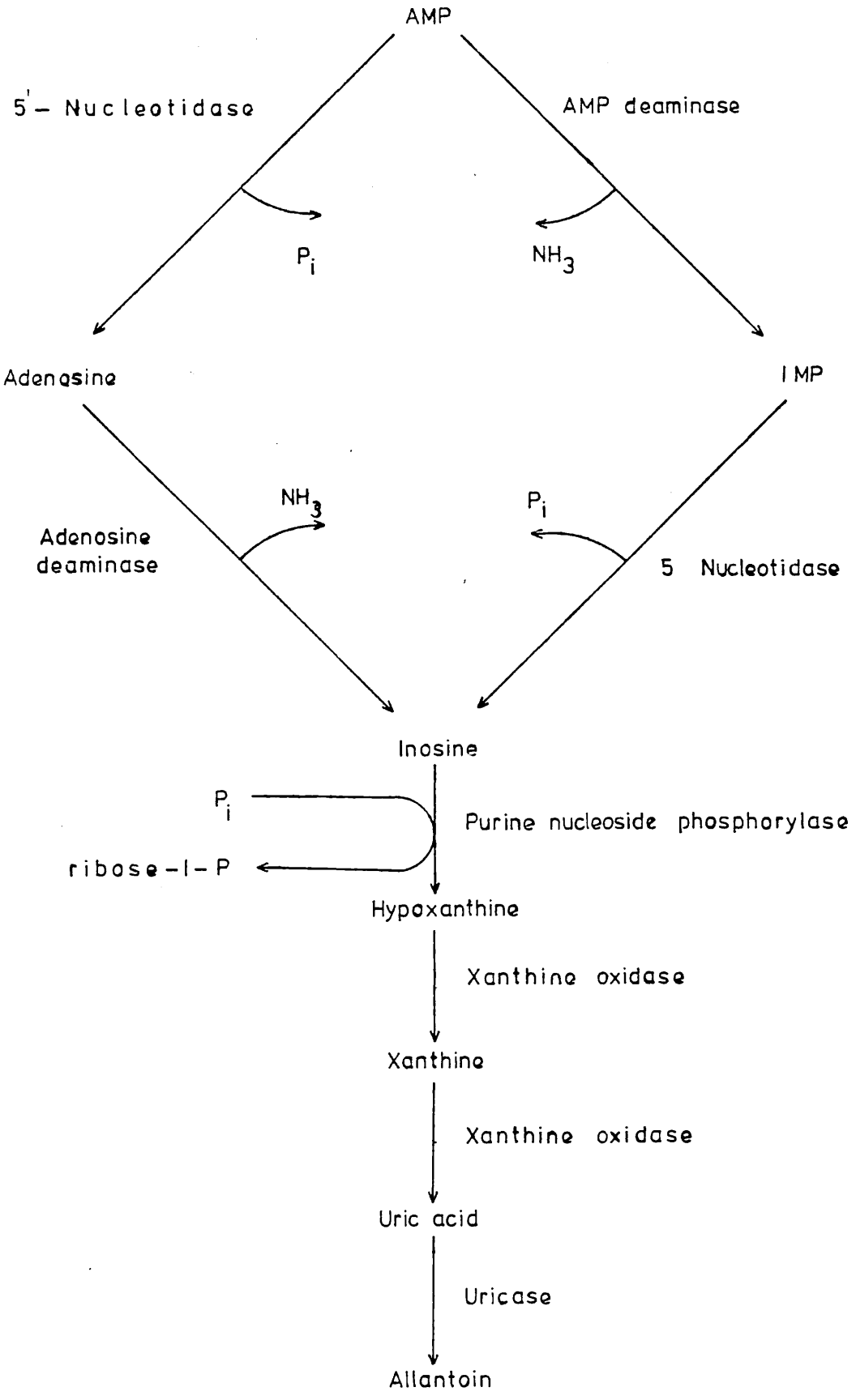


Fig. 6. Pathways of AMP degradation (Woods et al., 1970).

of physiological concentrations of ATP, P_i and GTP results in almost complete inhibition (95%) of enzyme activity (Van den Berghe et al., 1977a). Increasing the P_i concentration, from 1 to 20mM in the incubation medium of isolated hepatocytes, has been shown to result in a 30% decrease in allantoin production thereby providing supporting evidence that P_i is an inhibitor of AMP deaminase in vivo (Van den Berghe et al., 1980).

Three 5'-nucleotidaseshave been characterised, two are found in the subcellular fractions, either bound to the cell membrane or in the lysosomes,and the third is present in the cytosol. The cytosolic enzyme is considered to play the major role in the degradation of purine nucleotides and Woods et al. (1970) have proposed that the enzyme dephosphorylates IMP in addition to AMP. Van den Berghe et al. (1977b) showed that the partially purified enzyme displayed hyperbolic kinetics with IMP as substrate. Despite the enzyme having the same V_{max} with AMP as substrate as with IMP, the kinetics with AMP were sigmoidal. The increased affinity for IMP is reflected in the different K_m values and enzyme activities for each substrate (Table 2). The K_m for AMP is decreased to 1mM at

Table 2. Behaviour of 5'-nucleotidase towards IMP (1mM) or AMP (1mM) as substrate (Van den Berghe et al., 1977b).

Substrate	K_m (mM)	Enzyme activity (nmol/min/mg protein)
IMP	1.2	18.3
AMP	10.0	0.5

physiological concentrations of ATP. GTP is also a stimulator of enzyme activity and P_i is an inhibitor. At physiological concentrations of the effectors, the effects of activators and inhibitors cancel each other precluding the dephosphorylation of physiological concentrations of AMP (0.1 - 0.2mM) by 5'-nucleotidase whilst allowing the breakdown of similar concentrations of IMP. Thus, the degradation of adenine nucleotides to allantoin is proposed to occur through IMP and inosine by the sequential operation of AMP deaminase and 5'-nucleotidase. Under normal physiological concentrations, however, adenine nucleotide catabolism would be expected to be minimal since AMP deaminase is inhibited.

Van den Berghe et al., (1980) have shown that in isolated hepatocytes, in which the adenine nucleotide pool was prelabelled using 1μM -[^{14}C] adenine, 30% of the label incorporated into adenine nucleotides was lost after 1h. The loss could be fully accounted for by the production of [^{14}C] allantoin, which occurs at rates approaching 32 nmol/min/g cells. However, these workers also showed that GTP concentrations in isolated hepatocytes were only 0.2mM compared to 0.5mM in liver in vivo (Van den Berghe et al., 1977a). Since GTP is the major inhibitor of AMP deaminase such a fall in GTP concentration, without any change in ATP or P_i levels, would be expected to result in a 6-fold increase in IMP and, consequently, allantoin levels (Van den Berghe et al., 1977a). The decrease in basal GTP levels in isolated hepatocytes has been attributed to the transient hypoxia which occurs during the preparation of isolated hepatocytes. ATP levels are rapidly returned to normal during a 15 min preincubation

period by oxidative phosphorylation, however, GTP levels appear to be restored at a slower rate by the transfer of P_i from ATP to GDP by nucleoside diphosphate kinase.

Van den Berghe et al. (1980) were also able to confirm that AMP degradation to allantoin is via AMP deaminase, and not adenosine deaminase, from experiments with coformycin (an inosine analogue). Adenosine deaminase is maximally inhibited by 0.1μM - coformycin whereas AMP deaminase requires 50μM of the antibiotic for maximal inhibition. Addition of 0.1μM - coformycin to isolated hepatocytes, in which the adenine nucleotide pool was prelabelled using [^{14}C] adenine, had no effect on the production of allantoin. However, 50μM - coformycin decreased total allantoin levels by 85% and [^{14}C] allantoin production completely. The lack of total inhibition of allantoin production is attributed to guanosine catabolism, which is unaffected by coformycin.

Recently, Bontemps et al. (1983) have detected a 5'— nucleotidase activity, with physiological concentrations of AMP, of 8nmol/min/mg protein compared to an AMP deaminase activity of 26nmol/min/mg protein under similar conditions. These workers reported that isolated hepatocytes, prelabelled with 1μM — [^{14}C] adenine and incubated with 0.5mM — adenosine exhibited a 2-fold increase in ATP concentrations and a 5-fold increase in adenine nucleotide catabolism which they attributed to a 2-fold stimulation of AMP deaminase and a 2.5-fold increase in 5'nucleotidase activity. The addition of 5'-iodotubercidin, an inhibitor of adenosine kinase activity, resulted in a 2 to 3-fold decrease in ATP levels, an accumulation of [^{14}C] adenosine and, in the absence of coformycin,

a 2.5-fold increase in the production of allantoin. Thus, they proposed the existence of a futile cycle between AMP and adenosine in isolated hepatocytes which exceeds the basal rate of allantoin production (12nmol/min/g cells) and reaches levels of 20nmol/min/g cells.

b. Mechanism of fructose effect

Maenpää et al. (1968) were the first to report a 60% decrease in ATP and P_i levels in rat liver 5 min after the intravenous injection of fructose. They were unable to demonstrate any compensating increase in ADP or AMP levels. Instead a 53% fall in total adenine nucleotides occurred, which was attributed to adenine nucleotide degradation since 4-fold increases in plasma uric acid and allantoin levels were observed 15 min after the fructose load. Bode et al. (1973) confirmed these observations and also showed that injection of an equimolar amount of a 1.5M — solution of P_i with the fructose (1g/kg body weight), as well as depleting ATP levels, resulted in a 2-fold increase in AMP concentration. Total adenine nucleotide levels fell in the first 10 min by only 30% compared to 50% in controls, injected with fructose alone. The depletion of ATP can be explained by the rapid hepatic phosphorylation of fructose to Fru-1-P by ATP-dependent fructokinase (Adelman et al., 1967a). The P_i concentration also decreased as a result of its utilisation for the regeneration of ATP.

Van den Berghe et al. (1977a), using mice, reported a 30% fall in hepatic GTP concentration within 2 min of intravenous infusion of fructose. Depletion of GTP in the intact liver after a fructose load has been attributed to its use

as an alternative phosphate donor to ATP in the triokinase reaction (Frandsen and Grunnet, 1971). Van den Berghe et al. (1980) have also shown that GTP levels in isolated rat hepatocytes, which are already significantly lower than levels in the intact liver, are not depleted further by incubation with fructose.

Woods et al. (1970) showed that perfusion of rat liver with 10mM — fructose also results in a 77% decrease in ATP and a 58% fall in P_i and total adenine nucleotide levels within the first 10 min of perfusion. Whereas P_i levels were restored to normal by 40 min, ATP and total adenine nucleotides were at 50% of control levels after 80 min perfusion. They also reported a 7-fold increase in IMP concentration (to 1.14μmol/g) during the initial 10 min of perfusion, followed by a gradual fall to 0.37μmol/g after 40 min. This provided further evidence that AMP degradation was via the IMP pathway since no adenosine could be detected under the same conditions.

Isolated hepatocytes, in which the adenine nucleotide pool was prelabelled with [^{14}C] adenine, have been incubated with 28mM-fructose (Smith et al., 1977) or 6mM-fructose (Van den Berghe et al., 1980). In addition to the expected rapid decrease in labelled adenine nucleotides, there was an increase in [^{14}C]allantoin production reported by both groups. No accumulation of labelled adenosine was detected in the presence or absence of the adenosine deaminase inhibitors: 9-erythro-(2-hydroxy-3-nonyl) adenine (Smith et al., 1977) and 1μM-coformycin (Van den Berghe et al., 1980). Accumulation of label in both IMP and inosine was shown during the first

10 min incubation. Coformycin (50μM), whilst not affecting the fructose-induced breakdown of ATP, inhibited the rate of depletion of total adenine nucleotides with little change in either the basal rate of allantoin production or IMP levels. However, there was a 14-fold increase in AMP concentration and [^{14}C] adenosine was produced (Van den Berghe et. al., 1980). The detection of labelled adenosine suggests that AMP levels, at 1μmol/g cells, are high enough to allow AMP hydrolysis by 5'-nucleotidase instead of the normal degradation by AMP deaminase.

From these experiments the profound effects of fructose metabolism on purine degradation are apparent. They can be explained in terms of the previously described kinetics of AMP deaminase and 5'-nucleotidase. The fall in GTP and P_i concentrations, following a fructose load, will result in stimulation of AMP deaminase and a rapid synthesis of IMP at rates initially exceeding 1μmol/min/g liver. Since ATP can overcome the inhibition by P_i, the decrease in P_i concentration cannot alone account for the rapid degradation of adenine nucleotides following a fructose load. The simultaneous utilisation of GTP will,however, result in a transient activation of AMP deaminase which will be counteracted as activator (ATP) concentrations fall. Thus, the degradation of AMP appears to be under strict metabolite control of AMP deaminase through the relative concentrations of the enzyme's activator (ATP) and inhibitors (GTP and P_i). The transient accumulation of IMP occurs since its rate of synthesis is 10-fold faster than its rate of degradation (0.1μmol/min/g liver). As a result there is a delay in uric acid and allantoin production.

2. Effect of fructose on metabolite concentrations

a. Accumulation of Fru-1-P

The accumulation of Fru-1-P after a fructose load is unexpected in view of the similar activities of fructokinase and aldolase B activity (both approx. 3U/g). Woods et al. (1970) reported that aldolase B activity, with Fru-1-P as substrate, was sensitive to inhibition by a range of cell constituents. After 10 min of perfusion with 10mM-fructose Fru-1-P levels reach 8.7µmol/g liver. Of the metabolites tested only IMP and glycerol-3-phosphate concentrations were sufficiently increased, at 1.14 and 1.06µmol/g respectively, to play a physiological role in the inhibition of aldolase B activity. The inhibition by 1mM-IMP was 90% compared to only 34% with 1mM-glycerol-3-phosphate in vitro. Woods et al. (1970) showed IMP to be a competitive inhibitor (K_i 0.1mM) of the aldolase reaction and proposed that it could fully account for Fru-1-P accumulation.

This explanation was disputed by Van den Berghe et al. (1977a) who studied nucleotide and metabolite levels during the first few minutes following a fructose load. Their work revealed that within 30s there was a rapid depletion of P_i with a simultaneous increase in Fru-1-P concentration. ATP and GTP concentrations fall more slowly, with ATP levels decreasing by 60% and GTP by only 30% within 2 min. IMP, however, was only detectable after 1 min and its accumulation could not therefore be responsible for the increase in Fru-1-P levels. Furthermore, these workers proposed, from a consideration of the equilibrium constant for the cleavage of Fru-1-P to dihydroxyacetone phosphate and glyceraldehyde and the concentrations of these intermediates following a fructose

load, that the reaction was at thermodynamic equilibrium. Hence, inhibition of aldolase activity cannot explain the initial accumulation of Fru-1-P.

Perfusion of liver with 10mM-fructose results in a glucose output of 1.90µmol/min/g liver and lactate production of 1.86µmol/min/g liver (Woods et al., 1970). However, Van den Berghe et al. (1977a) showed that in the first 30s following a fructose load Fru-1-P was formed at rates approaching 12µmol/min/g liver, the potential for fructose phosphorylation is thus considerably greater than the total rate of flux from triose phosphates to glucose and lactate, 5.08 µmol triose phosphate/min/g liver (Woods et al., 1970). Hence, these workers proposed that Fru-1-P accumulated because fructokinase and aldolase metabolise fructose faster than the pathways converting the triose phosphates to glucose or lactate. However, they failed to explain how Fru-1-P could be formed at an initial rate 4-times greater than the maximum fructokinase activity, quoted previously as 3µmol/min/g liver.

Fru-1-P has been reported to directly affect the activities of two key glycolytic enzymes, pyruvate kinase and PFK. Eggleston and Woods (1970) demonstrated Fru-1-P activation of a crude liver preparation of pyruvate kinase, at sub-saturating phosphoenolpyruvate concentrations (< 0.8mM). However, Mapungwana and Davies (1982) reported activation of pyruvate kinase only after incubation of isolated hepatocytes with less than 3mM-fructose. Higher fructose concentrations (up to 10mM) resulted in inhibition of pyruvate kinase, which they attributed to accumulation of allantoin and alanine in the extrahepatocyte medium, since washing the hepatocytes

resulted in stimulation of enzyme activity at all fructose concentrations.

Purified yeast PFK, assayed at less than 1mM-Fru-6-P, has been shown to be activated by up to 0.3mM-fructose or 1mM-Fru-1-P. Higher concentrations of fructose or Fru-1-P were reported to be competitive inhibitors with respect to Fru-6-P and non-competitive inhibitors with respect to MgATP (Kreuzberg, 1978). No studies have been reported on the effects of Fru-1-P on the key regulatory enzymes of gluconeogenesis: pyruvate carboxylase, phosphoenolpyruvate carboxykinase and Fru-1,6-P_2ase.

Fru-1-P has been shown to inhibit the *in vitro* conversion of Fru-6-P to Glc-6-P by Glc-6-P isomerase (Zalitis and Oliver, 1967). These workers proposed that inhibition of Glc-6-P isomerase by Fru-1-P following a fructose load could account for the fructose-induced hypoglycaemia in patients with hereditary fructose intolerance.

b. Effects on other metabolites

In addition to accumulation of Fru-1-P and depletion of ATP, the levels of several other metabolites are altered following a fructose load. Woods *et al.* (1970) have studied the concentrations of many intermediatry metabolites at intervals during liver perfusion with 10mM-fructose. They reported an 8-fold increase in hepatic glycerol-3-phosphate content within 10 min and a 4-fold increase in both dihydroxyacetone phosphate and glyceraldehyde-3-phosphate concentrations, with smaller increases in Fru-1,6-P_2, Glc-6-P and 2- and 3—phosphoglycerate. Lactate and pyruvate concentrations both increased 4-fold after 80 min perfusion, with

the lactate / pyruvate ratio remaining fairly constant at 10 throughout. Burch et al. (1969) reported similar results following the intraperitoneal injection of fructose (40mmol/kg body weight) into rats previously starved for 24h. In addition these workers demonstrated a 5-fold increase in Fru-6-P levels within 15 min, with increases of 11-fold for Glc-6-P and 6-fold for glucose being reported to occur after 60 min. The observed increase in Fru-6-P levels was not confirmed by other workers (Woods et al., 1970; Sestoft, 1974) who were unable to show any affect of fructose on Fru-6-P concentration. Sestoft (1974) has proposed inhibition of Fru-1,6-P_2ase activity in the fed animal following perfusion with 10mM-fructose since Fru-1,6-P_2 levels were shown to increase without any compensating change in Fru-6-P.

3. Other physiological effects of fructose loading.

a. Effects on enzymes of glycogen metabolism.

The two regulatory enzymes in the control of glycogen metabolism are glycogen synthase and phosphorylase a, both of which exist in active and inactive forms. The forms are interconvertible through phosphorylation-dephosphorylation reactions which are catalysed by kinases and phosphatases (Fig. 7). The active form of glycogen synthase is the dephosphorylated form whereas the active form of phosphorylase is phosphorylated. Hence, stimulation of the kinases will result in activation of phosphorylase and inhibition of synthase activity leading to a stimulation of glycogen breakdown, whereas stimulation of the phosphatases will have the opposite effect resulting in the stimulation of glycogen synthesis. Glucose loading has been reported to stimulate glycogen

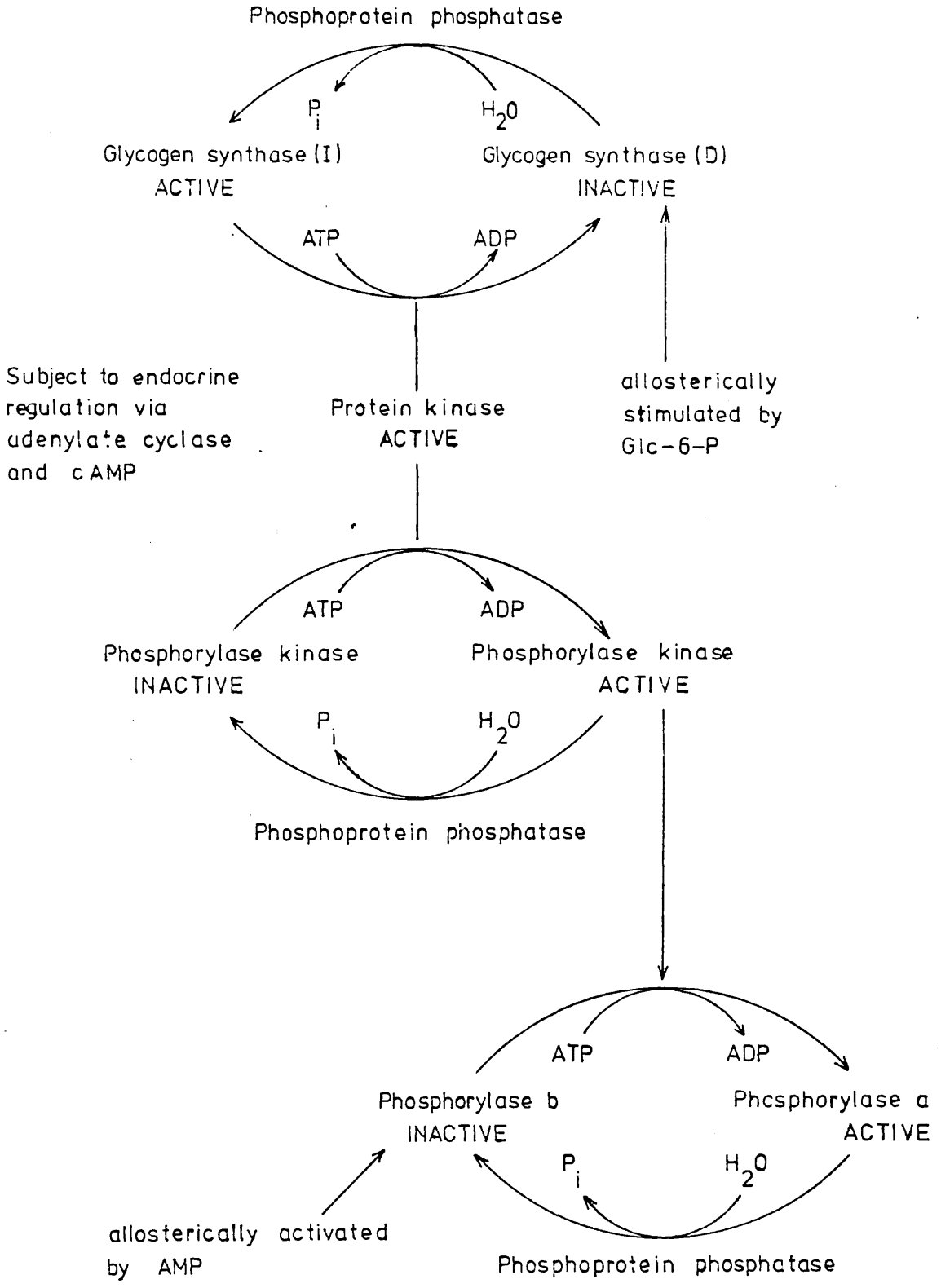


Fig. 7. Regulation of hepatic glycogen synthesis and breakdown

synthase phosphatase activity, and hence increase the proportion of glycogen synthase in the active (I) form, and to decrease the proportion of active phosphorylase a (Regan et al., 1980).

Miller (1978) failed to observe any significant change in glycogen synthase activity in livers from fasted rats perfused with 28mM-fructose. Similar experiments using livers from fed rats increased glycogen synthase activity by 5% over control values. However, compared to glucose, which increases synthase activity by 17%, the effect was minimal. Recent experiments by Regan et al. (1980) have demonstrated that a large intravenous dose of fructose (0.5g/kg body weight) decreases glycogen synthase phosphatase activity leading to a fall in the proportion of active glycogen synthase (I), which is significant within 30s of the fructose load. The mechanism by which fructose induces changes in synthase activity has not been elucidated.

Miller (1978) also reported a 2 to 3-fold increase in phosphorylase activity, following liver perfusion with 28mM-fructose, which he attributed to increases in intracellular cAMP levels and protein kinase activity. Van de Werve and Hers (1979) confirmed the fructose-induced activation of phosphorylase activity in cell-free extracts, isolated hepatocytes and in the liver in vivo. In addition they reported the effect to be dose- and K^+— dependent but independent of Fru-1-P or added Ca^{2+}. They were, however, unable to demonstrate any change in phosphorylase kinase activity on incubation of isolated hepatocytes with 1 — 28mM-fructose and an irregular activation was observed with 30mM-fructose.

Recent work by Regan et al. (1980) also failed to demonstrate any change in phosphorylase kinase or cAMP levels following intravenous injection of fructose (0.5g/kg body weight). The activation of phosphorylase parallels the depletion of ATP levels and thus it is proposed that a fall in the ATP : Mg ratio following a fructose load stimulates phosphorylase kinase activity without the conversion of phosphorylase b to a (Van de Werve and Hers, 1979; Regan et al., 1980). Optimal phosphorylase kinase activity in vitro is proposed to occur at an ATP : Mg ratio of 1 : 2. Since the majority of Mg^{2+} is bound to ATP in the cell the amount of free Mg^{2+} is estimated as only 0.5mM. Thus any fall in ATP levels which would increase the amount of free Mg^{2+} could be potentially important in stimulating phosphorylase kinase activity. Since the K_m for ATP of phosphorylase kinase is only 0.05mM and ATP levels only fall to around 1mM following a fructose load, changes in ATP levels would be expected to have only a limited effect on enzyme activity. However, fructose has been reported to increase Ca^{2+} uptake in pancreatic islet cells (Van den Berghe et al., 1973) which has in turn been shown to stimulate both phosphorylase a and b and this could account for the activation of phosphorylase (Regan et al., 1980). Recently Fru-1-P, at concentrations reached in the liver following a fructose load, has been shown to inhibit the dephosphorylation of phosphorylase a by phosphatase (Bot et al., 1982). This would result in an increase in the level of phosphorylase a without any effect on the phosphorylase kinase reaction.

Fru-1-P has also been reported to competitively inhibit

phosphorylase a activity (Wood et al., 1981; Bot et al., 1982). This could account for the failure to observe any change in liver glycogen concentration, despite the increase in the amount of phosphorylase a, following a fructose load. Since P_i is a substrate for phosphorylase a the depletion of P_i following a fructose load (Woods et al., 1970) could also limit liver glycogen breakdown.

b. Effect on respiration

Inhibition of oxygen uptake by glucose, known as the Crabtree effect, and high aerobic glycolysis were originally reported to occur in Ehrlich ascites tumour cells (Ibsen et al., 1960). Fructose (5-25mM) induces an initial stimulation, lasting less than 1 min, followed by an inhibition of respiration and stimulation of glycolytic flux in perfused livers (Hassinen and Ylikahri, 1970; Sestoft, 1974) and isolated hepatocytes (Chico et al., 1978) or hypothyroid rats (Hassinen et al., 1971). Respiratory rates were restored to normal within 10 min according to Hassinen et al. (1971). Under similar conditions fructose stimulated respiration in perfused livers from hyperthyroid rats (Hassinen et al., 1971) or in perfused regenerating liver (Martinez and Núñnez de Castro, 1980).

Sestoft (1974) reported 10 and 40% increases in oxygen uptake after 4.25 or 35 min perfusion with 10mM-fructose of livers from 48h starved rats. These results were confirmed using isolated hepatocytes from 16h fasted rats incubated with 20mM-fructose by Seglen (1974). However, Chico et al. (1978) failed to observe any change in oxygen consumption by

hepatocytes from 48h starved rats, incubated with 5 to 20mM-fructose for 60 min. Clarke et al. (1979) incubated hepatocytes from 24h starved rats for 6 min with different concentrations of fructose in the presence of 5mM-glucose. Physiological concentrations of fructose (2.5mM) stimulated oxygen uptake by 50%. However, incubation with 7.5 or 25mM-fructose inhibited oxygen consumption by 14 and 37% respectively. All these workers incubated or perfused liver preparations, from rats starved for between 16-48h, with various fructose concentrations for different incubation periods in the absence or presence of added substrates. It is therefore not possible to draw any clear conclusions from these results as to the effect of fructose on cellular respiration in the starved rat.

The redox state of the respiratory carriers is subjected to acute changes during fructose-induced inhibition of respiration in fed rats. Cytochromes a, c and b were reported to be oxidised compared with cells metabolising endogenous substrates (Chico et al., 1978). Hassinen and Ylikahri (1970) had earlier reported the oxidation of cytochromes a and c, and a small reduction in cytochrome b. Furthermore they showed that NAD^+ and the fluorescent flavoproteins are reduced following a fructose load. From this they concluded that inhibition of H^+ transfer between cytosolic NADH and the respiratory chain occurs at the first phosphorylation site. The fall in P_i concentrations following a fructose load was postulated to account for the resulting inhibition of respiration (Hassinen and Ylikahri, 1970). However, Hassinen et al. (1971) showed that P_i levels fall in both the hyper-

and hypo—thyroid, as well as in the fed, rat liver irrespective of any inhibition of respiration. Increasing the perfusate P_i concentration from 1 to 5mM also failed to affect the inhibition of respiration by fructose (Sestoft, 1974).

c. Effects on lipid metabolism.

The liver is the major source of serum triglycerides, present as very low density lipoproteins (VLDL), which are formed either by the esterification of plasma free fatty acids (FFA) or the de novo synthesis of long chain fatty acids. Diets high in fructose have been reported to increase serum triglyceride concentrations to a greater extent than glucose-rich diets (Sleder et al., 1980). The rate of VLDL secretion from the liver has been shown to be increased by 75% following fructose feeding (Kannan et al., 1981) or by 30% after liver perfusion with fructose (Topping and Mayes, 1972; 1976). Lipoprotein lipase activity, and hence removal of VLDL by adipose tissue, is unaffected by fructose-rich diets (Kannan et al., 1981).

Pereira and Jangaard (1971) proposed that fructose-induced hyperlipidaemia arises from the higher rate of fructolysis than glycolysis. This results in a fall in ATP levels and an increase in pyruvate concentrations, either of which could be responsible for the reported 2 to 3-fold activation of pyruvate dehydrogenase following perfusion with 10mM-fructose (Topping and Mayes, 1977) leading to a 40-60% increase in acetyl-CoA concentrations (Söling et al., 1970). Perfusion with 0.25mM-non-esterified fatty acid and 1.5mM-fructose does not significantly affect ATP concentrations but leads to production of VLDL-triacylglycerol fatty acid at a higher rate

(6.0μmol/h/g liver) than in similar perfusions with 8.9mM-fructose (2.3μmol/h/g liver) when ATP levels are depleted (Laker and Mayes, 1979). These workers showed that oxidation to CO_2 and ketone bodies was 471 or 124 μmol/h/g liver following incubation with 8.9mM or 1.5mM-fructose respectively. It therefore appears that following a physiological fructose load (1.5mM), when pyruvate dehydrogenase activity (Topping and Mayes, 1977) and ATP levels are not significantly affected, lipogenesis is stimulated possibly via activation of pyruvate kinase leading to an increase in pyruvate concentrations (Mapungwana and Davies, 1982). However, 8.9mM-fructose, despite increasing acetyl-CoA concentrations, appears to inhibit lipogenesis and to stimulate oxidation to ketones and CO_2 (Laker and Mayes, 1979). In the long-term fructose feeding stimulates the synthesis of several lipogenic enzymes, for example acetyl-CoA carboxylase, fatty acid synthase and ATP-citrate lyase (Van den Berghe, 1977). However, induction appears to be a consequence rather than cause of the increased rate of lipogenesis.

Glucagon inhibits lipogenesis through a fall in malonyl-CoA levels and an inhibition of acetyl-CoA carboxylase thereby leading to a stimulation of β-oxidation. In addition, glucagon has been proposed to decrease triacylglycerol synthesis by lowering glycerol-3-phosphate concentrations, as opposed to affecting enzyme activity (Declercq et al., 1982a). Glycerol-3-phosphate concentrations have been reported to limit esterification in both fed and fasted rats, in the absence of triglyceride precursors (Debeer et al., 1981; Declercq et al., 1982b). Declercq et al. (1982b) were able to show that, in fed animals, changes in glycerol-3-phosphate concen-

tration between 0.08 and 0.40μmol/g liver cells altered rates of esterification dramatically. Glycerol-3-phosphate concentrations have been reported to be increased following intravenous injection of fructose (Zakim and Herman, 1968) or perfusion of isolated rat liver with up to 25mM-fructose (Woods et al., 1970; Sestoft, 1974). Controls are quoted as having a glycerol-3-phosphate content between 0.13 and 0.32μmol/g liver whilst perfusion with 10mM-fructose leads to reported concentrations of 0.60μmol/g liver (Woods et al., 1970) and 0.96μmol/g liver (Sestoft, 1974) at 40 min. Increases such as these, in addition to providing substrate, will directly stimulate fatty acid esterification. Fructose has also been shown to increase by 30% the incorporation of [^{14}C] labelled fatty acids into triglycerides and inhibit β-oxidation by 36% in the perfused liver (Topping and Mayes, 1972; 1976). The rate of uptake of perfusate FFA by the liver remained within the control range of 30-35% (Topping and Mayes, 1976).

In summary, fructose appears to stimulate the release of triglycerides by the liver, as VLDL, via increased esterification of fatty acids and a coincident inhibition of catabolism. Depending on the concentration of fructose a stimulation or inhibition of fatty acid synthesis will occur (Fig. 8).

C. The Fru-6-P/Fru-1,6-P_2 substrate cycle.

Recycling between Fru-6-P and Fru-1,6-P_2 involves the enzymes, PFK and Fru-1,6-P_2ase and the apparently wasteful hydrolysis of ATP to ADP and P_i. The system oscillates between two conditions; a gluconeogenic state, in which PFK is inhibited and Fru-1,6-P_2ase is activated, and a glycolytic

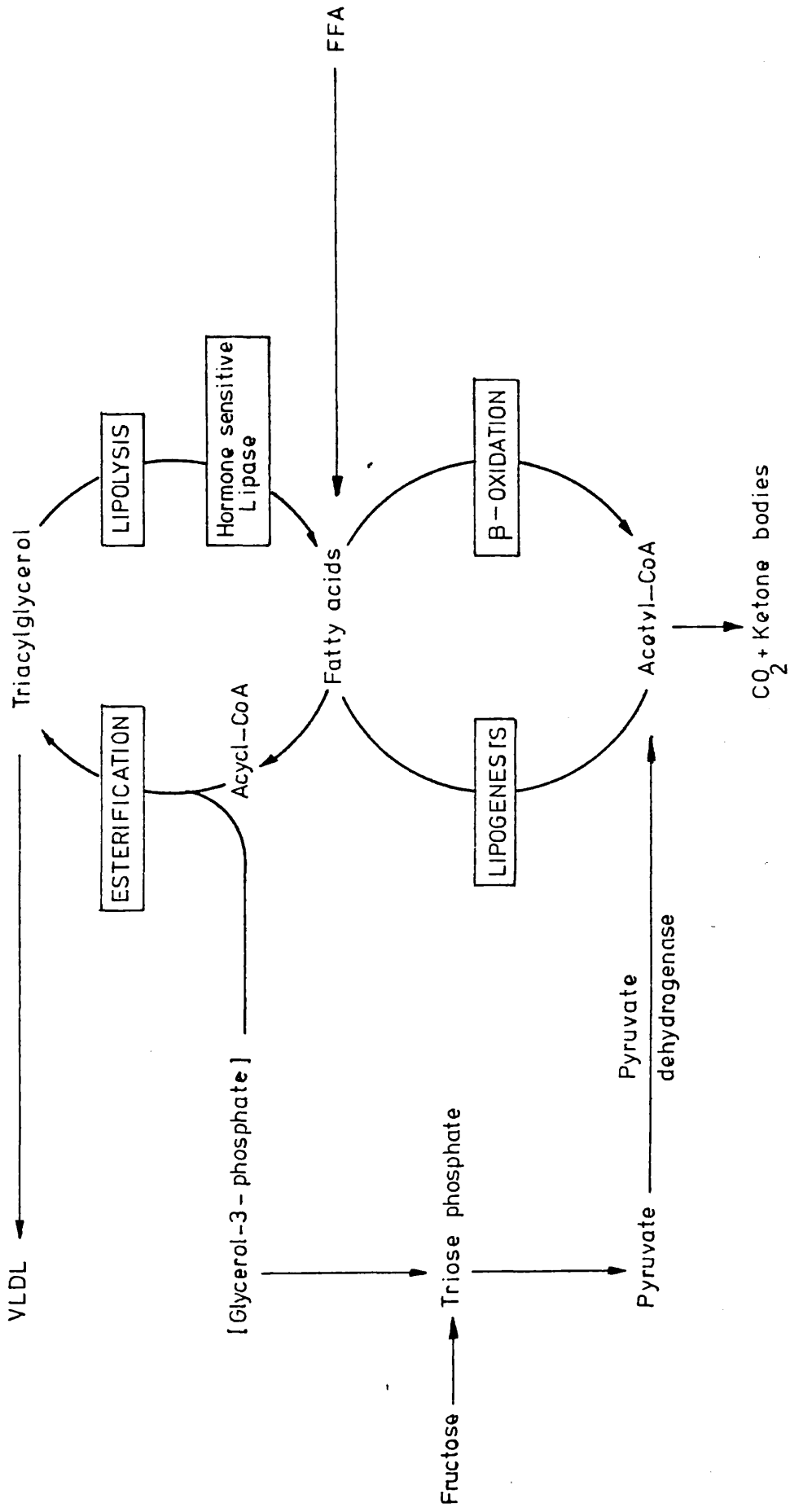


Fig. 8. Proposed scheme to account for the effects of fructose in promoting the esterification and decreasing the oxidation of FFA by the liver and promoting the secretion of VLDL (Topping and Mayes, 1976).

state, where PFK is activated and Fru-1,6-P_2ase is inhibited. Both enzymes are under tight allosteric control and are possibly regulated by covalent modifications (Hers and Hue, 1983).

The occurrence of significant substrate cycling, under physiological conditions, has been the subject of extensive study over recent years. In 1967, Newsholme and Gevers concluded that the operation of the substrate cycle, between Fru-6-P and Fru-1,6-P_2, although wasteful in terms of energy utilisation, would allow a rapid response to altering physiological conditions.

Isotope studies using [5-^{3}H] and [U-^{14}C] glucose (Clark et al., 1974) or [1-^{14}C] galactose (Rognstad and Katz, 1976) demonstrated the existence of a substrate cycle between Fru-6-P and Fru-1,6-P_2 in isolated rat hepatocytes. Furthermore, both groups showed that glucagon enhanced gluconeogenesis and inhibited Fru-6-P phosphorylation.

Koerner et al. (1977) have proposed that the anomeric specificities of PFK for β-D-Fru-6-P_2, would prevent substrate cycling. Computer modelling experiments have also led to the conclusion that the physiological concentration and distribution of activators and inhibitors are such as to prevent substrate cycling (Garfinkel et al., 1979).

Recent experiments have shown that up to 30% recycling occurs between Fru-6-P and Fru-1,6-P_2 in hepatocytes from fed rats (Van Schaftingen et al., 1980a,b; Rognstad and Katz, 1980). Glucagon was shown to reduce cycling by 50% under these conditions. Recycling was almost negligible in hepatocytes from starved rats but was greatly increased following

incubation with high concentrations of glucose (Van Schaftingen et al., 1980a). These changes were shown to be paralleled by variations in PFK activity which were later shown to be due to changes in the concentration of Fru-2,6-P_2 (Van Schaftingen et al., 1980a,b; Richards and Uyeda, 1980; Claus et al., 1981a). Glucose increases the concentration of Fru-2,6-P_2 with the resulting inhibition of Fru-1,6-P_2ase and activation of PFK. However, Fru-1,6-P_2ase remains active as a result of a 10-fold increase in the concentration of its substrate, Fru-1,6-P_2, and thus substrate cycling is observed under these conditions (Van Schaftingen et al., 1980a). Glucagon is proposed to reduce Fru-2,6-P_2 concentrations through the cAMP-dependent phosphorylation of PFK-2/Fru-2,6-P_2ase (Van Schaftingen et al., 1981a, 1982; Sakakibara et al., 1984). Recent experiments by Bartrons et al. (1983) have shown that PFK-2/Fru-2,6-P_2ase is a poor substrate for cAMP-dependent protein kinase compared to glycogen phosphorylase or pyruvate kinase. Hence, Fru-2,6-P_2 concentrations were shown to fall relatively slowly, from 15 to 2nmol/g cells in a 10 min period, with Fru-1,6-P_2 levels falling even more slowly. From this the initial effect of glucagon appears to be the cAMP-dependent phosphorylation of both pyruvate kinase and glycogen phosphorylase leading to the inactivation of both enzymes and the stimulation of gluconeogenesis and glycogenolysis. The phosphorylation of PFK-2/Fru-2,6-P_2ase occurs as a secondary effect and results in a decrease in Fru-2,6-P_2 concentration. This will in turn lead to an inhibition of PFK and hence Fru-1,6-P_2 levels will fall.

1. Phosphofructokinase (EC 2.7.1.11)

PFK catalyses the ATP-dependent phosphorylation of Fru-6-P to Fru-1,6-P_2. The properties of the enzyme from various sources have been extensively reviewed by Bloxham and Lardy (1973), Hofman (1976), Goldhammer and Paradies (1979), Uyeda (1979), Hers and Hue (1983). Two isoenzymes of PFK from rat liver have been isolated and characterised by Dunaway and Weber (1974). The major isoenzyme, L2, is present only in parenchymal cells and shows greater susceptibility to effector control and to nutritional and hormonal influences compared to the minor isoenzyme, L1, which is only found in Kupffer cells (Dunaway et al., 1978).

The purified rat liver enzyme exists as a tetramer with a molecular weight of 325·000 - 365·000 (Dunaway and Weber, 1974; Brand and Söling, 1974). The enzyme readily forms higher molecular-weight aggregates at physiological protein concentrations (> 1µg/ml) and dissociates on dilution or in the absence of substrates into dimers and monomers with a concomitant loss of activity (Reinhart and Lardy, 1980b). MgATP promotes tetramer formation more efficiently than Fru-6-P and the aggregated enzyme has a higher affinity for Fru-6-P than the dissociated enzyme (Reinhart and Lardy, 1980b,c). Reinhart (1983) has recently reported that Fru-2,6-P_2 stabilises the high molecular-weight aggregated forms of the enzyme against inactivation by dilution. It also promotes reaggregation of the dissociated enzyme into high molecular-weight forms even in the presence of MgATP, which stabilises the tetramer form. This led to the proposal that Fru-2,6-P_2 regulates PFK activity in vivo through changes in aggregation state of the enzyme.

PFK activity in rat liver has been reported to be 0.89µmol/min/g liver at 30°C (Brand and Söling, 1974). Kinetic studies have revealed an ordered Bi-Bi reaction mechanism with Fru-6-P binding first, followed by MgATP, then the release of MgADP and subsequently Fru-1,6-P_2 (Brand and Söling, 1974).

a. Properties of PFK

The enzyme displays typical Michaelis-Menten kinetics at alkaline pH with K_m values of 0.08mM for Fru-6-P and 0.11mM for MgATP reported (Brand and Söling, 1974). However, at neutral pH the substrate saturation curve for Fru-6-P is sigmoidal, with the K_m for Fru-6-P increased to 6mM, and the enzyme is allosterically influenced by effectors (Reinhart and Lardy, 1980a). Activators such as AMP, P_i, Fru-1,6-P_2 at low concentrations (Reinhart and Lardy, 1980a), Glc-1, 6-P_2, Fru-2,6-P_2 (Van Schaftingen et al., 1981b) and 6-phosphogluconate (Sommercorn and Freedland, 1981) alter the substrate saturation curve from sigmoidal to hyperbolic with a concomitant decrease in K_m value but no change in V_{max}. Negative effectors including excess MgATP, citrate, H^+ (Reinhart and Lardy, 1980a), oleate, palmitate (Ramadoss et al., 1976) and glycerol-3-phosphate (Claus et al., 1982) decrease the affinity of the enzyme for Fru-6-P.

In 1976, Pogell et al. proposed a direct interaction between PFK and Fru-1,6-P_2ase which resulted in an inactivation of PFK potentiated by ATP and 3-phosphoglycerate. Söling et al. (1977) failed to show any inhibition of PFK at Fru-1,6-P_2 concentrations above 2µM or if Glc-1,6-P_2,

which is not cleaved by Fru-1,6-P_2ase, was used to activate PFK. From this they concluded that Fru-1,6-P_2ase affects PFK activity by altering the Fru-1,6-P_2 concentration.

When the enzyme is assayed under conditions of high sensitivity for its allosteric effectors the same stimulation can be achieved with a concentration of Fru-2,6-P_2 1000-fold smaller than of either Glc-1,6-P_2 or Fru-1,6-P_2 (Pilkis et al., 1981a; Van Schaftingen et al., 1981b). Fru-2,6-P_2 potentiates AMP activation of PFK and acts synergistically with AMP to relieve ATP inhibition (Van Schaftingen et al., 1981b; Uyeda et al., 1981). At physiological concentrations of ATP (2.5mM), Fru-6-P (50μM) and P_i (5mM) PFK activity is too low to account for its proposed role in glycolysis. However, the presence of physiological concentrations of AMP (0.25mM) and Fru-2,6-P_2 (2.5μM) results in enzyme activity at 65% of V_{max} which accounts for the regulatory role of PFK. Under these conditions V_{max} is obtained by increasing the Fru-6-P concentration to 5mM (Van Schaftingen et al., 1981b).

Fru-2,6-P_2 is now considered to be the major regulator of PFK since factors influencing PFK activity also affect Fru-2,6-P_2 levels. Hepatic Fru-2,6-P_2 concentrations have been reported to be in the 10-20μM range in normal fed rats (Van Schaftingen et al., 1980a,b; Hers and Hue, 1983) and fall by 90% following a 72h fast (Neely et al., 1981). Incubation of isolated hepatocytes, from fasted rats, with 20 to 50mM-glucose (Richards and Uyeda, 1980; Hue et al., 1982) or intravenous injection of glucose (Kuwajima and Uyeda, 1982) results in the restoration of hepatic Fru-2, 6-P_2 levels.

b. Covalent modification

Taunton et al. (1972, 1974) reported the glucagon-induced inactivation of PFK and proposed that this was due to cAMP-dependent phosphorylation of the enzyme. The inactivation could also be shown to occur in isolated hepatocytes treated with glucagon (Pilkis et al., 1979; Castaño et al., 1979) or exogenous cAMP (Castaño et al., 1979) and it was claimed that the change in enzyme activity was stable to gel filtration (Castaño et al., 1979). PFK inactivation was accompanied by an increase in the K_m value for Fru-6-P, from 0.09 to 0.31mM, without any change in V_{max} and in an enzyme more sensitive to ATP inhibition (Pilkis et al., 1979). The phosphorylation of PFK by glucagon (Kagimato and Uyeda, 1979) and cAMP-dependent protein kinase (Claus et al., 1980) has been demonstrated, however, in neither case did this coincide with a decrease in enzyme activity.

Van Schaftingen et al. (1980a,b) showed that glucagon (10^{-7}M) exerts its effect on PFK by decreasing the concentration of Fru-2,6-P_2 and that gel filtration treatment abolished the hormonal effect. Recent experiments have shown that glucagon exerts its effect on PFK activity through cAMP-dependent phosphorylation of PFK-2/Fru-2,6-P_2ase thereby inhibiting Fru-2,6-P_2 synthesis (Van Schaftingen et al., 1981a; El-Maghrabi et al., 1982) and stimulating its degradation (Van Schaftingen et al., 1982). Furuya and Uyeda (1980) reported that cAMP-dependent phosphorylation of PFK resulted in an enzyme with a lower affinity for Fru-2,6-P_2.

Recently, Sakakibara and Uyeda (1983) have purified low and high phosphate forms of PFK from rat liver. Kinetic studies showed the high phosphate form to be more sensitive to ATP inhibition and less sensitive to Fru-2,6-P_2 activation than the low phosphate form. Binding studies however, demonstrated that ATP binding to the inhibitory site was increased following phosphorylation of the enzyme and that Fru-2,6-P_2 binding was unaffected (Kitajima et al., 1983). The previously reported increases in K_m values for Fru-2,6-P_2, following phosphorylation of PFK (Furuya and Uyeda, 1980; Sakakibara and Uyeda, 1983), were explained by the presence of MgATP in the assay mixture (Kitajima et al., 1983). Since the binding of ATP to the phosphorylated enzyme was greater at lower pH values, Kitajima et al. (1983) proposed that PFK exists in two conformational forms; a protonated phosphorylated form which binds ATP and an unprotonated dephosphorylated form which binds Fru-6-P and the activators (Fru-2,6-P_2 and AMP).

The occurrence and significance of conformational changes in vivo remains to be established especially in view of the fact that Claus et al. (1982) failed to demonstrate any significant effect on PFK activity in isolated hepatocytes following phosphorylation. Since both glucose and glucagon increased ^{32}P—incorporation into PFK, despite having opposite effects on Fru-2,6-P_2 levels and enzyme flux, these workers concluded that changes in the concentration of effectors, primarily Fru-2,6-P_2, are responsible for the regulation of PFK activity.

Cyclic AMP-independent phosphorylation of PFK, leading to an increase in V_{max}, has been reported and the enzymes responsible for the phosphorylation and dephosphorylation have been isolated (Brand and Söling, 1975). The proportion of the enzyme in the phosphorylated, active form has been shown to decrease on fasting and increase in the presence of 30mM-glucose (Brand et al., 1976) and these changes parallel the disappearance or appearance of Fru-2,6-P_2 (Van Schaftingen et al., 1980a,b). Söling et al.(1981) showed that Fru-2,6-P_2 inhibits the inactivation of PFK by a PFK phosphatase. From this and more recent experiments (Brand and Söling, 1982) they concluded that the degree of phosphorylation of PFK is controlled by the metabolic state, exogenous substrates and hormones.

In conclusion, PFK has been shown to be phosphorylated under conditions when its activity is both enhanced (by glucose) and inhibited (by glucagon) and hence the physiological significance of such a modification has not been demonstrated. Glucagon has now been conclusively shown to decrease Fru-2,6-P_2 levels, via cAMP-dependent phosphorylation of PFK-2/Fru-2,6-P_2ase, whereas glucose increases Fru-2,6-P_2 concentrations by increasing the availability of Fru-6-P to PFK-2. Thus, Fru-2,6-P_2 appears to be the major regulator of PFK.

2. Fructose-1,6-bisphosphatase (EC 3.1.3.11)

Fru-1,6-P_2ase is the first regulatory enzyme involved in the conversion of fructose to glucose whereas fructolysis to lactate bypasses the Fru-6-P/Fru-1,6-P_2 cycle. This latter fact has been proposed to account for the faster rate

of glycolysis from fructose than from glucose.

The properties of Fru-1,6-P_2ase have been extensively reviewed by several groups (Pontremoli and Horecker, 1971; Horecker et al., 1975; Söling and Kleineke, 1976; Pilkis et al., 1978; Benkovic and deMaine, 1982; Hers and Hue, 1983). At least two isoenzymes of Fru-1,6-P_2ase exist in animal tissues, one in the liver and kidney, the other in muscle where the enzyme is more sensitive to AMP inhibition. The existence of a third isoenzyme in the intestine has been proposed from experiments on mice by Mizunuma et al. (1980). Sato and Tsuiki (1969) reported that whilst liver Fru-1,6-P_2ase activity is retained in well-differentiated tumours, it is replaced by the muscle isoenzyme in rapidly dividing, poorly-differentiated hepatic tumours. Rat liver enzyme activity has been reported to be 4.15 U/g liver at 23°C by Traniello (1974). Orengo and Patenia (1981) have recently reported that whilst the majority of the hepatic Fru-1,6-P_2ase activity is located in the cytosol, 5% appears to be microsomal bound.

Microdissection studies on rat liver tissues have shown 2 to 3-fold more Fru-1,6-P_2ase activity in the periportal zone than in the perivenous zone of the liver parenchyma (Katz et al., 1977; Schmidt et al., 1978). This supports the metabolic compartmentation theory, in which glycolytic enzymes are proposed to predominate in the perivenous zone and gluconeogenic enzymes in the periportal region.

a. Native and 'alkaline' enzyme

In 1971, Traniello et al. showed that many of the earlier experiments on the liver enzyme had not been carried out on

the native enzyme, with a neutral pH optimum, but on what is now known as the 'alkaline' enzyme, having a pH optimum of 9. The native enzyme has a molecular weight of 140 000 (Traniello et al., 1971, 1972) and consists of four identical subunits with molecular weight 35-36 000, determined by SDS-disc gel electrophoresis (Traniello et al., 1972; Riou et al., 1977; Claus et al., 1981b). The 'alkaline' enzyme is less sensitive to AMP inhibition, has a molecular weight of 29-30 000 and is proposed to arise from the proteolytic cleavage of a 6 000 dalton peptide, containing tryptophan, from the N-terminal region of the native enzyme (Traniello et al., 1971; Humble et al., 1979). Incubation of the native enzyme with subtilisin or liver lysosomes yielded an enzyme with properties similar to 'alkaline' Fru-1,6-P_2ase (Horecker et al., 1975).

Heating the enzyme, at 60°C and pH 7.2 for 2 min, in the purification procedure prevents the release and activation of these lysosomal proteases (Traniello et al., 1971). Since the native enzyme, from rabbit liver, was shown to contain one molecule of tryptophan per subunit the absence of tryptophan residues in the 'alkaline' enzyme was exploited in early structural investigations (Horecker et al., 1975). However, later experiments showed that the enzyme from livers of fasted rabbits whilst lacking tryptophan, still retained a neutral pH optimum. Pontremoli et al. (1978, 1979) provided evidence that the tryptophan residues arise from liver aldolase, which under certain conditions will co-purify with Fru-1,6-P_2ase.

Three lysosomal proteases, responsible for converting the native to the 'alkaline' enzyme form, have recently been partially purified and characterised by Melloni et al. (1981) - Table 3. Incubation of Fru-1,6-P_2ase with each 'converting enzyme' was shown to result in an increased activity at pH 9 and a decrease in sensitivity to AMP measured at neutral pH.

Table 3. Properties of the 'converting enzymes' isolated from rabbit liver (Melloni et al., 1981).

Converting enzyme	Molecular weight	Location	pH_{max}
CE-I	150 000	Soluble fraction	5.0
CE-II	70 000	30-40% membrane bound	6.5
CE-III	30 000	Soluble fraction	5.0

The site of subtilisin attack, in the rabbit liver enzyme has been proposed to be the peptide bonds Ala^{60}-Gly^{61} and Thr^{63} - Asn^{64} which are contained within an exposed peptide loop between residues 57 and 67 (Botelho et al., 1977). Dzugaj et al. (1976) reported that subtilisin cleaved regions corresponded to 1/6 and 5/6 of the original peptide, with the larger polypeptide product being resistant to further subtilisin digestion and showing enhanced activity at pH9.

b. Properties of the native enzyme

Numerous regulators of Fru-1,6-P_2ase have been found, however, their physiological significance has, in many cases,

not been established. The presence of divalent metal ions (Mg^{2+} or Mn^{2+}) and a metal chelator (EDTA, citrate or histidine), to remove inhibitory Zn^{2+} ions, has been shown to be essential for optimal enzyme activity at neutral pH (Datta et al., 1974). In 1976, Tejwani et al. proposed two Zn^{2+} binding sites per enzyme subunit. Binding to the high affinity site caused a rapid inhibition of enzyme activity which was uncompetitive with respect to Mg^{2+} or Mn^{2+} (K_i 0.3μM). Higher concentrations of Zn^{2+} (10μM) bind to the low affinity site, activating Fru-1,6-P_2ase. Pedrosa et al. (1977) confirmed these results, however, their experiments revealed a further Zn^{2+} binding site which was inhibitory. Binding constants for both the high affinity (< 0.01 M and 0.4μM) and low affinity (1.5μM) sites were determined and the latter was shown to be equivalent to the Mg^{2+} binding site, with a K_m of 5μM (Pedrosa et al., 1977). Homocystine, in the presence of Mg^{2+} or Mn^{2+}, has been shown to activate Fru-1,6-P_2ase in vitro, presumably as a result of sulphydryl modification of the protein molecule (Nakashima et al., 1970). Such a modification has not been demonstrated in vivo and thus is unlikely to play a physiological role.

Purified Fru-1,6-P_2ase exhibits Michaelis-Menten kinetics with a K_m for Fru-1,6-P_2 of 1-3μM (Taketa and Pogell, 1965; Traniello, 1974). Maximum enzyme activity is observed with 50μM-Fru-1,6-P_2 and higher substrate concentrations have been shown to be inhibitory (Traniello, 1974). However, the majority of cellular Fru-1,6-P_2 is protein-bound and therefore changes in the physiological concentration of the substrate (5-50μM) could affect enzyme activity (Hers and Hue, 1983).

Fru-2,6-P_2 and AMP are now considered to be the major regulators of Fru-1,6-P_2ase. Both are potent inhibitors with K_i values of 20μM (Taketa and Pogell, 1965) and 2.5μM (Pilkis et al., 1981b) respectively. The effect of Fru-2,6-P_2 is synergistic with that of AMP (Van Schaftingen and Hers, 1981; Pilkis et al., 1981b), an allosteric non-competitive inhibitor of the enzyme (Taketa and Pogell, 1965). Fru-2,6-P_2 changes the saturation curve from hyperbolic to sigmoidal at low substrate concentrations indicating that a threshold concentration of Fru-1,6-P_2 exists below which Fru-6-P formation is inhibited (Van Schaftingen and Hers, 1981). AMP (25μM) and Fru-2,6-P_2 (1μM) together inhibit Fru-1,6-P_2ase by 80%, whereas individually they inhibit enzyme activity by 35% and 20% respectively (Van Schaftingen and Hers, 1981). Since AMP does not affect the shape of the saturation curve it was proposed that AMP and Fru-2,6-P_2 bind to different sites on the enzyme. Pilkis et al. (1981c) confirmed these results, additionally showing that Fru-1,6-P_2 potentiates AMP inhibition, at concentrations considerably higher than with Fru-2,6-P_2. Furthermore, from acetylation experiments they claim that Fru-2,6-P_2 binds to both the catalytic and allosteric sites of the enzyme, through tyrosine residues present at each site.

Meek and Nimmo (1983) proposed that Fru-2,6-P_2 interacts with the catalytic site, as a competitive inhibitor, and with an allosteric site, which also binds Fru-1,6-P_2 at high concentrations. The allosteric site was postulated to be different from the AMP binding site to account for the synergism between AMP and Fru-2,6-P_2 which requires their

simultaneous binding. François et al. (1983) compared the effect of Fru-2,6-P_2 with that of other ligands of Fru-1, 6-P_2ase which bind to either the catalytic or allosteric sites. Whilst concluding that Fru-2,6-P_2 binds to an allosteric site different from the AMP binding site, their results suggested that the catalytic site had no affinity for Fru-2,6-P_2. However, McGrane et al. (1983) have reported from covalent modification and substrate-analog binding studies, that Fru-2,6-P_2 binds to the catalytic site thereby inducing a conformational change similar to that induced by AMP at the allosteric site. The evidence from these workers supports the theory that Fru-2,6-P_2 binds to a regulatory site different from that of AMP. Whether this is an allosteric site or the catalytic site has not been firmly established.

c. Effects of hormones on native Fru-1,6-P_2ase

In 1972, Taunton et al. reported that portal vein injection of glucagon or adrenalin increased cAMP levels within 30s and caused a 3-fold stimulation of Fru-1,6-P_2ase within 4 min in rat liver. Insulin counteracted the latter effect but cAMP levels were unchanged. This led to the suggestion that Fru-1,6-P_2ase activity was regulated by a phosphorylation — dephosphorylation mechanism. Riou et al (1977) demonstrated the in vivo incorporation of ^{32}P from $^{32}P_i$ into the rat liver enzyme and the in vitro phosphorylation of purified Fru-1,6-P_2ase by the catalytic subunit of cAMP-dependent protein kinase. Acid hydrolysis of the immunoprecipitated, in vitro phosphorylated enzyme revealed that only seryl residues were labelled.

The amino acid sequence of the rat liver phosphorylation site was reported by Humble et al. (1979) to be Ser-Arg-Tyr-(^{32}P) Ser P-Leu-Pro-Leu-Pro. Pilkis et al. (1980) identified a similar sequence, however, they failed to detect any tyrosine residues in several preparations of the tryptic peptide and reported a proline residue instead at position 3. Proteolytic modification of the enzyme with subtilisin decreased the subunit molecular weight by 25% (Humble et al., 1979). However, the ^{32}P-labelling still coincided with the major protein band on polyacrylamide gels indicating that the rat liver enzyme is not phosphorylated in the N-terminal region, which is cleaved by subtilisin.

Ekman and Dahlqvist-Edberg (1981) showed that the in vitro phosphorylation of Fru-1,6-P_2ase, using the catalytic subunit of cAMP-dependent protein kinase, decreased the K_m for Fru-1,6-P_2 from 21μM to 11μM. Treatment of the phosphorylated enzyme with trypsin or a partially purified protein phosphatase increased the K_m to 22μM. The phosphorylated and dephosphorylated enzymes both had a pH optima of 7. However, treatment with subtilisin, which attacks the unphosphorylated N-terminal of the enzyme, increased the pH optimum more than similar treatment with trypsin, which attacks at the phosphorylated C-terminal region of the enzyme. The phosphorylated enzyme was 4-times more sensitive to attack by trypsin than the dephosphorylated enzyme and hence phosphorylation was proposed to regulate enzyme activity in vivo by rendering it susceptible to hydrolytic cleavage and inactivation. Glucagon has been

shown to stimulate the incorporation of ^{32}P from $^{32}P_i$ into Fru-1,6-P_2ase in isolated hepatocytes (Claus et al., 1980). However, the concentration required for half-maximal stimulation of ^{32}P incorporation was 1.5nM, compared to only 0.3nM for half-maximal stimulation of gluconeogenesis. Mörikofer-Zwez et al. (1981) showed that following intraperitoneal injection of glucagon the K_i for Fru-2,6-P_2 is increased 2-fold indicating that the enzyme is also less sensitive to Fru-2,6-P_2 under these conditions. Furthermore, these workers reported a 44% increase in enzyme activity, at subsaturating Fru-1,6-P_2 concentrations, within 20 min of glucagon administration.

The addition of glucagon to isolated hepatocytes has been shown to decrease Fru-2,6-P_2 levels by up to 90% thus relieving Fru-2,6-P_2 inhibition of Fru-1,6-P_2ase (Claus et al., 1981b; Richards and Uyeda, 1980; Richards et al., 1981; Van Schaftingen et al., 1980a). Phosphorylation of the enzyme may play a regulatory role by altering the enzyme's ability to respond to changes in effector levels; however, such an effect remains to be established.

d. Fru-1,6-P_2ase deficiency

Fru-1,6-P_2ase deficiency was first described in 1970 and the genetic inheritance is proposed to be autosomal recessive (Gitzelmann et al., 1982). It is characterised by metabolic acidosis, fasting hypoglycemia, hyperventilation, ketosis and hepatomegaly (Rallison et al., 1979). Fructose, sorbitol and glycerol also induce hypoglycemia which tends to be less severe than in patients with hereditary fructose

intolerance. Providing patients avoid fasting and the intake of any of the above sugars the condition is symptomless. Fru-1,6-P_2ase activity is absent, or present in only trace amounts, in the liver of 60% of patients. Enzyme levels in the others are less than 20% of normal (Gitzelmann et al., 1982).

D. Summary and Aims of Thesis

The fate of fructose carbon is dependent on the activities of Fru-1,6-P_2ase and pyruvate kinase which are the first regulatory enzymes, of gluconeogenesis and glycolysis, encountered by the products of fructose metabolism. It might be expected that PFK would be inactive during fructose metabolism to prevent recycling between Fru-6-P and Fru-1,6-P_2. However, at the start of this work, the published evidence suggested that the hormonal and allosteric control of PFK and pyruvate kinase are very similar and thus it was decided to investigate the effects of fructose on the activities of Fru-1,6-P_2ase and PFK. The effects of fructose on pyruvate kinase activity had previously been investigated in this laboratory (Mapungwana, 1982). The present study reveals an unexpected activation of PFK by fructose concentrations up to 8mM which can be attributed to the previously unknown activator, Fru-2,6-P_2.

Hepatic fructose metabolism is characterised by an accumulation of Fru-1-P and a depletion of adenine nucleotides. The accumulation of Fru-1-P is proposed to be due to the inhibition of liver aldolase activity by one of the products of adenine nucleotide degradation, IMP. However,

IMP levels are initially too low to account for the rapid increase in Fru-1-P concentration observed. It was therefore decided to reinvestigate the initial products of [U-^{14}C] fructose metabolism in order to explain the inhibition of aldolase and accumulation of Fru-1-P. Physiological fructose concentrations ($\sim$ 2mM) were used in the present study in contrast with the unphysiological fructose concentrations (10mM or greater) used by previous workers to study this problem. Under these conditions an unknown compound was shown to accumulate in addition to Fru-1-P. This thesis reports an attempt to characterize and identify this product.

II. MATERIALS AND METHODS

A. Materials

Enzymes and substrates were obtained from Boehringer Corporation, Lewes, Sussex or Sigma Chemical Company, Poole, Dorset. Solvents and inorganic reagents were from BDH Chemicals, Poole, Dorset and were of AnalaR grade. All solutions were made up in glass-distilled deionised water. [U-^{14}C] Fructose was purchased from Radiochemical Centre, Amersham, Bucks.

Krebs-Ringer bicarbonate (KRB) buffer contained 0.12M-NaCl, 4.85mM-KCl, 1.21mM-KH_2PO_4, 1.21mM-$MgSO_4$ and 0.025mM-$NaHCO_3$ in glass-distilled deionised water (Krebs and Henseleit, 1932). The buffer was adjusted to pH 7.4 with CO_2 and stored in the cold prior to use.

B. Methods

1. Preparation of hepatocytes

Hepatocytes were prepared by the method of Berry and Friend (1969), with later modifications of Wagle and Ingebretson (1975) and Krebs et al. (1973). Adult male Wistar rats (200-300g), fed on a laboratory chow diet, were anaesthetised by intraperitoneal injection of Nembutal (60 mg/ml sodium pentobarbitone, 0.1 ml/100 g body weight).

The peritoneal cavity was opened, the portal vein cannulated and the inferior vena cava divided below the right renal vein. The liver was perfused via the portal vein cannula with KRB buffer, continuously gassed with O_2/CO_2 (19:1 v/v). The thoracic cavity was opened and the

inferior vena cava anterior to the diaphragm cannulated through the right atrium. The inferior vena cava was tied off proximal to the right atrial cannulae.

The rat was then transferred to a perfusion cabinet, maintained at 37°C, and the liver perfused with collagenase (30 mg/100 ml KRB buffer plus 2.3mM-$CaCl_2$) for approximately 15 min with continuous gassing. When the liver turned pale and started to fragment, the perfusion was stopped. The liver was transferred to a plastic beaker containing KRB buffer and finely minced using scissors. The cell suspension was then filtered through a plastic funnel covered with nylon mesh (Nybolt, No. 10; 132 micron) into 50 ml centrifuge tubes. Cells were sedimented at 50g for 50s in a bench centrifuge and the pellet resuspended in KRB buffer containing 2.3mM-$CaCl_2$ and BSA (1.5% w/v). The hepatocytes were transferred to siliconised conical flasks (25 ml) and preincubated in a shaking water bath (100 strokes/min) at 37°C for 10 min with continuous gassing. Cell concentration and viability was determined microscopically by trypan blue dye exclusion using a haematocrit chamber. All flasks used for incubating hepatocytes were siliconised by rinsing thoroughly with dimethyldichlorosilane solution (2% v/v in 1,1,1-trichloroethane) and leaving them to dry overnight.

2. Incubation of hepatocytes

a. For enzyme assays

An equal vol. of glucose, fructose, glycerol, galactose or dihydroxyacetone was added to the incubated cells, at the end of the preincubation period, to give final hepatocyte

concentrations between 60 and 80 mg wet wt./ml. The substrates were made up in KRB buffer containing 2.3mM-$CaCl_2$ and 1.5% (w/v) BSA. The flasks were returned to the shaking water bath and gassed continuously throughout the appropriate incubation time. Incubations were stopped by freezing samples of the mixture in liquid N_2 prior to the assay of Fru-1,6-P_2ase (Section 6.a). For the assay of PFK activity an aliquot of the hepatocyte incubation mixture was spun at maximum speed in a bench centrifuge. The supernatant was discarded and the pellet resuspended in ice-cold homogenising buffer containing 50mM-Tris/HCl buffer, pH 7.6, 2.5mM-DTT, 0.1mM-EDTA, and 50mM-KF and homogenised, using a tight fitting Potter-Elvejhem glass homogeniser. The homogenate was centrifuged, at 27 000g for 30 min at 4°C, and the supernatant assayed for PFK activity (Section 6b).

b. For investigation of products of fructose metabolism

An equal vol. of fructose in KRB buffer (plus Ca^{2+} and BSA) containing 8.8 x 10^6dpm [U-^{14}C] fructose/ml was added to the pre-incubated cells to give a final hepatocyte concentration of between 60 and 80 mg wet wt./ml. The hepatocytes were incubated in the shaking water bath as before and reactions were stopped by one of three methods:

(i) Cells were harvested by centrifugation for 2 min at maximum speed in an MSE microcentrifuge, resuspended in 80% ethanol and frozen in liquid N_2. Samples could then be stored at -20°C prior to analysis.

(ii) After centrifugation as above, cells were suspended in an equal vol. of 50mM-Hepes buffer, pH 7.4, containing 100mM-KF and 15mM-EGTA, and frozen in liquid N_2. Extracts were thawed, heated to 90°C for 5 min, centrifuged and the supernatants kept frozen until analysed.

(iii) Cell suspensions (1 ml) were added to centrifuge tubes on ice, containing 4.2M-perchloric acid (0.05 ml). Samples were centrifuged for 10 min at maximum speed in a bench centrifuge and the supernatant neutralised with 4.2M-potassium carbonate (0.05 ml) and frozen at -20°C before analysis.

Cell extracts were analysed by paper chromatography. Samples (0.2ml) were applied to Whatman No. 3 chromatography paper and chromatographed for 16h in solvent A or B, to separate sugar phosphates from sugars. Glucose and fructose were separated by chromatographing samples (0.05 ml) applied to Whatman No. 1 chromatography paper, for 48h in solvent D. (For solvent details see Section 7.a). Similar samples were applied to Whatman No. 1 paper and subjected to high voltage electrophoresis in buffer H for 45 min (Section 6

Hepatocyte extracts were also applied to Dowex 1X4-400 (borate) and Dowex 1X8-400 (acetate) anion exchange columns (Section 8). In some experiments samples were treated with charcoal and precipitated with barium acetate before further analysis (Section 4).

3. Liver perfusion

Livers of male Wistar rats, fed on a laboratory chow diet, were cannulated and cleared of blood as detailed in Section 1. Once transferred to the perfusion cabinet, livers were perfused for 10 min with a non-recirculating medium containing 2mM-fructose in KRB buffer with 2.3mM-$CaCl_2$. After 10 min perfusion the livers were washed with 30 ml KRB buffer, containing 2.3mM-$CaCl_2$, removed and homogenised with a pestle and mortar in 2 vol. of 0.21M ice-cold perchloric acid. Liver extracts were then centrifuged for 10 min at maximum speed in a bench centrifuge. The supernatant was neutralised with 4.2M-potassium carbonate and, after further centrifugation for 10 min, the supernatant was stored frozen prior to analysis. In perfusions, in which [U-^{14}C] fructose was included, the procedure was as above except that a recirculating system with 50 ml of 2mM-fructose containing 1.32 x 10^6dpm/µmol was used. Liver extracts were applied to anion exchangers (Section 8) before and after charcoal treatment and barium acetate precipitation (Section 4).

4. Removal of nucleotides and barium precipitation

a. Removal of nucleotides by charcoal treatment

Hepatocyte and liver extracts, prepared as described in Sections 1, 2b and 3, were treated with activated charcoal (5% w/v extract). The mixture was shaken from time to time and after 10 min the charcoal was removed by centrifugation for 15 min at maximum speed in a bench centrifuge (Van Schaftingen et al., 1980b).

b. Barium precipitation

Charcoal-treated supernatants were mixed with 0.2 vol. of M-barium acetate and a few drops of phenolphthalein (1% in absolute ethanol). M-NaOH was added slowly until the solution retained a pink colour. The mixture was left for 40 min, and then centrifuged at maximum speed in a bench centrifuge for 15 min. The water-soluble precipitate contained sugar diphosphates and inorganic phosphate. Glycogen was precipitated from the supernatant by the addition of one vol. of ethanol (95% v/v). The mixture was left for 30 min, centrifuged as before and a further 3 vol. of ethanol (95% v/v) added to the supernatant. After 40 min the precipitate, collected by centrifugation, contained hexose, pentose and triose monophosphates. The supernatant contained water-and alcohol-soluble neutral sugars (Cardini and Leloir, 1957; Van Schaftingen et al., 1980b). Barium salts of the sugar monophosphates were solubilised by one of two methods:

(i) Precipitates were mixed with distilled water (equal to half the initial sample volume) and shaken, on ice, with 1g Amberlite IRC-50(H^+) resin per 5 ml for 15 min. After centrifugation the resin was extracted again with an equal vol. of water, centrifuged and the two supernatants combined and passed through an anion exchange column (Section 8).

(ii) Precipitates were mixed with distilled water (equal to the initial sample vol.) and 0.5M-H_2SO_4 (0.13 ml acid/ml extract) and shaken, on

ice, for 10 min (Cardini and Leloir, 1957). Samples were centrifuged and supernatants neutralised with M-NaOH prior to being applied to an anion exchange column (Section 8).

5. Purification of rat liver fructokinase.

Fructokinase was purified by the methods of Sánchez et al. (1971a) and Adelman et al. (1967a). Three male Wistar rats (250g) were killed and their livers removed, weighed, and homogenised, using a Potter-Elvejhem glass homogeniser with a teflon pestle, in 3 vol. of ice-cold 0.15M-KCl. The homogenate was centrifuged for 15 min at 20 000g (0 - 4°C). The resulting supernatant was heated at 65°C for 2 min, with constant stirring. The solution was cooled on ice to 4°C and centrifuged at 20 000g for 15 min. M-Sodium acetate buffer, pH 5.0, was added to the supernatant (1:19 v/v) followed by solid $(NH_4)_2SO_4$ to 45% saturation. The supernatant was stirred for 30 min, in the cold, and the precipitate formed collected by centrifugation for 15 min at 20 000g. The pellet was dissolved in 4 ml ice-cold water and dialysed for 3h against 500 vol. of 0.1M-Tris/HCl buffer, pH 7.5.

The enzyme was diluted to 30 ml with 0.01M-Tris/HCl buffer, pH 7.5, and applied to a DEAE-cellulose ion-exchange column (2 x 25 cm) previously equilibrated with the same buffer. The column was washed with 50 ml 0.01M-Tris/HCl buffer, pH 7.5 and fructokinase was eluted with a linear gradient of 0 to 1M-KCl in 0.01M-Tris/HCl buffer, pH 7.5 (total vol. 360 ml). Fractions (2 ml) were collected at a rate of 1.5 ml/min and assayed for fructokinase activity

(Section 6.d).

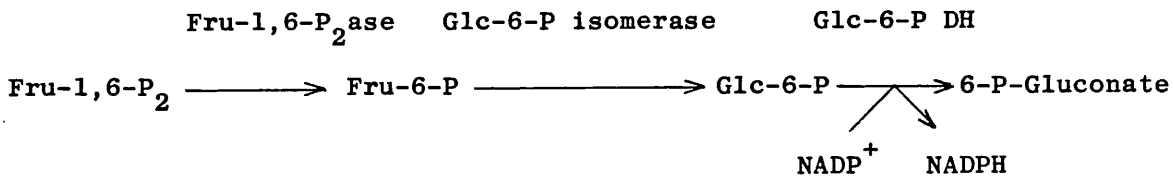
The fractions containing most of the fructokinase activity were pooled and the volume reduced to 5 ml, by ultrafiltration through an Amicon PM10 filter, prior to application to a Sephadex G-100 column (2.5 x 90 cm). The enzyme was eluted with 0.01M-Tris/HCl buffer, pH 7.5 at a rate of 0.5 ml/min. Fractions (2 ml) were collected and assayed for protein (Section 11), myokinase and fructokinase (Section 6c,d).

The purified enzyme stored at -20°C in 0.01M-Tris/HCl buffer, pH 7.5, was stable for several months.

6. Enzyme Assays

a. Assay of fructose-1,6-bisphosphatase (EC.3.1.3.11)

Test Principle

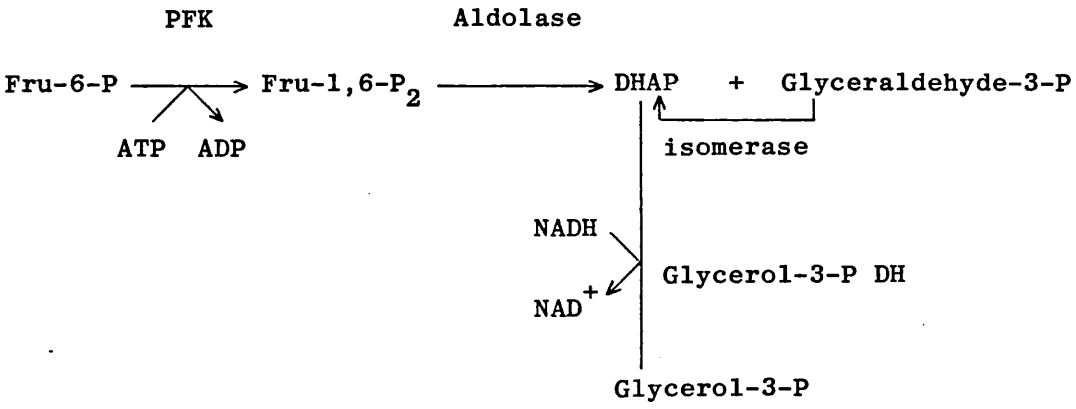


Fru-1,6-P_2ase was assayed spectrophotometrically at 340nm, following the rate of formation of Fru-6-P by the reduction of $NADP^+$ in the presence of excess Glc-6-P dehydrogenase and Glc-6-P isomerase (Traniello, 1974). The assay (final vol. 1 ml) contained: 50mM-Tris/HCl buffer, pH 7.6, 2.5mM-$MgCl_2$, 0.1mM-EDTA, 0.1mM-$NADP^+$, 0.54 units Glc-6-P dehydrogenase, 1.26 units Glc-6-P isomerase and 0.31 ml hepatocyte suspension diluted in 50mM-Tris/HCl

buffer, pH 7.6, to give approximately 1.4 mg cells per assay. The incubation mixture was preincubated for 5 min at 25°C to utilise all endogenous substrates present in the cell extract. Fru-1,6-P_2 was added at the appropriate concentration (0.02 to 0.20mM) to start the reaction and the rate of formation of NADPH followed at 340 nm and 25°C. One unit of enzyme activity is defined as the amount which catalyses the hydrolysis of 1µmol Fru-1,6-P_2 per min at 25°C.

b. Assay of phosphofructokinase (EC.2.7.1.11)

Test Principle



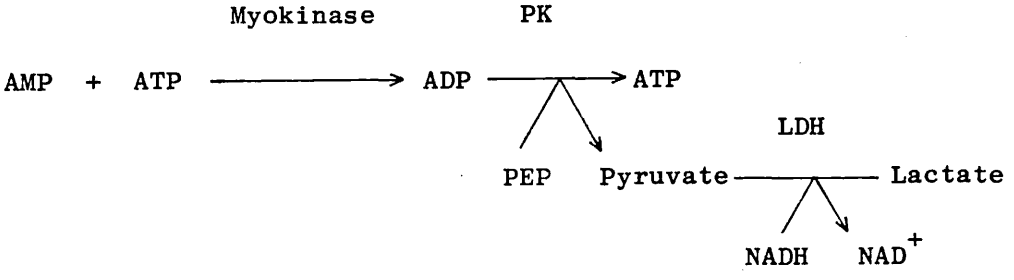
PFK was assayed by following the enzyme coupled formation of glycerol-3-phosphate spectrophotometrically with NADH and excess aldolase, triose phosphate isomerase and glycerol-3-phosphate dehydrogenase (Brand and Söling, 1974). The incubation mixture (final vol. 1 ml) contained 50mM-Tris/HCl, pH 7.6, 1mM-EDTA, 5mM-$MgCl_2$, 2.5mM-DTT, 0.16mM-NADH, 0.45 units muscle aldolase, 2.7 units triose phosphate isomerase, 0.4 units glycerol-3-phosphate dehydrogenase, 1mM-ATP and 0.20 ml hepatocyte extract, containing approximately 16 mg cells. A 5 min preincubation, at 25°C, was

found to be sufficient to utilise all endogenous Fru-6-P present in the extract. Fru-6-P, at varying concentrations, was then added to start the reaction and the rate of formation of NAD^+ was followed by measuring the decrease in absorbance at 340 nm. In experiments in which fructose was added to hepatocytes Fru-1-P was formed, which caused a high endogenous rate. The inclusion of 5mM-IMP in the assay, to inhibit liver aldolase activity, allowed a more accurate measurement of enzyme activity under these conditions. One unit of enzyme activity is defined as the amount that catalyses the phosphorylation of 1µmol Fru-6-P per min at 25°C.

In some experiments, cell extracts (1 ml) were also applied to a Sephadex G-25 gel-filtration column (1.2 x 10 cm) and eluted with 50mM-Tris/HCl buffer, pH 7.6, containing 0.1mM-EDTA, 2.5mM-DTT and 50mM-KF, at a rate of 1 ml/min. Fractions (1 ml) were collected and the peak of PFK activity (between fractions 4 and 8) pooled and assayed as described above.

c. Assay of myokinase (EC.2.7.4.3)

Test Principle

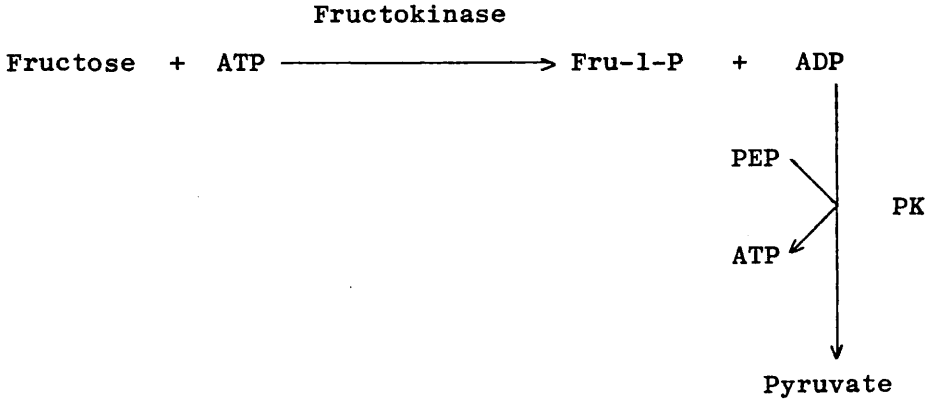


Myokinase activity was determined spectrophotometrically, by the enzyme coupled reaction usually used to determine

ADP and AMP, using pyruvate kinase and lactate dehydrogenase as detailed above (Jaworek et al., 1974). The assay (final vol. 1 ml) contained: 70mM-Triethanolamine buffer, pH 7.6, 1mM-$MgSO_4$, 0.13M-KCl, 2mM-AMP, 0.6mM-phosphoenolpyruvate, 0.2mM-NADH, 20 units pyruvate kinase, 25 units lactate dehydrogenase and enzyme extract. 1.2mM-ATP was added to start the reaction and the ATP-dependent reduction of NADH to NAD^+ was followed at 340 nm and 25°C. One unit of enzyme activity is defined as the amount that catalyses the hydrolysis of 1µmol ATP per min at 37°C.

d. Colorimetric assay of fructokinase (EC.2.7.1.4)

Test Principle



Fructokinase was assayed by following the fructose-dependent formation of ADP, using pyruvate kinase and phosphoenolpyruvate, and measuring the production of pyruvate by forming the dinitrophenylhydrazone derivative as described by Sánchez et al., (1971a). The incubation mixture (total vol. 0.1 ml) contained: 0.1M-Tris/HCl buffer, pH 7.4, 0.5mM-KCl, 6mM-$MgCl_2$, 5mM-phosphoenolpyruvate, 5mM-fructose, 6mM-ATP, 1 unit pyruvate kinase and 0.01 ml enzyme preparation. After incubation at 37°C for 15 min, the reaction was stopped

by the addition of 0.16 ml 10% trichloroacetic acid and 0.30 ml dinitrophenylhydrazine (0.1% in 2M-HCl). Following a further 5 min incubation at 37°C, 0.80 ml of 2.5M-NaOH was added and the resulting mixture centrifuged for 4 min in a micro-centrifuge. The absorbance of the supernatant was read at 520 nm and 37°C against controls, prepared by incubation in the absence of fructose or ATP. Enzyme activity was calculated using the Beer-Lambert Law, taking the molar extinction coefficient for dinitrophenylhydrazone as 1.8×10^4 litre/mol/cm at 520 nm. One unit of fructokinase is defined as the amount which catalyses the formation of 1μmol ADP per min at 37°C.

e. Assay of fructokinase using [U-^{14}C] fructose

Two methods were used for the assay of fructokinase activity using [U-^{14}C] fructose. The method used depended on the KCl concentration in the incubation mixture. Both assays, however, involved the assay of [^{14}C] Fru-1-P formed.

(i) The enzyme was incubated with 2mM fructose, 6mM-MgATP or 6mM-MgGTP, 0.1M-Tris/HCl buffer, pH 7.4, 0.5M-KCl and [U-^{14}C] fructose (4.4×10^6 dpm/ml). All reactions were carried out at 37°C and stopped with 4.2M-perchloric acid (0.5 ml/ml assay). Following centrifugation in a micro-centrifuge for 5 min, extracts were neutralised with 4.2M-potassium carbonate, recentrifuged, and aliquots of the supernatant applied to Whatman No. 3 paper and chromatographed in solvent A (Section 7.a). The region corresponding to standard Fru-1-P was cut out and the radio-

activity bound to the paper assayed, with 10 ml of toluene/PPO scintillant (5g PPO/litre toluene), using a Beckman scintillation counter. One unit of fructokinase activity is defined as the amount which catalyses the formation of 1μmol Fru-1-P per min at 37°C.

(ii) The incubation mixture (total vol. 0.05 ml) contained: 0.01M-Tris/HCl buffer, pH 7.4, 0.1M-KCl, 2.2 x 10^5dpm [U-^{14}C]fructose and enzyme. Specified concentrations of fructose and MgATP or MgGTP were added and incubations were carried out at 37°C. Reactions were usually started by the addition of enzyme. However, the order of additions made no significant difference to the observed rate of reaction. In assays involving the study of inhibitors, MgADP or MgGDP were included in the incubation mixture. At the end of the incubation time samples (0.05 ml) were spotted on Whatman DE81 discs (2.5 cm). After drying, each disc was washed with 50 ml distilled water using a Buchner funnel. The radioactivity bound to the disc was assayed using 10 ml of toluene/PPO scintillant as before. One unit of fructokinase activity is defined as in (i) above.

7. Separation methods

a. Paper chromatography

The following paper chromatography solvents were used:

Solvent A

Methoxyethanol: methylethylketone: 3N-ammonia (7:2:3 v/v) (Mortimer, 1952).

Solvent B

Isopropyl alcohol: ammonia (sp.gr. 0.88) : water (7:1:2 v/v) (Pontis and Fischer, 1963).

With these solvents samples were chromatographed on Whatman No. 3 chromatography paper for between 16 and 20h.

Solvent C

Ethanol-ammonium acetate pH 3.8. (7.5 : 3 v/v) (Paladini and Leloir, 1952).

Solvent D

Ethyl acetate: acetic acid: formic acid: water (18:3:1:4 v/v) (Hirst and Jones, 1955).

Solvent E

Butanol: pyridine: water (10:3:3 v/v) (Hirst and Jones, 1955).

Samples were chromatographed on Whatman No. 1 paper in solvent C or E for 18h and in solvent D for between 36 and 48h.

b. Development of chromatography paper

Standard sugar and sugar phosphates were located by dipping the chromatograms in PABA reagent (7g 4-amino-

benzoic acid and 4 ml phosphoric acid dissolved in 1000 ml of methanol), drying and heating at 110°C for 5 min.

A Birchover model 406 spark chamber was used to locate the position of radioactive material (20 min at 1.9 kV with 3 litre/min/ argon/methane. 90:10 v/v). The regions of the chromatogram containing radioactive material were cut into 0.5 or 1.0 cm strips. The amount of fructose remaining, and of label incorporated into each product was determined using 10 ml of toluene/PPO scintillant (5g PPO/ litre toluene).

c. Thin-layer chromatography (t.l.c.)

Glucose and fructose were separated by t.l.c. using cellulose acetate t.l.c. plates and developing three times in solvent F - ethyl acetate: butanol: pyridine: water (30: 30:25:20 v/v). Alternatively separation was achieved using silica gel G t.l.c. plates and developing twice in solvent G - ethyl acetate: pyridine: acetic acid: water (60:30:10: 10 v/v) Menzies et al. (1978).

Standard sugars could be located on the dried plates using a modified PABA reagent (7g 4-aminobenzoic acid and 17.5 ml phosphoric acid dissolved in 482.5 ml methanol) and heating at 110°C for 5 min. Radioactivity was located as detailed in Section 7, strips (0.5 cm) of absorbent material being scraped off the plate and the radioactivity associated with each area of the plate determined by suspension in 10 ml of toluene/PPO scintillant (5g PPO/litre toluene).

d. Electrophoresis

Samples were applied to Whatman No. 1 chromatography paper (10 cm wide) and run for 45 min in a Shandon model L24 high voltage electrophoresis tank (80mA, 2kV) in buffer H: 0.1M-ammonium formate: formic acid buffer, pH 3.6 (Smith, 1968).

8. Anion-exchange chromatography

a. Dowex 1X4-400 (borate) column

Samples were adjusted to pH 8.0 with dilute ammonia prior to application to a Dowex 1X4-400 (1 x 20 cm) anion exchanger in the borate form. Sugars and sugar phosphates were eluted with a linear gradient of 0.1 to 0.4M-triethylammonium borate (TEAB) in a total vol. of 360 ml at a rate of 1 ml/min (Lefewinae et al., 1964). Fractions (2 ml) were collected and samples (1 ml) assayed for radioactivity in 10 ml of Triton-toluene-PPO cocktail (5g PPO/litre toluene: Triton X-100, 2:1, v/v). Borate was removed from pooled fractions by washing with 50 ml methanol and rotary evaporating to dryness at 50°C at least three times or until the borate was removed. Samples were resuspended in a minimum vol. of distilled water.

b. Dowex 1X8-400 (acetate) column

Neutralised samples were diluted to 30 ml with distilled water and applied, at a rate of 0.4 ml/min, to a Dowex 1X8-400 (1 x 6 cm) anion exchanger in the acetate form (Rognstad, 1982a). Fractions (1.4 ml) were collected and neutral sugars were eluted by washing with 20 ml distilled water. Lactate, pyruvate and other carboxylic acids were

eluted with 2M-acetic acid (20 ml) applied at a rate of 1 ml/min. Sugar phosphates were subsequently eluted with 4M-formic acid (50 ml) at a similar rate. Aliquots (0.5 ml) of each fraction were assayed for radioactivity as described above. Formic acid was removed from the pooled sugar phosphate fractions by rotary-evaporation to dryness at 50°C. Samples were washed twice with 50 ml distilled water and resuspended in water for further analysis.

In some liver perfusion experiments neutralised perchloric acid extracts were applied directly to a Dowex 1X8-400 (acetate) column (1 x 28 cm) and eluted successively with water (100 ml), 2M-acetic acid (100 ml) and 4M-formic acid (300 ml).

9. Bio-Gel P-2 Chromatography

Following anion-exchange chromatography (Section II. B.8), neutralised samples (1 ml) were applied to a Bio-Gel P-2 column (1.2 x 103 cm) and eluted with water (210 ml) at a rate of 0.5 ml/min. The void vol. determined with Blue Dextran 2000, was 48.5 ml and fractions (2 ml) were then collected. Aliquots (1 ml) were assayed for radioactivity after addition of 10 ml of a Triton-toluene-PPO cocktail (5 g PPO/l toluene : Triton X-100, 2:1 v/v). Peak fractions were pooled and rotary-evaporated to dryness prior to further analysis.

10. Metabolite determinations

a. Enzymatic assays

Enzyme-coupled reactions were used to determine glucose,

Fru-1-P, Fru-2,6-P_2 and Glc-6-P concentrations.

(i) Glucose

The concentration of free glucose was determined spectrophotometrically following the reaction of glucose with glucose oxidase and peroxidase as described below (Bergmeyer and Bernt, 1974).

Test Principle

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} H_2O_2 + \text{Gluconate}$$

$$H_2O_2 + DH_2 \xrightarrow{\text{peroxidase}} 2H_2O + D$$

(DH_2-orthodianisidine)

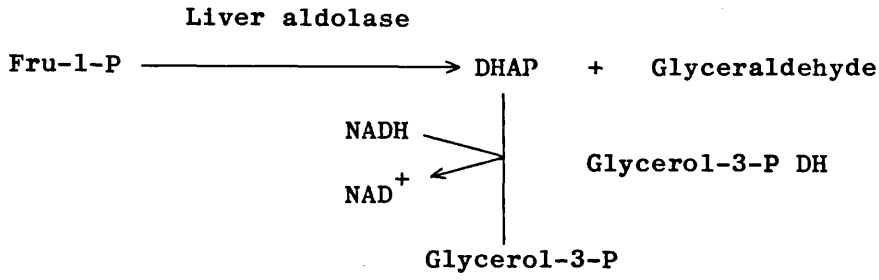
The stock buffer solution (166 ml) contained 100 ml 0.5M-Tris/HCl buffer, pH 7.0 and 66 ml glycerol. Working buffer solutions were made up daily by adding 3 mg glucose oxidase, 0.3 mg peroxidase and 1 mg ortho-dianisidine to 10 ml of stock buffer solution.

To 0.5 ml of sample, containing up to 20µg glucose, 1.0 ml of working buffer solution was added and samples were incubated at 37°C for 60 min. Reactions were stopped by adding 1.0 ml 9M-H_2SO_4 and absorbances were then read at 540 nm. Glucose concentrations were determined from a standard curve covering the range 0 to 20µg glucose.

(ii) Fru-1-P

Fru-1-P levels were determined according to the method of Eggleston (1974) using liver aldolase.

Test Principle



Fru-1,6-P_2 reacts with both liver and muscle aldolases whereas Fru-1-P reacts very slowly with muscle aldolase. Since hepatocytes contain both Fru-1,6-P_2 and Fru-1-P it is necessary to have muscle aldolase in the preincubation mixture to remove all Fru-1, 6-P_2. Liver aldolase preparations are frequently contaminated with lactate dehydrogenase and lactate dehydrogenase was therefore also routinely included in the preincubation mixture,to remove any pyruvate in the sample.

The assay mixture (1.5 ml) contained: 50mM-Tris/ HCl buffer, pH 7.4, 0.07mM-NADH, enzyme cocktail (containing 5 units lactate dehydrogenase, 0.14 units muscle aldolase, 0.1 units glycerol-3-phosphate dehydrogenase and 1.4 units triose phosphate isomerase) dialysed against water to remove ammonium sulphate, and 0.575 ml sample or water. Each sample was run in conjunction with a water blank to take account of extinction changes on the addition of liver

aldolase. The preincubation reaction was followed spectrophotometrically for 5 min at 340 nm and 0.10 ml of liver aldolase preparation (for purification see below) was added to start the reaction. The reaction was followed for 10-15 min and the change in absorbance recorded. The absorbance change in the reagent blank was subtracted from this prior to the calculation of Fru-1-P levels.

Preparation of liver aldolase

Liver aldolase was prepared according to the method of Eggleston (1974). Livers from adult Wistar rats, reared on a laboratory Chow diet, were extracted, weighed, homogenised in 4 vol. of 0.15M-KCl and centrifuged at 30 000g for 20 min. To every 100 ml of supernatant 27.7g $(NH_4)_2SO_4$ was added with constant stirring and the pH adjusted to 7.8 with 2M-NaOH. After 60-90 min in the cold (2°C) the extract was re-centrifuged at 30 000g for 20 min. A further 6.5g $(NH_4)_2SO_4$ was added to every 100 ml of supernatant and, after another 90 min in the cold, the precipitate formed was collected by centrifugation (30 000g for 20 min). The pellet obtained was dissolved in water (0.3 ml/g original liver) and then dialysed in the cold against 200 vol. of distilled water. The water was changed every hour for 4h to remove as much of the $(NH_4)_2SO_4$ as possible. The resultant protein solution was cloudy and hence was centrifuged to give a clear supernatant to which was added 0.1 ml 0.1M-EDTA, pH 7.4. The mixture was left to stand at room temperature for 60 min and then stored at -20°C prior to use.

(iii) Fru-6-P, Fru-2,6-P_2 and Glc-6-P

Fru-6-P and Glc-6-P concentrations were determined in perchloric acid extracts of hepatocytes by the method of Hohorst (1963). Since Fru-2,6-P_2 is acid labile and readily breaks down to Fru-6-P it will increase measured Fru-6-P concentrations. The ratio of Glc-6-P to Fru-6-P has been established as 3:1 (equilibrium constant, $K_c \sim 2$). If the measured concentration of Fru-6-P is more than would be predicted from such a ratio, the difference between expected and measured levels of Fru-6-P can be attributed to the presence of Fru-2,6-P_2 in the hepatocytes.

Test Principle

1) Glc-6-P $\xrightarrow[NADP^+ \rightarrow NADPH]{\text{Glc-6-P DH}}$ 6-P-Gluconate

2) Fru-6-P $\xrightarrow{\text{Glc-6-P isomerase}}$ Glc-6-P

The assay mixture (total vol. 1.02 ml) contained 0.2M-triethanolamine buffer pH 7.6, 0.5 ml extract, 0.2mM-$NADP^+$ and 5mM-$MgCl_2$. Having measured the absorbance spectrophotometrically at 340 nm, 1µg Glc-6-P dehydrogenase was added and the reaction followed to completion against a buffer blank. From the change in absorbance the concentration of Glc-6-P can be calculated and, by adding 1µg phosphoglucose isomerase and noting the further absorbance change, total Fru-6-P concentrations can also be determined.

b. Colorimetric assays

Colorimetric assays were used to determine total carbohydrate, fructose, reducing sugar and phosphate concentrations.

(i) Total carbohydrate

The phenol-sulphuric reaction of Dubois et al. (1956) was used to determine total carbohydrate. Standard curves were prepared for both glucose and fructose, in the 0 to 100 µg range, since the specificities for each sugar are slightly different.

To 1.0 ml of sample, containing up to 100µg sugar, 0.025 ml 80% (w/v) redistilled phenol and 2.5 ml conc. H_2SO_4 (specific gravity 1.84) were added. The test-tubes were shaken vigorously and left for 30 min at room temperature prior to absorbances being measured spectrophotometrically at 480 nm. Carbohydrate concentrations were determined from the standard curves.

(ii) Fructose

Fructose concentrations were determined according to the modified resorcinol — HCl method of Roe (Roe et al., 1949). Two stock solutions were made up as follows. Solution A contained 0.1 g resorcinol and 0.25 g thiourea in 100 ml glacial acetic acid. This solution was stable if stored in the dark. Concentrated HCl was diluted 5 to 1 with water to make solution B.

To 0.2 ml of the sample, containing less than 40µg fructose, 0.1 ml solution A and 0.7 ml solution B was added. Samples were mixed and then placed in a water bath, at 80°C, for 10 min. Following cooling on ice, for 5 min, the absorbance at 520 nm was measured spectrophotometrically within the next 30 min. Fructose concentrations were determined from a standard curve covering the range 0 to 40µg fructose.

The cysteine-carbazole reaction, described by Dische and Borenfreund (1951), was also used for fructose determinations. To 0.5 ml of sample, containing less than 50µg fructose, 0.1 ml 1.5% (w/v) cysteine-HCl solution was added followed by 3 ml 75% (v/v) H_2SO_4 and 0.1 ml carbazole reagent (0.12%, w/v, carbazole in ethanol). After thorough mixing, samples were left at room temperature for 24h prior to absorbances being measured spectrophotometrically at 560 and 750 nm. The difference in absorbance at the two wavelengths was proportional to the concentration of fructose in the sample which was determined by reference to a standard curve.

(iii) Reducing sugar

The arsenomolybdate method described by Nelson (1944) was modified to allow the determination of 5 to 40µg of reducing sugar. Three stock reagent solutions were made up as follows. Reagent A contained 25 g anhydrous sodium carbonate, 25 g Rochelle salt, 25 g sodium bicarbonate and 200 g sodium

sulphate in a litre of distilled water. Reagent B was a 15% (w/v) copper sulphate solution containing a few drops of conc. H_2SO_4. Reagent C contained 25g ammonium molybdate dissolved in 450 ml distilled water, to which 21 ml conc. H_2SO_4 was added slowly followed by 25 ml 12% (w/v) sodium hydrogen arsenate. Reagent C was incubated for 24h at 37°C and stored in the dark prior to use. Reagent D was made up daily from 50 ml of reagent A and 2 ml of reagent B. To 0.2 ml of sample, containing less than 40µg of reducing sugar, 0.8 ml of water and 1.0 ml of reagent D were added. The mixture was boiled for 20 min at 100°C and, after cooling, 1.0 ml of reagent C was added and the absorbance measured spectrophotometrically at 520 nm. The concentration of reducing sugar was determined by reference to a standard curve. When acid-revealed reducing sugar was to be determined samples were boiled with H_2SO_4 (final concentration 2M) at 100°C for 20 min. Following neutralisation with 2M-NaOH samples were assayed as described above.

(iv) Phosphate

Phosphate concentrations were determined by the method of Fiske and Subbarow (1925).

The sample (0.5 ml), containing up to 40µg phosphate, was boiled with 0.5 ml 4M-H_2SO_4 at 100°C for 30 min. Incubations were neutralised with 0.8 ml 5M-NaOH and reactions stopped with 5 ml 0.2% (w/v) ammonium molybdate in 0.2M-H_2SO_4. Colour was developed by

adding 0.20 ml of 1-amino-2-napthol-4-sulfonic acid solution, made up freshly by dissolving 0.2g sodium bisulphite and 6.0g sodium sulphite in 100 ml water. If cloudy, the incubation was centrifuged at maximum speed on a bench centrifuge for 5 min and kept on ice until the absorbance at 680 nm was measured spectrophotometrically. Phosphate concentrations were determined by reference to a standard curve.

11. Protein determination

Protein was routinely assayed, using Coomassie Brilliant Blue G-250 dye, according to the method of Bradford (1976). The protein reagent contained 100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% (v/v) ethanol, to which 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was made up to one litre with distilled water and, when stored in the dark, was stable for several months.

To 0.05 ml of sample, containing up to 50µg protein, 2.5 ml of protein reagent was added and the samples were mixed thoroughly. The absorbance at 595 nm was measured spectrophotometrically against a reagent blank after 2 min and within 60 min. BSA (0-50µg) was used to obtain absorbance values for a standard curve from which the amount of protein in the samples could be determined.

12. Statistical analysis

Statistical analysis of experimental results was routinely performed using the formulae described below to

calculate the standard deviation (S.D.) and standard error of the mean (S.E.M.) - Ballentine, 1974.

1. $$S.D. = \sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}}$$

where $\sum$ = sum of

X = observation or observed value

$\bar{X}$ = mean of observed values of the sample

i.e. $\sum \frac{X}{n}$

n = number of observations

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

A t-test for small populations was used to test the significance of the results obtained from different incubations.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sigma} \qquad \text{Degrees of freedom} = n_1 + n_2 - 2$$

where t = test of significance

σ^2 = variance of mean estimated from pooled variances

s = standard deviation of the observation of the population

Since $$\sigma^2 = SD^2 \left(\frac{n_1 + n_2}{n_1 n_2} \right)$$

and $$SD = \sqrt{\frac{\sum (X_1 - \bar{X}_1)^2 + \sum (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 2}}$$

Hence $$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(X_1 - \bar{X}_1)^2 + (X_2 - \bar{X}_2)^2}{n_1 + n_2 - 2}} \cdot \frac{n_1 + n_2}{n_1 n_2}}$$

13. Amino acid analysis

Amino acid analysis was performed according to the procedures of Moore and Stein (1963) by the Botany Department at Royal Holloway College. Samples were hydrolysed with 2M-TFA at 100°C for one hour. After rotary-evaporation at 50°C the hydrolysates were dissolved in water and the pH of the resulting solution was adjusted to 2 with 1M-HCl. The analysis was performed using an amino acid analyser (JLC-6AH/Jeol Ltd.) previously calibrated by analysing a mixture of pure amino acids.

Hydrolysed samples were also analysed qualitatively for amino acids by t.l.c. in solvent G (Section 7.c) and the amino acids located using ninhydrin reagent (5% v/v ninhydrin in acetone with 1.5 ml of pyridine added per 100 ml of reagent.

14. Mass Spectrometry

Purified samples of the unknown metabolite were analysed by both electron impact (e.i.) and chemical ionisation (c.i.) mass spectrometry by the Chemistry Department at Royal Holloway College using a VG Analytical 12F Mass Spectrometer. The operating conditions are as detailed below;

Ionisation energy 70eV
Source temperature 180°C
Accelerating voltage 4000 volts

For c.i. the ionisation energy was reduced to 50eV, the reagent gas was isobutane and the source pressure was 0.1 torr, other conditions were as described above.

III. RESULTS AND DISCUSSION

The aims of this thesis, as described in Section I.D, were to attempt to explain the rapid accumulation of Fru-1-P following a fructose load and to investigate the effects of fructose on PFK and Fru-1,6-P_2ase.

A. Studies on the accumulation of Fru-1-P

1. Fructose utilisation

Isolated perfused liver and isolated heptocytes were shown to be capable of high rates of fructose utilisation. The utilisation of a physiological concentration of fructose (2mM) by the perfused liver is shown in Fig. 9. An initial rate of fructose removal of at least 1.57µmol/min/g was calculated. The results of a similar study using isolated hepatocytes, from both fed and 24h starved rats, incubated with 2mM-fructose are shown in Fig. 10. The initial rates of fructose utilisation and glucose production are reported in Table 4. These results correlate well with a reported

Table 4. Initial rate of fructose utilisation and glucose production in isolated hepatocytes incubated with 2mM-fructose.

For full experimental details see Fig. 10

Hepatocytes from	Fructose utilisation µmol/min/g cells	Glucose production µmol/min/g cells
Fed rats	1.31	0.81
24h starved rats	2.21	1.64

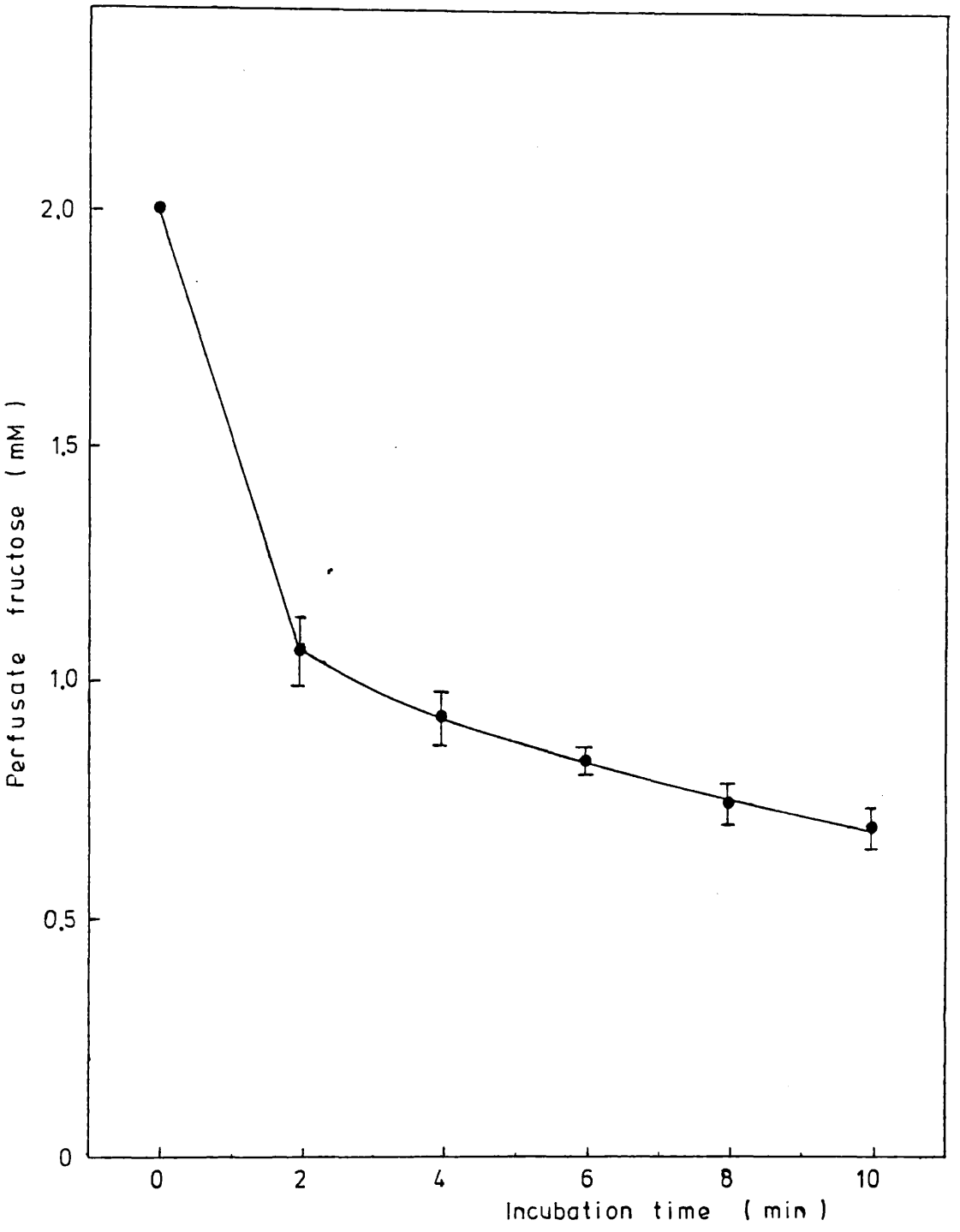


Fig. 9. Effect of incubation time on the utilisation of 2mM-fructose by perfused liver.

Livers (15g) from fed male rats were perfused with 2mM-fructose for 10 min as detailed in Section II.B.3. Aliquots (0.2 ml) were taken from the perfusate at 2 min intervals and reactions were stopped with 4.2M-perchloric acid (0.01 ml). Neutralised samples were assayed for fructose content by the method of Roe (Section II.B.10.b.ii). Results plotted are mean of 3 different perfusions with s.e.m. as shown by the bars.

Fig. 10. Utilisation of [U-^{14}C] fructose (●—●) and production of [^{14}C] glucose (o—o) by isolated hepatocytes prepared from fed (A) and 24h starved (B) rats.

Hepatocytes were incubated with 2mM-[U-^{14}C] fructose and reactions were stopped with 4.2M-perchloric acid as detailed in Section II.B.2.b.iii. Neutralised samples were subjected to paper chromatography in solvent D and the separated hexoses assayed by liquid scintillation counting (Section II.B.7.a,b). Results plotted are means of 2 different cell preparations. The mean hepatocyte concentration was 52 mg cells/ml (A) and 112 mg cells/ml (B).

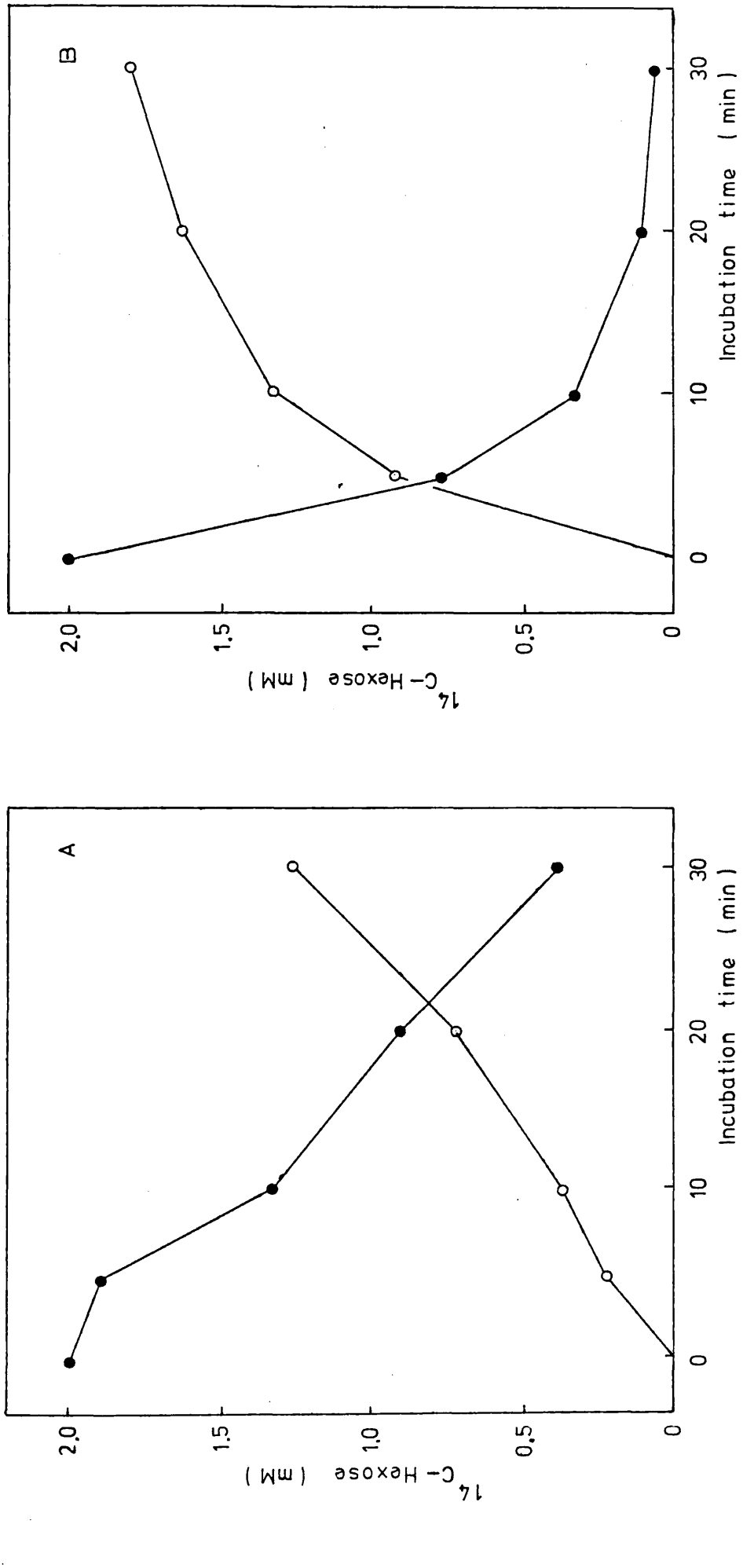


Fig. 10. Utilisation of [U-^{14}C] fructose (●—●) and production of [^{14}C] glucose (o—o) by isolated hepatocytes prepared from fed (A) and 24h starved (B) rats.

activity for fructokinase of 2.58μmol/min/g liver (Sánchez et al., 1971a) and are in agreement with an initial rate of fructose removal of 2.63μmol/min/g liver and a glucose output of 1.90μmol/min/g liver, during perfusion with 10mM-fructose, reported by Woods et al. (1970). The results indicate that in the starved rat fructose is more rapidly taken up and metabolised by the liver cells than in fed animals. After 30 min incubation of the hepatocytes from starved animals 90% of the fructose has been converted to glucose, 3% remains unconverted and the remaining 7% is presumably converted to lactate and other metabolites. Hepatocytes from fed rats show slower rates of fructose utilisation and of glucose production with 63% of the fructose converted to glucose after 30 min, 18% converted to other products and 19% unmetabolised confirming that under conditions of fasting gluconeogenesis predominates. As discussed in Section I.C.1.a, following a 24h fast hepatic Fru-2,6-P_2 concentrations have been shown to be significantly depleted. Since Fru-2,6-P_2 is a major regulator of glycolysis and gluconeogenesis a fall in its concentration will result in an inhibition of PFK and activation of Fru-1,6-P_2ase and consequently gluconeogenesis will predominate under these conditions.

2. Products of fructose metabolism

The products of fructose metabolism were investigated using liver preparations from fed rats unless otherwise stated. Hepatocytes were incubated with [U-^{14}C] fructose for 20 min, the cell extracts were heated to 90°C for 5 min to precipitate protein (Section II.B.2.b.ii), centrifuged

and the supernatant applied to a Dowex 1X4-400 (borate) column (Fig. 11A). The radioactive peak eluting between fractions 80-100 was shown to contain fructose (Roe determination) and was identified as Fru-1-P by three criteria. Firstly the material co-chromatographed with standard Fru-1-P on Whatman No. 3 chromatography paper in solvent A (Fig. 12). Secondly it reacted with liver aldolase yielding dihydroxyacetone phosphate and thirdly it eluted in the same position as standard Fru-1-P from the Dowex 1X4-400 (borate) column (data not shown). The first major peak contained fructose (determined by the method of Roe) and it eluted in the same position as fructose standard (results not shown). Since Fru-1-P is reported to be the major initial product of fructose metabolism (Cori et al., 1951; Hers, 1955) the appearance of a peak eluting immediately after the fructose peak was unexpected. In order to show that the appearance of this peak was not due to an artifact of the extraction procedure the hepatocyte incubation was repeated and products extracted with 80% ethanol (Fig. 11B) or 4.2M-perchloric acid (Fig. 11C) as described in Section II.B.2.b.i,iii. As shown in Fig. 11, following incubation with 2mM-[U-^{14}C] fructose, in addition to Fru-1-P an unknown metabolite accumulates irrespective of the extraction method used.

The fractions containing the unknown metabolite were concentrated by rotary-evaporation and the sample applied to Whatman No. 3 chromatography paper and chromatographed with solvent B. As shown in Fig. 12, the unknown metabolite separated from both standard Fru-1-P and [^{14}C] Fru-1-P obtained from the same Dowex 1X4-400 (borate) column. It

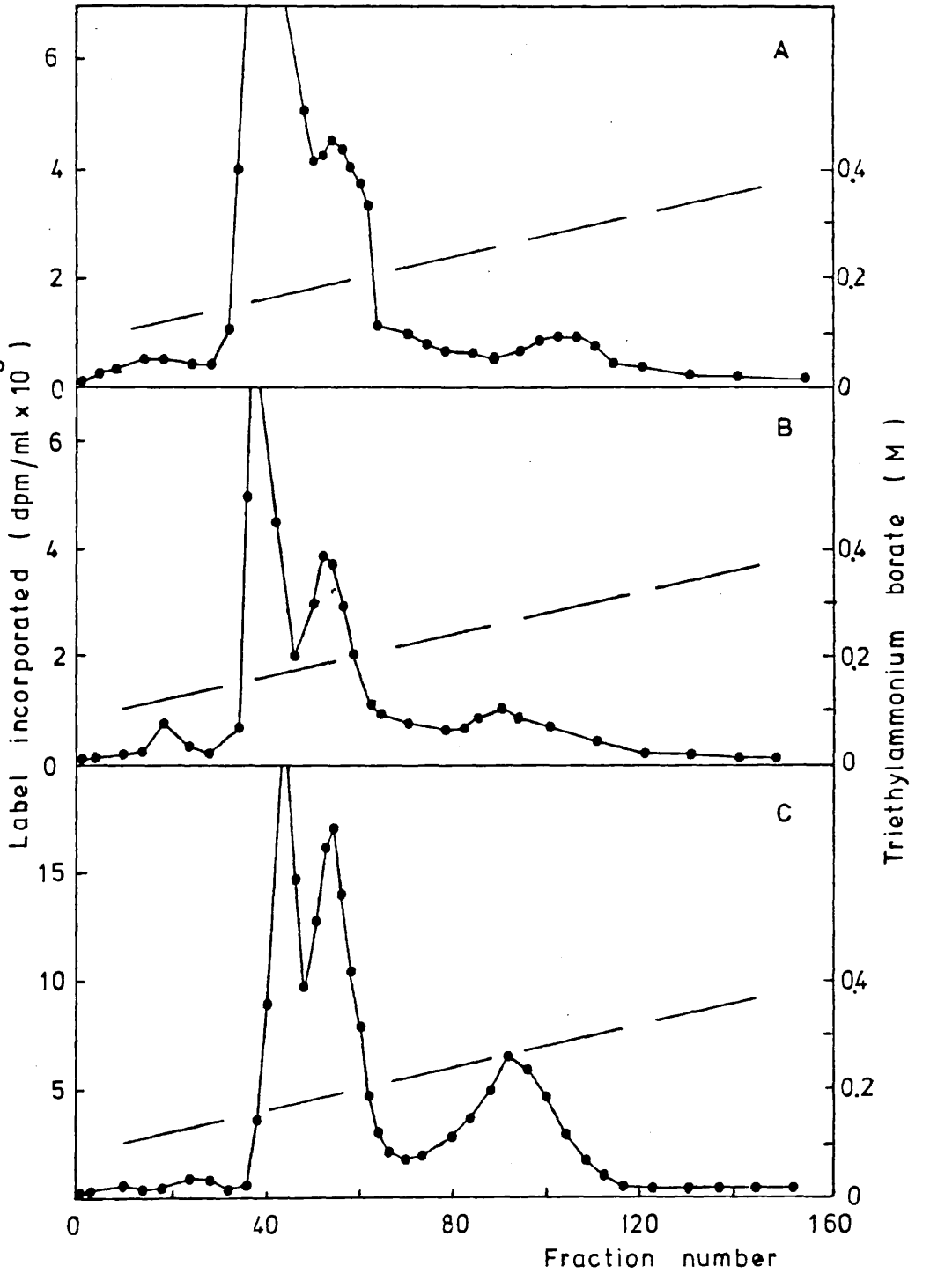


Fig. 11. Dowex 1X4-400 (borate) chromatography of the products of 2mM-[U-^{14}C] fructose metabolism in isolated hepatocytes.

Hepatocytes were incubated for 20 min (A,B) and 10 min (C), the reactions stopped and the products extracted by: A, heat (90°C); B, 80% ethanol or C, 4.2M-perchloric acid precipitation (Section II.B. 2.b.). Samples were applied to a Dowex 1X4-400 (borate) column and eluted with a linear gradient of 0.1 - 0.4M - TEAB (Section II.B.8.a). Fractions (2 ml) were collected and aliquots (1 ml) assayed for radioactivity.

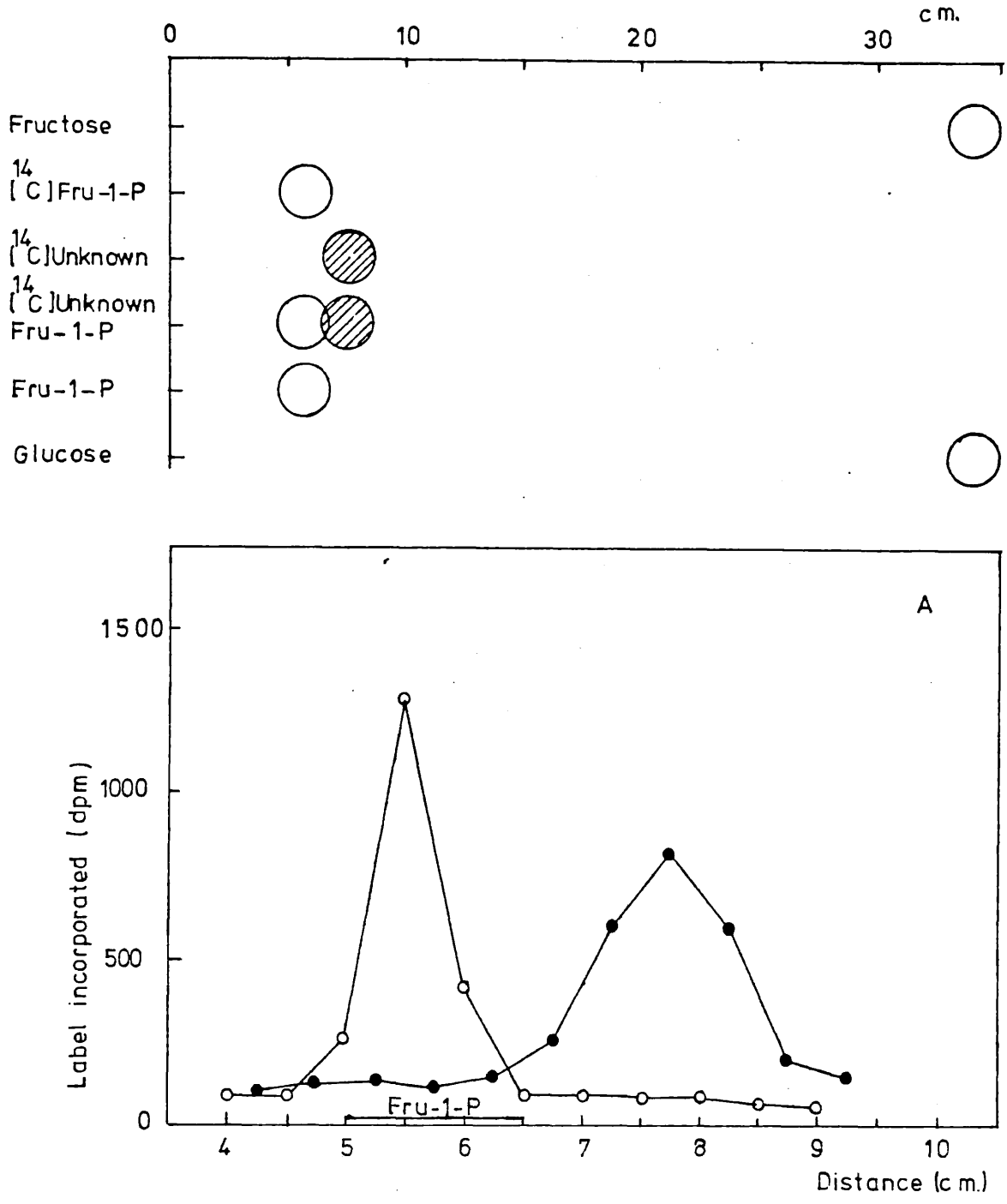


Fig. 12. Paper chromatographic analysis of the labelled products following separation by anion exchange chromatography.

Ethanol extracts of hepatocytes following a 20 min incubation with 2mM-[U-^{14}C] fructose (Section II.B.2.b.i) were applied to a Dowex 1X4-400 (borate) column and fractions containing Fru-1-P and the unknown metabolite were separately applied to Whatman No. 3 chromatography paper and run in solvent B (Section II.B.8.a and 7a,b). Fructose, glucose and unlabelled Fru-1-P standards were located using PABA reagent. The radioactive Fru-1-P and unknown metabolite were located using a spark chamber and then (Fig. 12A) by cutting the chromatography paper into 1 cm strips and assaying the radioactivity by liquid scintillation counting.

was also clearly separated from both glucose and fructose. Similar results were obtained using solvent A (results not shown). The unknown metabolite could also be separated from Fru-1-P and fructose when a hepatocyte extract was directly subjected to paper chromatography using solvents A (results not shown) and B (Fig. 13). The peak at the baseline was probably attributable to glycogen since products extracted with 80% ethanol, which precipitated glycogen, did not show a radioactive peak at the origin (Fig. 17). There was good agreement between the concentration of unknown metabolite determined after separation on either anion-exchange or paper chromatography (Table 5). It was therefore decided

Table 5. Hepatocyte concentration of Fru-1-P and unknown metabolite determined after separation by either anion-exchange or paper chromatography.

Hepatocytes (71 mg cells/ml) were incubated with 2mM-[U-^{14}C] fructose for 20 min and metabolites precipitated with 80% ethanol (Section II.B.2.b.i). Samples were applied either to Whatman No. 3. chromatography paper and run in solvent B or to a Dowex 1X4-400 (borate) column.

Chromatography	Unknown metabolite µmol/g cells	Fru-1-P µmol/g cells
Dowex 1X4-400	0.639	0.271
Paper (Solvent B)	0.631	0.329

to use paper chromatography to resolve Fru-1-P and the unknown metabolite in order to study the effects of incubation time

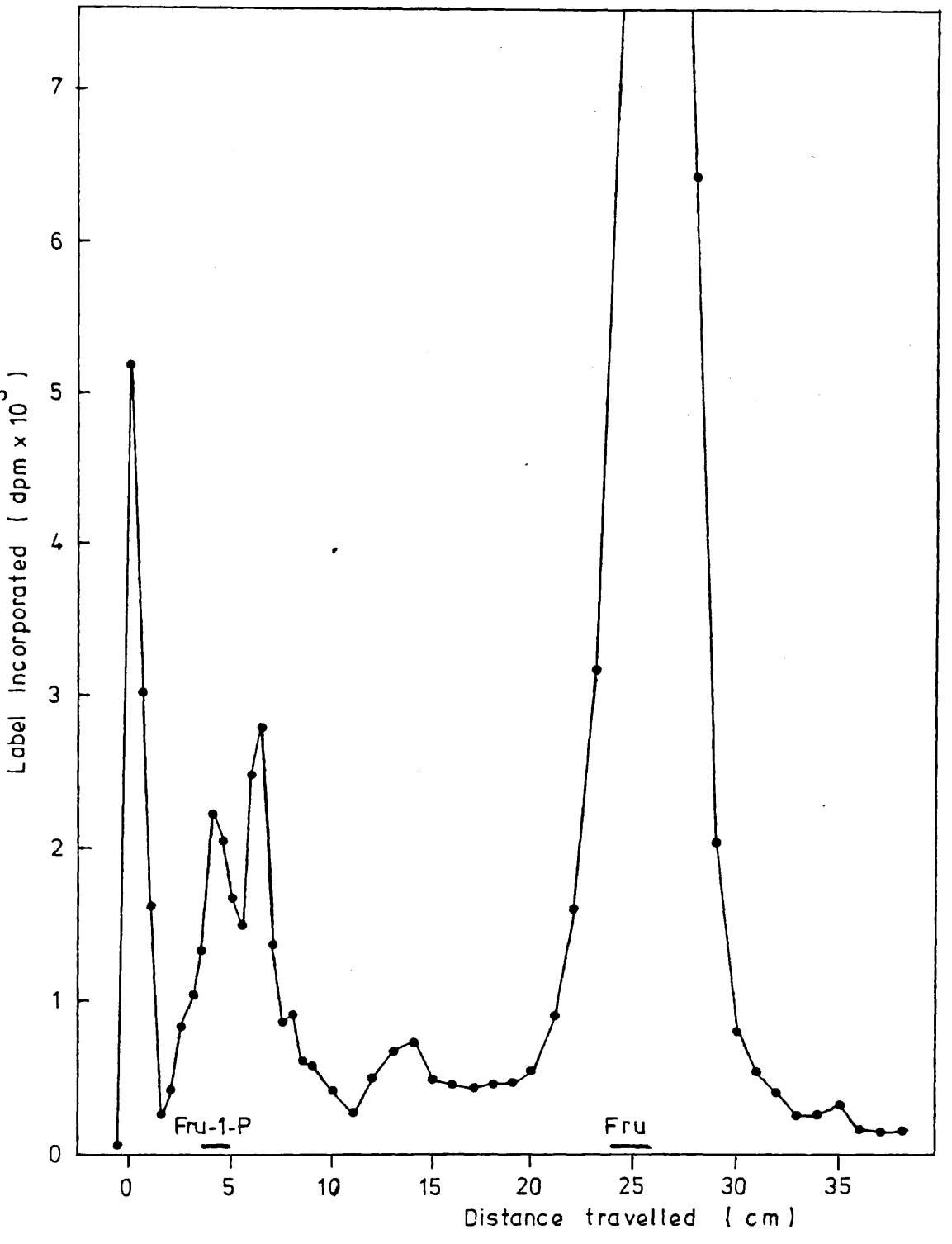


Fig. 13. Paper chromatographic separation of the radioactive products resulting from the incubation of hepatocytes with 2mM-[U-^{14}C] fructose.

Hepatocytes were incubated for 30 min with 2mM-[U-^{14}C] fructose. Reactions were stopped by heating to 90^{o}C as detailed in Section II.B.2.b.ii. Products were separated in solvent B and assayed by liquid scintillation counting with standards being located with PABA reagent (Section II.B.7.a,b).

(Fig. 14) and fructose concentration (Fig. 16) on metabolite production since several samples could be analysed simultaneously.

Fru-1-P and the unknown metabolite are synthesised rapidly by hepatocytes at rates of 105 and 173 nmol/min/g cells respectively in response to a 2mM-fructose load (Fig. 14). Both products reach a maximum concentration after 10-15 min incubation. However, the unknown disappears at a much faster rate over the next 15 min (21 nmol/min/g cells) than Fru-1-P (6 nmol/min/g cells). A similar experiment measuring Fru-1-P and unknown metabolite production during the first 180 sec of the incubation gave no further indication as to which was formed first (results not shown).

Various possibilities exist as to the pathway of fructose metabolism to the unknown metabolite. The unknown metabolite could be formed directly from fructose by an as yet undefined pathway. Alternatively, it could be either an intermediary in the production, or a metabolite, of Fru-1-P.

Purified fructokinase, 2mM - [U-^{14}C] fructose and a variety of nucleotide triphosphates (6mM) were incubated for 15 min at 37^{o}C. Separation of the products on paper chromatography (Solvent A) indicated there was no significant enzyme activity (< 5% of activity with MgATP) with MgCTP or MgUTP (data not shown). This was consistent with the earlier work on fructokinase (Parks et al., 1957; Adelman et al., 1967a). However, incubation of the enzyme with MgGTP was shown to result in the phosphorylation of fructose to a product which co-chromatographed with Fru-1-P on paper

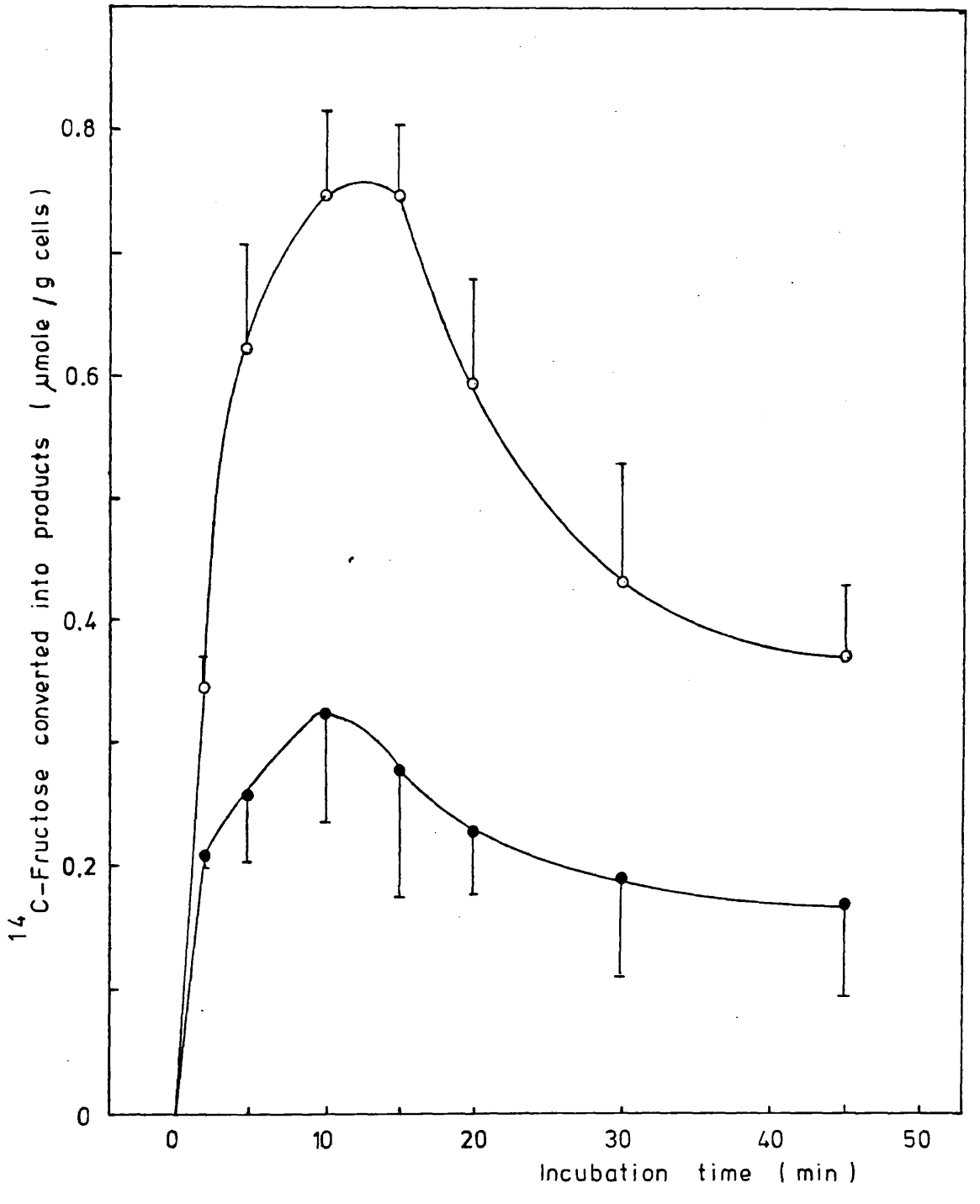


Fig. 14. Time-course of the formation of Fru-1-P (●—●) and the unknown metabolite (o—o) in isolated hepatocytes.

Hepatocytes were incubated with 2mM-[U-^{14}C] fructose and reactions stopped with 4.2M-perchloric acid as detailed in Section II.B.2.b. iii. The labelled products were separated by paper chromatography in solvent A and assayed by liquid scintillation counting (Section II.B.7.a,b). The results plotted are means of data from 3 different cell preparations with s.e.m. as indicated by the bars. The mean hepatocyte concentration was 70 mg cells/ml.

chromatography in solvent A (data not shown). Neutralised perchloric acid extracts of the MgGTP incubations, together with standard Fru-1-P, were passed down a Dowex 1X4-400 (borate) column. The resulting profiles confirmed that the sole labelled product of the reaction was Fru-1-P (Fig. 15). [^{14}C] Fru-1-P, produced by incubating the enzyme with MgATP and [U-^{14}C] fructose, also eluted at a similar TEAB concentration as shown in Fig. 15. The kinetics of the fructokinase reaction with MgGTP were investigated further and are reported in Section III.C.3. These results show that fructokinase, purified by an established procedure (Sánchez et al., 1971a), whilst clearly catalysing the formation of Fru-1-P does not form the unknown metabolite in the presence of the substrates and effectors included in the assay. The hepatic activity of fructokinase more than accounts for the rate of synthesis and degradation of Fru-1-P thus the existence of another enzyme responsible for the synthesis of the unknown metabolite from fructose seems unlikely. This was confirmed by the failure of a Sephadex G-25 filtrate, of a 100 000g supernatant of a liver homogenate from a fed rat, incubated with [U-^{14}C] fructose and either ATP or GTP to form the unknown under conditions of optimal fructokinase activity (results not shown). It also shows that the unknown metabolite is not formed as a result of a non-specific interaction of aldolase with either fructose or Fru-1-P. It therefore seems likely that the unknown metabolite is formed after Fru-1-P synthesis from fructose. Recent experiments in this laboratory have shown that the unfiltered extract of a 100 000g supernatant will form the unknown in the presence of [U-^{14}C] fructose and ATP suggesting that a low molecular

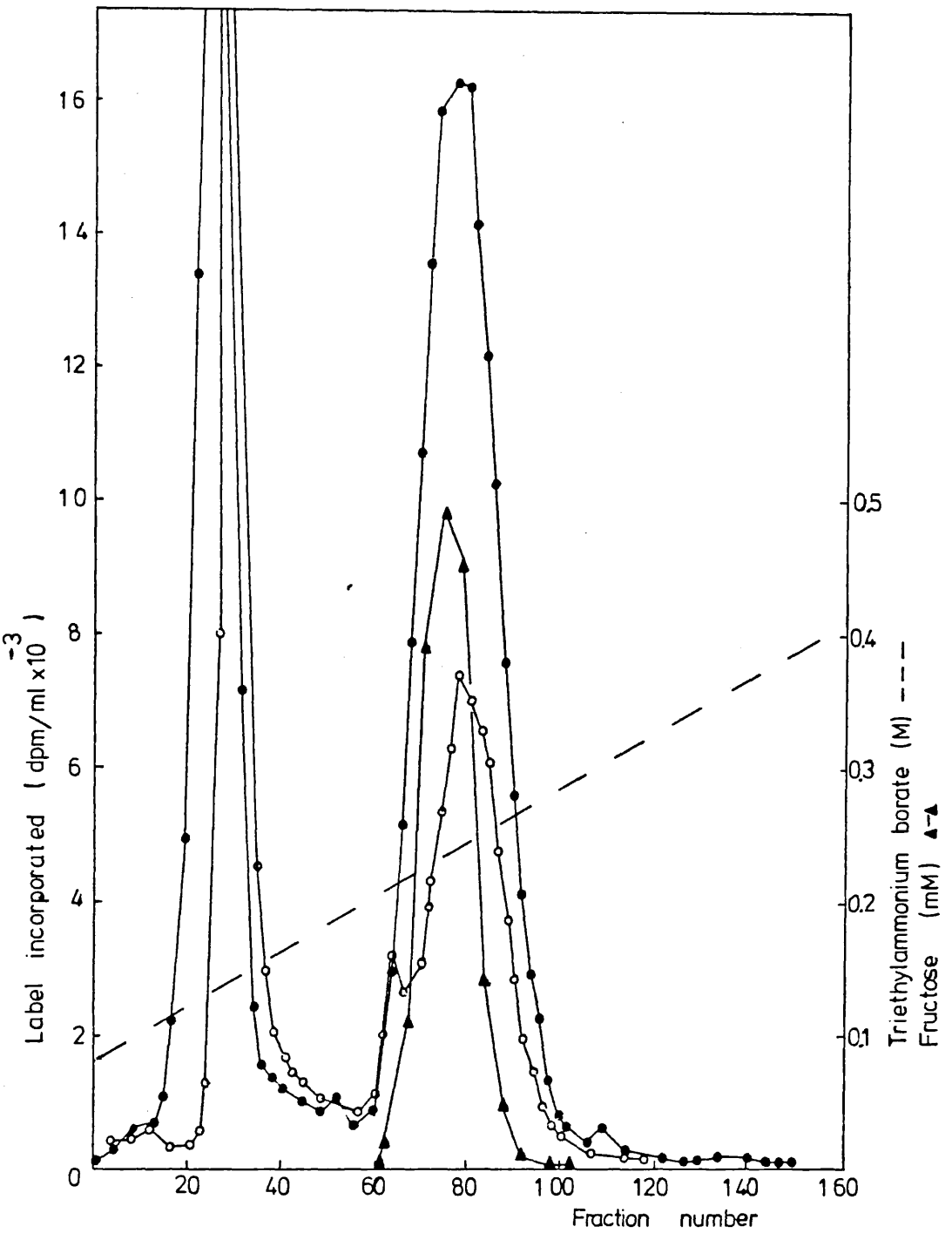


Fig. 15. Dowex 1X4-400 (borate) chromatography of the products of ATP (●—●) and GTP (o—o) dependent fructokinase activity.

The 0-45% $(NH_4)_2 SO_4$ fraction was incubated for 15 min at 37°C with 2mM- [U-^{14}C] fructose and 6mM- Mg ATP or 6mM-MgGTP as detailed in section II.B.6.e.i. Reactions were stopped with 4.2M-perchloric acid and the supernatants (pH to 8.0) applied to the Dowex 1X4-400 (borate) column (Section II.B.8.a) with standard Fru-1-P and eluted with a linear gradient of 0.1 to 0.4M-TEAB. Fractions (2ml) were collected and aliquots (1 ml) assayed for radioactivity. Standard Fru-1-P was detected using Roe's colorimetric assay for fructose (Section II.B. 10.b.ii).

weight compound, removed by Sephadex G-25 filtration, is necessary for the synthesis of the unknown metabolite by liver cells (personal communication).

As shown in Figs. 16 and 17 and in Table 6 the unknown metabolite is formed predominately, compared to Fru-1-P, following incubation with low fructose concentrations (< 4mM). At 4mM-fructose approximately equal amounts of both products accumulate whilst at higher fructose concentrations there is a rapid increase in Fru-1-P synthesis relative to the unknown metabolite, the concentration of the latter changed little between 4 and 10mM-fructose. The possibility that the unknown arises as a result of a [^{14}C] labelled contaminant of [U-^{14}C] fructose is unlikely since changes in the level of the unknown metabolite occur as a function of the concentration of unlabelled fructose.

These results suggest further that the unknown metabolite is formed from Fru-1-P, since when the unknown metabolite is limiting (following incubation with 4-10mM -fructose) Fru-1-P concentrations rapidly increase. At low fructose concentrations the level of Fru-1-P formed is low and this could be due to its conversion to the unknown metabolite. The concentration of Fru-1-P formed by hepatocytes incubated with fructose is lower than that reported by Mapungwana (1982), the largest discrepancy being at low fructose concentrations. Following incubation with 2mM-fructose for 20 min the experiments here showed the formation of only 0.25 µmol Fru-1-P/g cells whereas Mapungwana (1982) enzymatically estimated Fru-1-P concentrations under similar conditions as 3.2µmol/g cells. The enzymatic assay is not

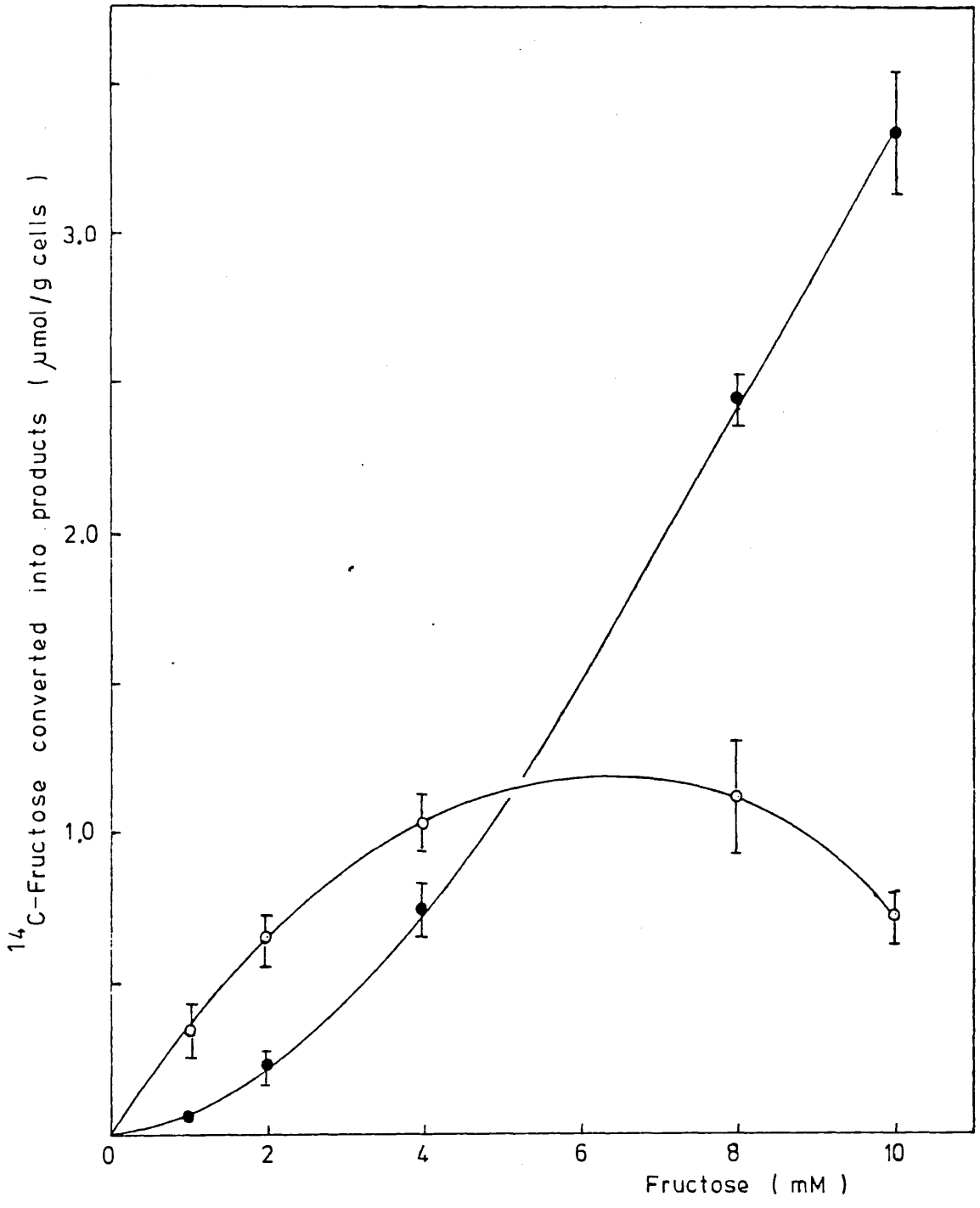


Fig. 16. Effect of fructose concentration on the formation of Fru-1-P (●—●) and unknown metabolite (o—o) in isolated hepatocytes.

Hepatocytes were incubated with various concentrations of fructose and 4.4 x 10^6 dpm [U-^{14}C] fructose/ml incubation for 20 min and reactions stopped with 4.2M-perchloric acid (Section II.B.2.b.iii). For other experimental details see Fig. 14. The mean hepatocyte concentration was 66 mg cells/ml.

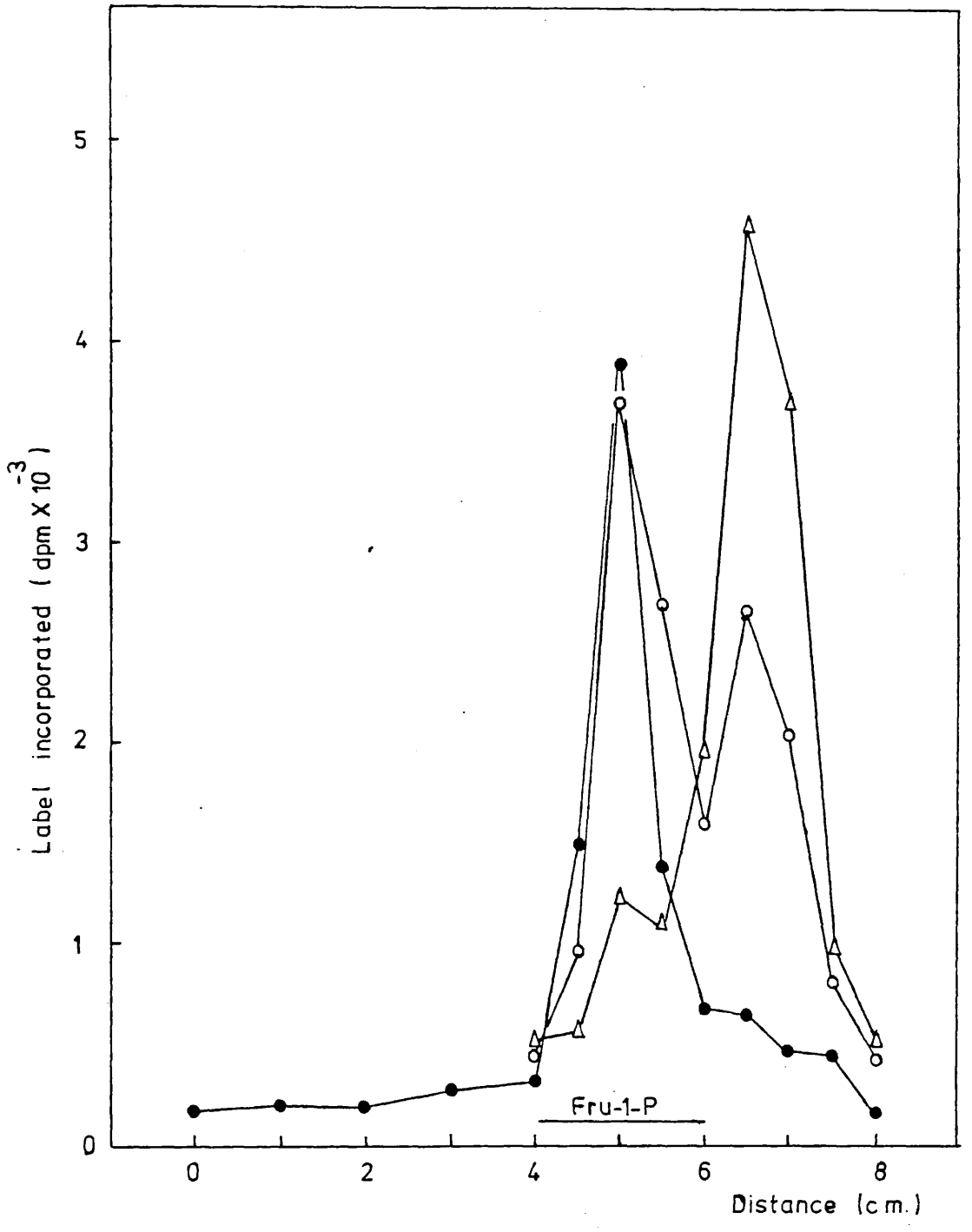


Fig. 17. Paper chromatographic separation of the radioactive produgts following incubation of hepatocytes with various fructose concentrations.

Hepatocytes were incubated for 20 min with 2mM-(Δ—Δ), 4mM-(o—o) or 10mM-(●—●)[U-^{14}C]fructose. Other experimental details are as described in Fig. 14 with the exception that ethanol was used for the extraction procedure and solvent A was used for the chromatographic separation. Fructose standard ran to 21.5 cm.

Table 6. The effect of fructose concentration on the concentration of Fru-1-P and unknown metabolite.

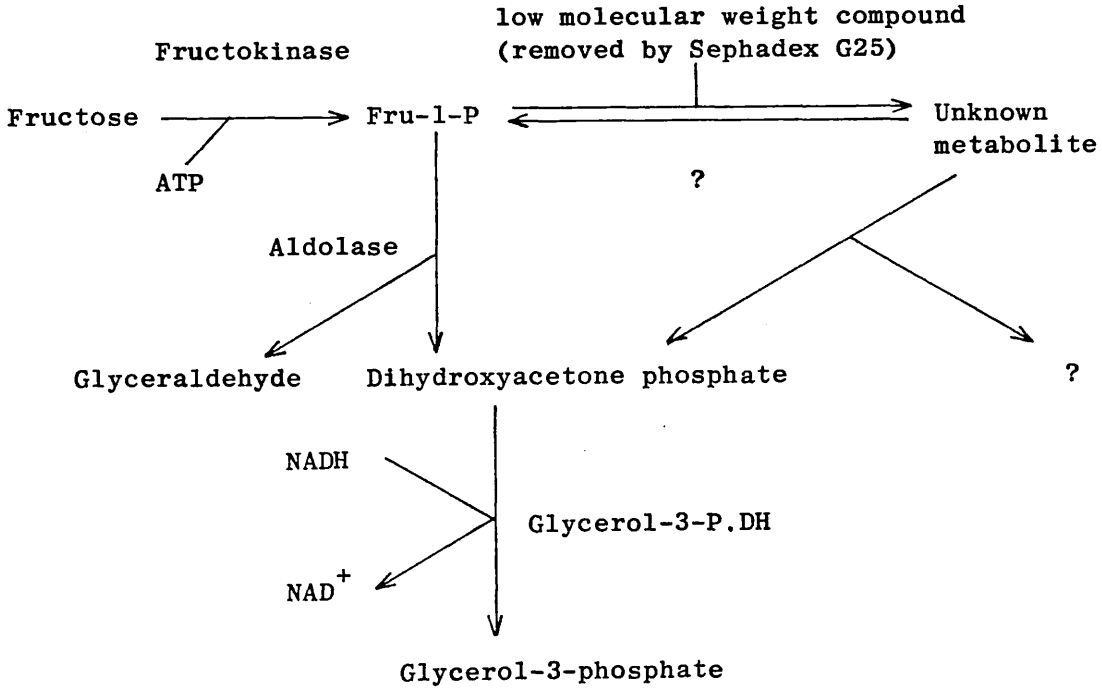
Experimental details are as in Fig. 14. Results are mean of 3 experiments ± s.e.m. Mean hepatocyte concentration 66 mg cells/ml.

Fructose	Time of incubation (min)			
	10		20	
(mM)	Fru-1-P (μmol/g)	Unknown (μmol/g)	Fru-1-P (μmol/g)	Unknown (μmol/g)
1	0.15 ± 0.06	0.50 ± 0.12	0.06 ± 0.02	0.34 ± 0.10
4	1.04 ± 0.95	0.95 ± 0.10	0.75 ± 0.11	0.73 ± 0.09
10	3.09 ± 0.65	1.24 ± 0.18	3.34 ± 0.21	1.07 ± 0.10

very accurate as it is hard to define the start and end of the reaction with low concentrations of Fru-1-P however, it is not so inaccurate as to explain the discrepancy revealed here. When Fru-1-P levels were estimated enzymatically in the preparations obtained here, concentrations close to the sum of the Fru-1-P and unknown metabolite levels were obtained suggesting that the unknown metabolite have been cleaved by liver aldolase to form dihydroxyacetone phosphate which was then reduced with NADH to glycerol-3-phosphate. Since only a crude preparation of liver aldolase is used in the assay of Fru-1-P this experiment does not distinguish between the unknown being broken down directly by aldolase or its conversion to Fru-1-P which is then aldolytically cleaved. The concentration of Fru-1-P, thus estimated, was of the same order as that reported by Mapungwana (1982).

Since the unknown metabolite accumulates faster than Fru-1-P, the enzyme responsible for the synthesis of the unknown metabolite must have a higher affinity for its substrate than fructokinase has for fructose. Assuming that the unknown metabolite is formed from Fru-1-P it is possible to plot from Fig. 16 the rate of synthesis of unknown metabolite as a function of Fru-1-P concentration in the cell (data not shown). Since under these conditions Fru-1-P concentrations are changing this is not strictly accurate, however, it does show that the enzyme responsible for the synthesis of the unknown metabolite has a K_m of approximately 0.2mM-Fru-1-P and a V_{max} of 1.1µmol/min/g cells. Compared to this fructokinase has a reported K_m for fructose of between 0.46mM and 0.80mM, depending on the

assay KCl concentration, and enzyme activity is reported as 2.5-3.0 U/g (Sánchez et al., 1971a), although higher activities have been claimed by Van den Berghe et al. (1977a) to occur immediately following a fructose load. The K_m value for Fru-1-P for liver aldolase has been reported as 0.35mM in rat liver (Woods et al., 1970) although a higher value (0.8mM) has been reported from studies on the rabbit liver enzyme (Rutter, 1964). Fru-1-P would be expected to accumulate when the enzyme responsible for synthesis of the unknown metabolite is saturated and/or liver aldolase 1B is inhibited, possibly by the unknown metabolite. If liver aldolase is responsible for the breakdown of both Fru-1-P and the unknown metabolite it must have a higher affinity for the latter since the unknown metabolite is broken down faster than Fru-1-P (Fig. 14). The proposed pathway of unknown metabolite synthesis is summarised below.



B. Further characterization of the unknown metabolite

Incubation of isolated hepatocytes with low concentrations of fructose has been shown to result in the accumulation of a previously uncharacterised fructose metabolite, in addition to Fru-1-P. The unknown metabolite was shown to elute from a Dowex 1X4-400 (borate) column immediately after fructose at a TEAB concentration of 0.18M (Fig. 11) whereas, following paper chromatography the unknown metabolite clearly separated from fructose but ran close to Fru-1-P (Fig. 13). The properties of the unknown metabolite, prepared by either of these methods from perchloric acid extracts of hepatocytes incubated with 2mM-$[U\text{-}^{14}C]$ fructose, were studied further. Unless otherwise stated the yield of radioactive material obtained following the chromatographic procedures described in this section was > 95%.

1. Chromatographic properties of the unknown

Following paper chromatographic separation of hepatocyte extracts, the region of the chromatogram containing Fru-1-P and the unknown metabolite was extracted with water and the eluate applied to a Dowex 1X4-400 (borate) column. As shown in Fig. 18A, both metabolites eluted at the same TEAB concentrations as previously observed when the hepatocyte extract was applied directly to this column (Fig. 11). However, when the unknown metabolite fractions (fractions 38-48, Fig. 18A) were pooled and applied to a Dowex 1X8-400 (acetate) column two peaks of $[^{14}C]$ labelled material appeared, the first was eluted in the flow-through and the second was eluted with formic acid (Fig. 18B). The possibility that the first peak

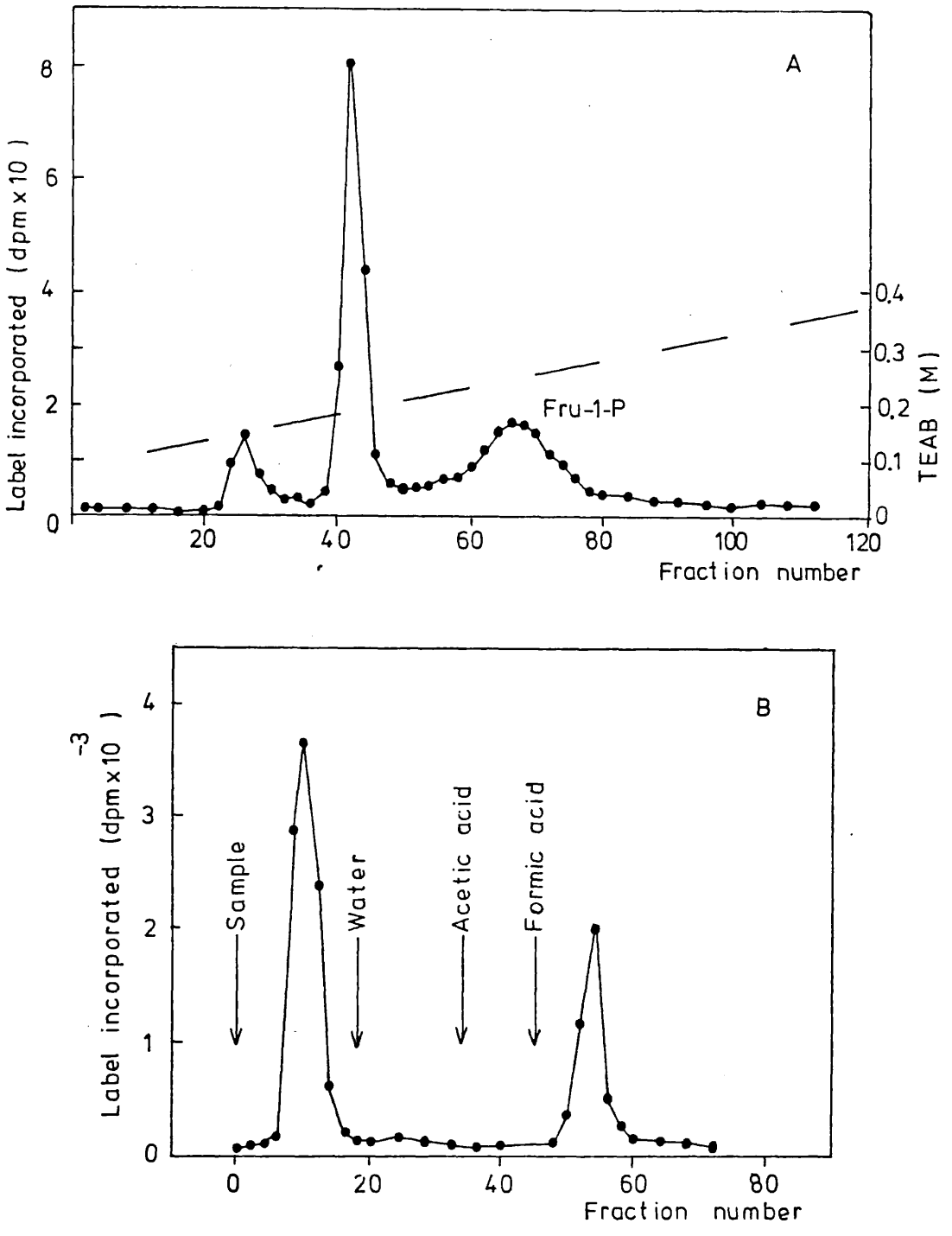


Fig. 18. Anion-exchange chromatography of the unknown metabolite previously separated by paper chromatography.

Hepatocytes (68mg/ml) were incubated for 10 min with 2mM- $[U-^{14}C]$ fructose as described in Section II.B.2.b.iii. Neutralised perchloric acid extracts were applied to Whatman No. 3 chromatography paper and run in solvent B (Section II.B.7.a). The area containing Fru-1-P and the unknown metabolite was eluted with water, applied to a Dowex 1X4-400 (borate) column and eluted with a linear gradient of 0.1-0.4M-TEAB (A). Fractions 38-48 were pooled, applied to a Dowex 1X8-400 (acetate) column and eluted successively with water, 2M-acetic acid and 4M-formic acid (B) as detailed in Section II.B.8.

was due to column overload was eliminated by reapplying this fraction to a second Dowex 1X8-400 (acetate) column. Again the radioactive material was eluted in the column flow-through but in this case no $[^{14}C]$ material was eluted by formic acid (data not shown). Rognstad (1982a) reported that when hepatocyte extracts were applied to a similar column, glucose, lactate and Glc-6-P were eluted with water, acetic acid and formic acid respectively. In the present study, it was confirmed that fructose and Fru-1-P were eluted in the flow-through and formic acid fractions respectively in this chromatographic system (results not shown). Thus, the fraction eluting with formic acid would be expected to contain phosphate esters, whereas that eluting immediately from the column is probably uncharged.

In order to establish that the unknown metabolite was breaking down, the sample eluted from the paper chromatogram was rechromatographed by paper chromatography (Fig. 19A). Rechromatography resulted in the appearance of a labelled product running near fructose standard in addition to a peak of radioactivity running near Fru-1-P which was assumed to be the unknown metabolite. The radioactive metabolite which co-chromatographed with fructose accounted for 52% of the radioactivity applied with the other peak accounting for 38%. The sample eluted from a paper chromatogram was also subjected to anion-exchange chromatography on a Dowex 1X8-400 (acetate) column (Fig. 20). In this case, three peaks of radioactive material were eluted with water (59%), acetic acid (16%) and formic acid (25%). When the region of the paper chromatogram between 4 and 9 cm (Fig. 19A) was eluted and the eluate applied to a Dowex 1X8-400 (acetate) column only two labelled products

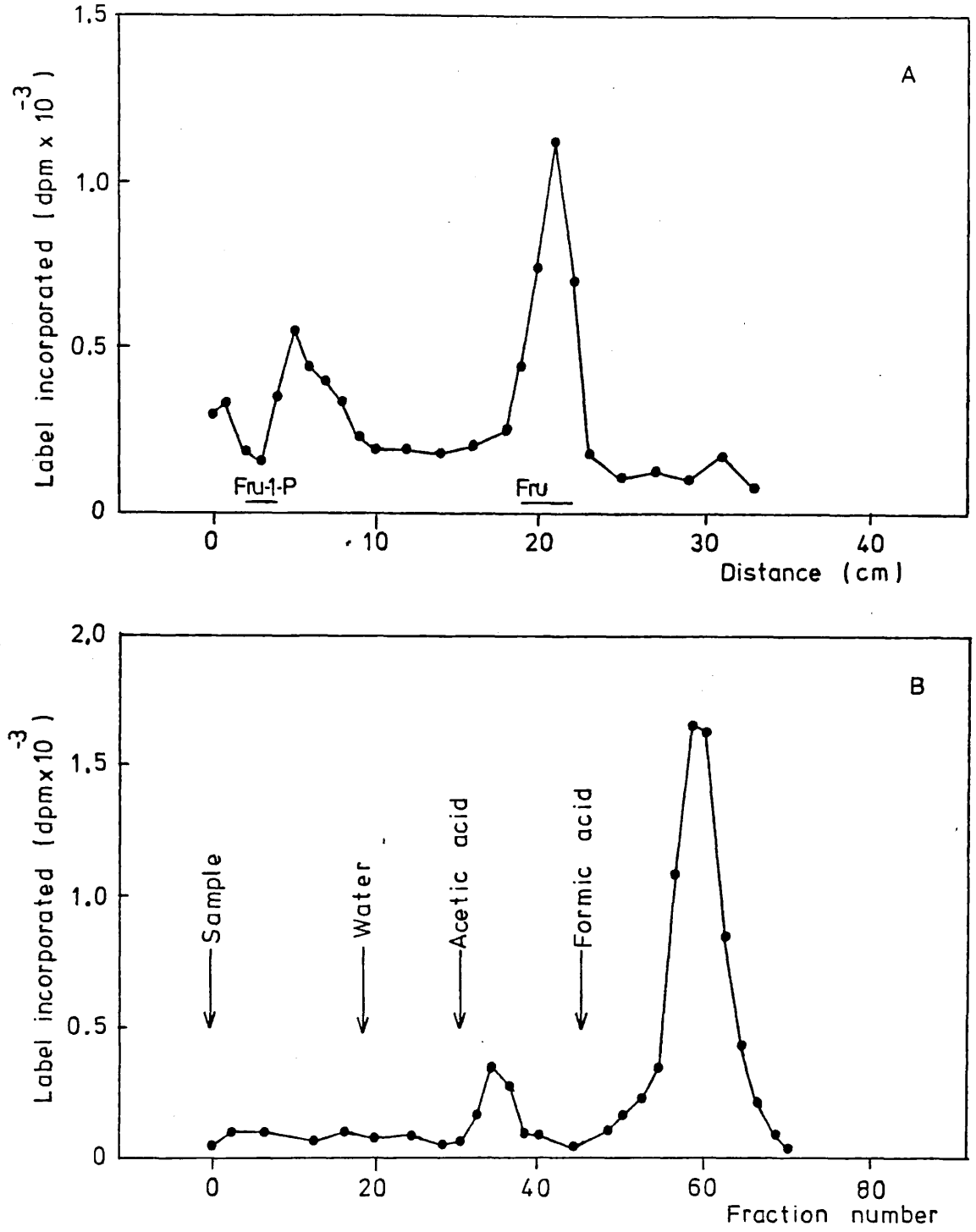


Fig. 19. Paper chromatography (A) followed by Dowex 1X8-400 (acetate) chromatography (B) of the unknown metabolite initially isolated by paper chromatography.

For experimental details see Fig. 18. Following paper chromatography of the hepatocyte extract in solvent B the region containing the unknown metabolite was eluted with water. The eluant was reapplied to Whatman No. 3 chromatography paper and rechromatographed in solvent B (A). The region corresponding to 4-9 cm was eluted with water and the eluant applied to a Dowex 1X8-400 (acetate) column and eluted successively with water, 2M-acetic acid and 4M-formic acid (B) as detailed in Section II.B.8.b.

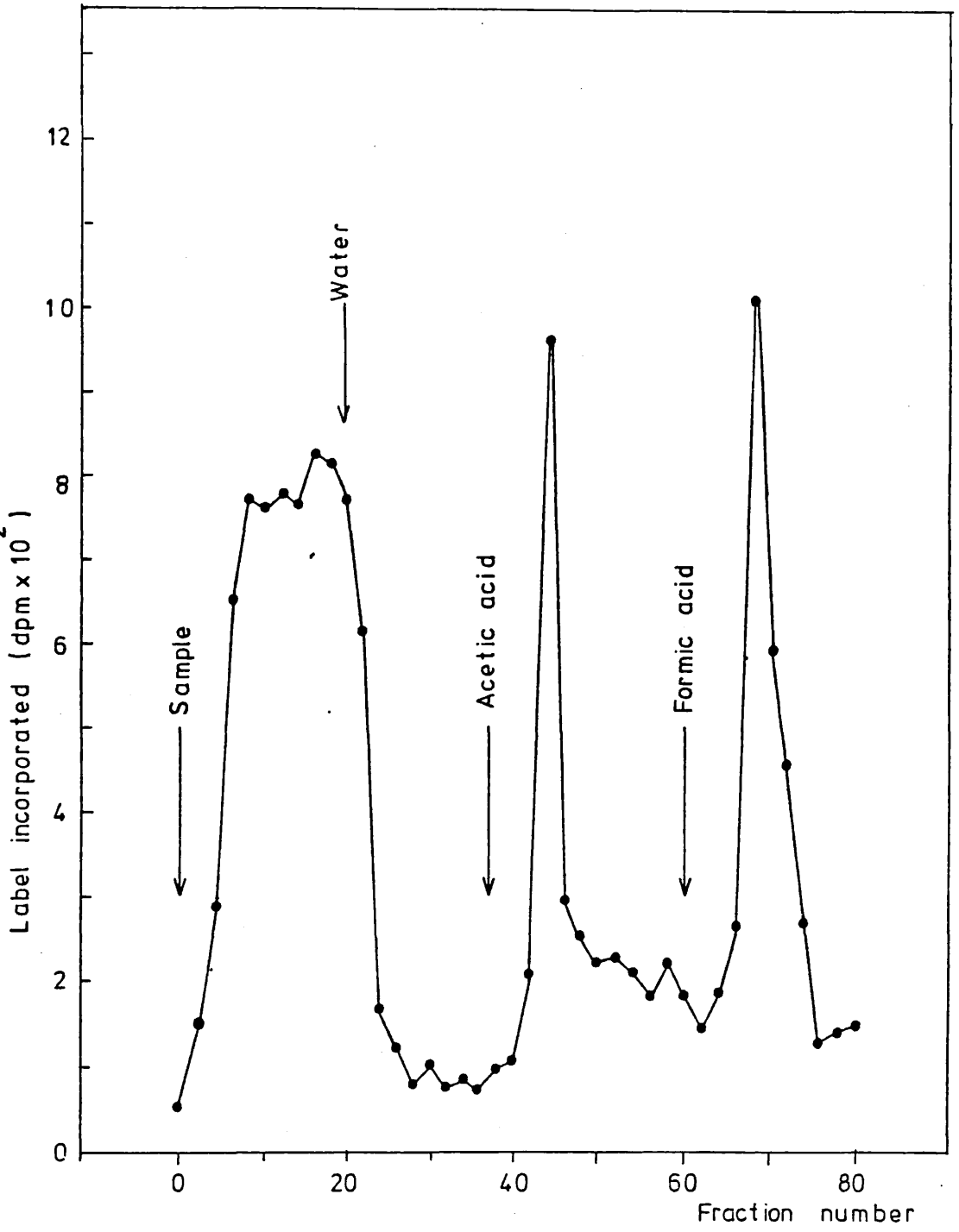


Fig. 20. Dowex 1X8-400 (acetate) chromatography of the unknown metabolite previously isolated by paper chromatography.

See Figs. 18 and 19 for full experimental details.

were obtained, which eluted with acetic acid and formic acid respectively (Fig. 19B). The lack of a peak eluting in the flow-through can be explained by its removal by the paper chromatographic procedure (Fig. 19A). The results from these experiments indicated that the unknown metabolite was broken down following the paper chromatographic separation of the hepatocyte extracts. It can also be concluded that the breakdown products and the unknown metabolite co-chromatograph on a Dowex 1X4-400 (borate) column, indicating that the latter separation procedure is an unsatisfactory method for differentiating between the unknown and its breakdown products.

When hepatocyte extracts were applied to a Dowex 1X8-400 (acetate) column, without prior paper chromatography, the radioactive products were found to be eluted in the flow-through, acetic acid and formic acid fractions (Fig. 21). If the formic acid fractions were pooled and applied to either a Dowex 1X4-400 (borate), Fig. 22, or Bio-Gel P-2, Fig. 23, column, labelled products corresponding to the unknown metabolite and Fru-1-P were obtained. The identity of Fru-1-P was confirmed by co-chromatography of the radioactive product with standard Fru-1-P on both columns (results not shown). The molecular weights of the unknown metabolite and of Fru-1-P, estimated from gel-filtration on Bio-Gel P-2, were 583 ± 20 ($n = 4$) and 270 ± 15 ($n = 3$) respectively. If the unknown metabolite region from the Dowex 1X4-400 (borate) column (Fig. 22) was pooled and reapplied to a Dowex 1X8-400 (acetate) column all the radioactivity was eluted with formic acid (Fig. 24). This indicated that anion-exchange chromatography, on either Dowex 1X4-400 (borate) or Dowex 1X8-400 (acetate)

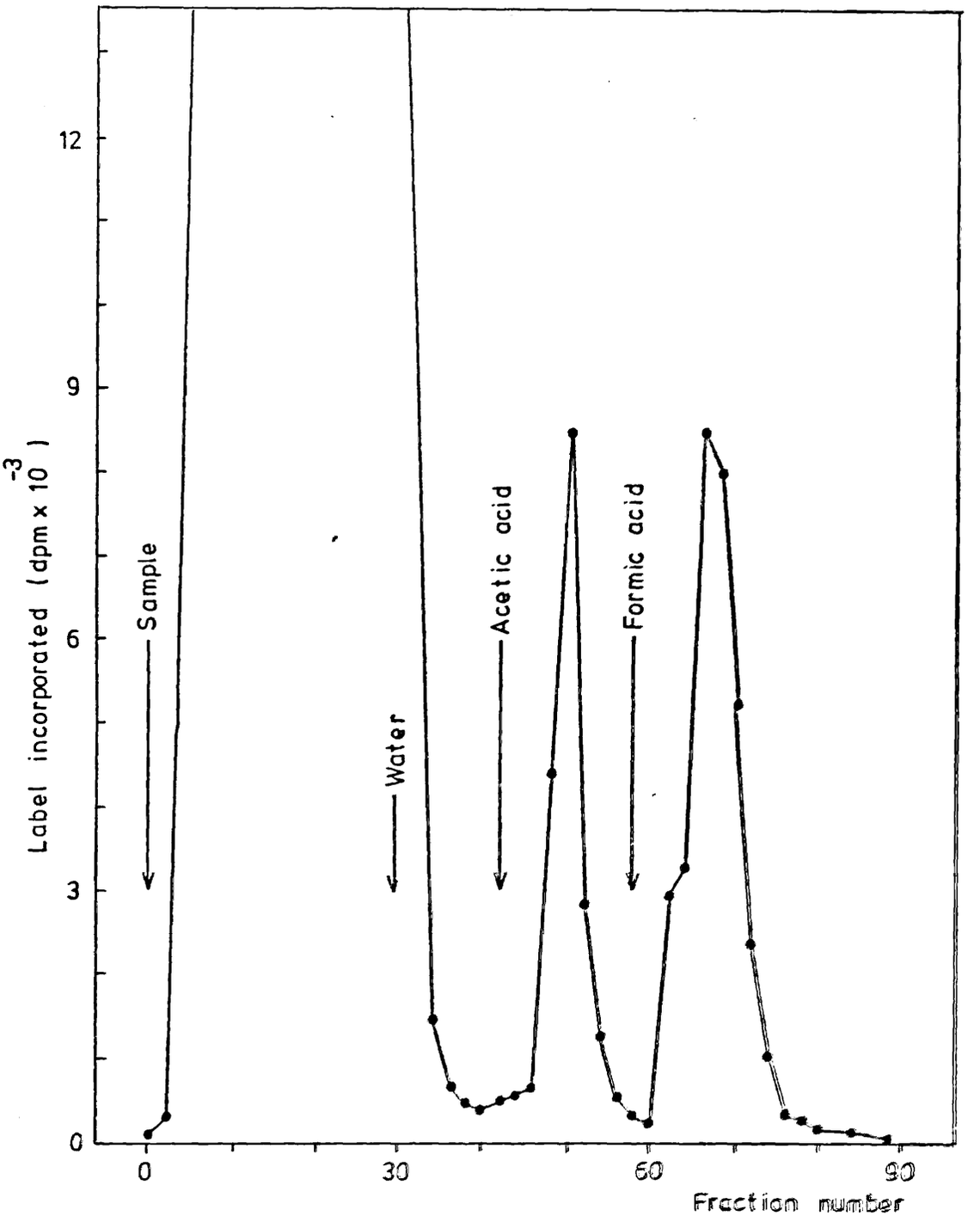


Fig. 21. Dowex 1X8-400 (acetate) chromatography of hepatocyte extracts.

Hepatocytes (64mg/ml) were incubated for 10 min with 2mM - [U-^{14}C] fructose (Section II.B.2.b.iii). Neutralised perchloric acid extracts were treated with activated charcoal (Section II.B.4.a), applied to a Dowex 1X8-400 (acetate) column and eluted successively with water, 2M-acetic acid and 4M-formic acid (Section II.b.8.b).

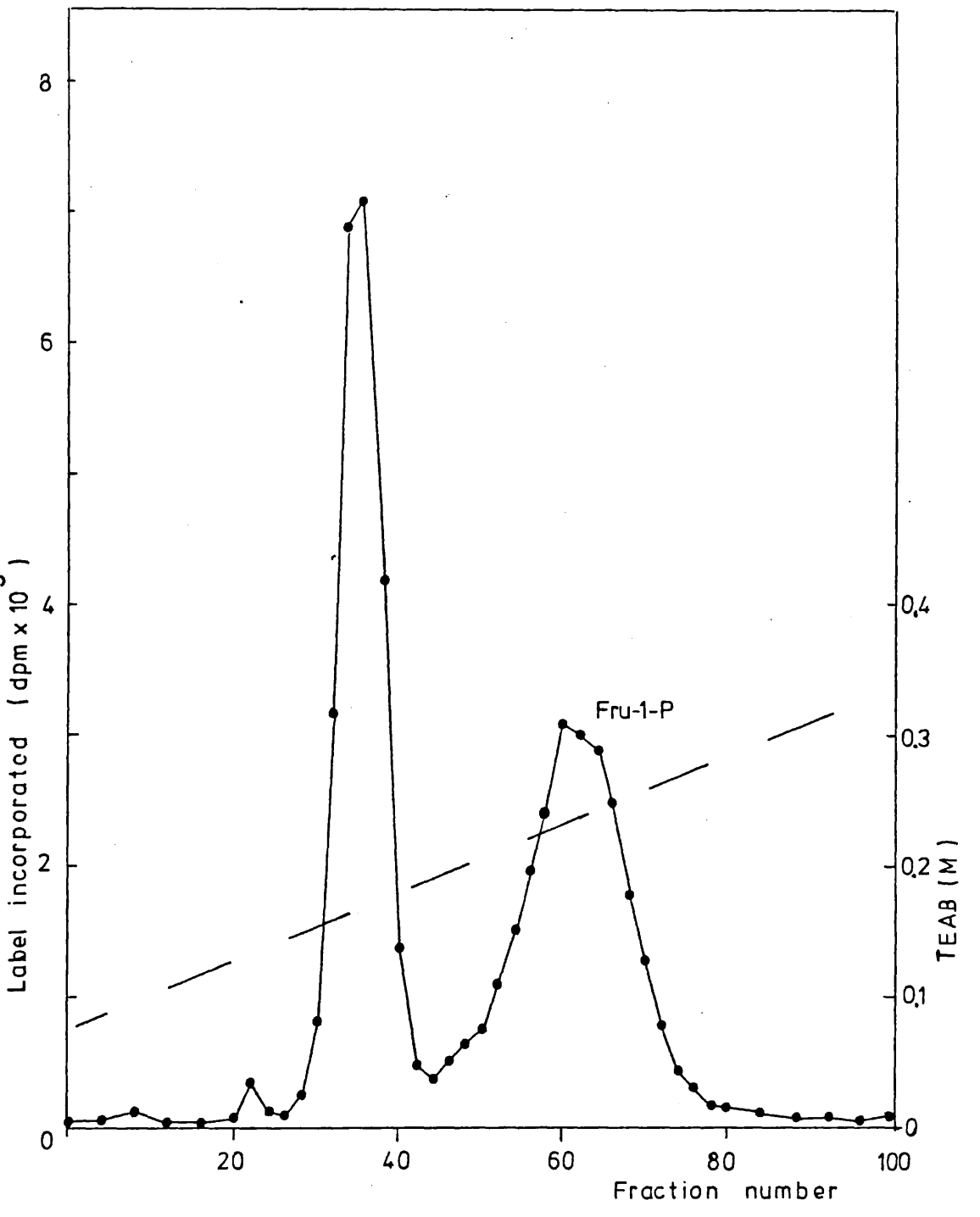


Fig. 22. Dowex 1X4-400 (borate) chromatography of formic acid fraction following Dowex 1X8-400 (acetate) chromatography.

See Fig. 21 for full experimental details. The formic acid eluted fractions were pooled, concentrated by rotary-evaporation to remove the acid, applied to a Dowex 1X4-400 (borate) column and eluted with a linear gradient of 0.1-0.4M-TEAB (Section II.B.8.a).

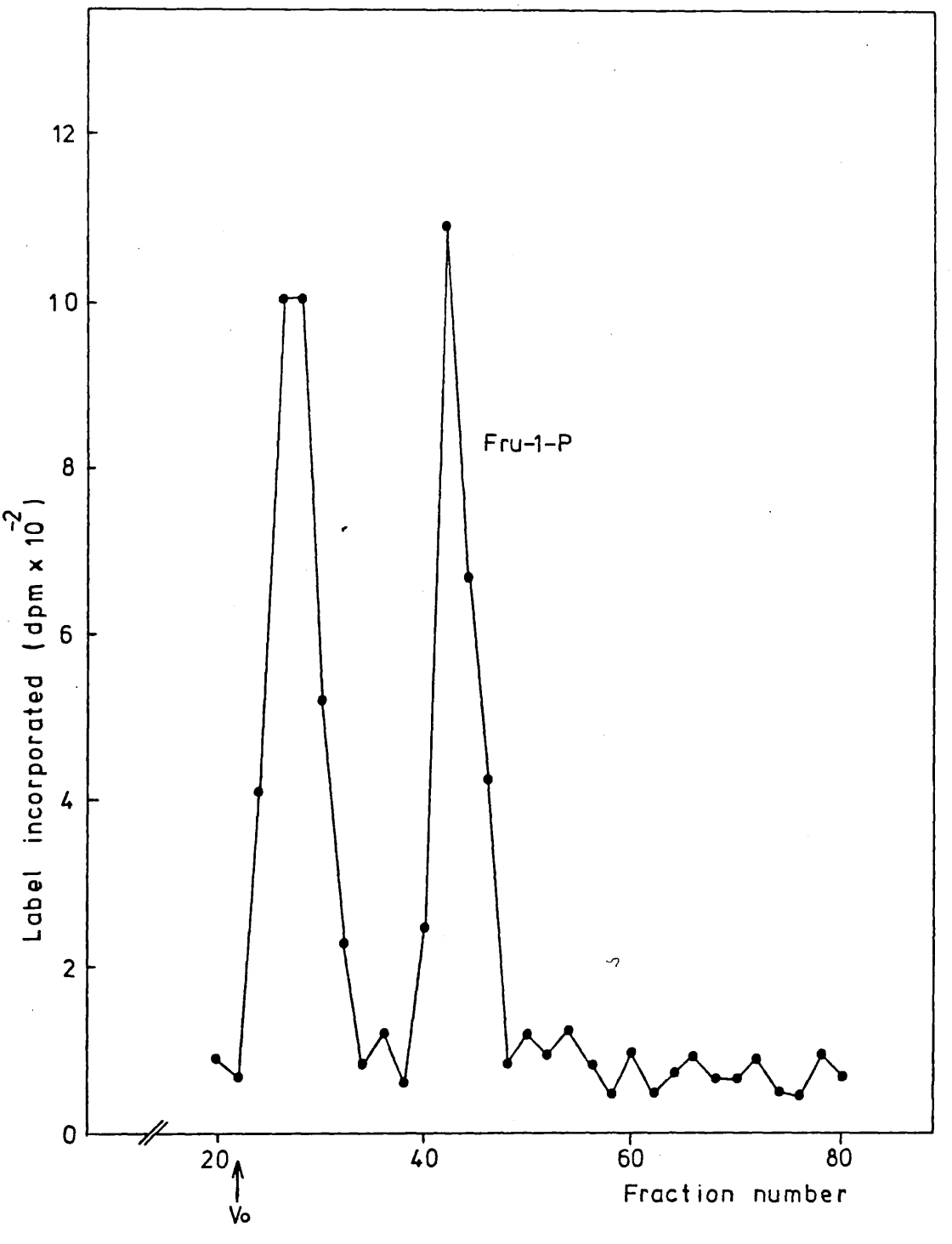


Fig. 23. Gel-filtration of formic acid fraction following Dowex 1X8-400 (acetate) chromatography.

See Fig. 21 for full experimental details. The formic acid eluted fractions were pooled, rotary-evaporated to remove the acid, redissolved in water (1 ml), applied to a Bio-Gel P-2 column and eluted with water (Section II.B.9).

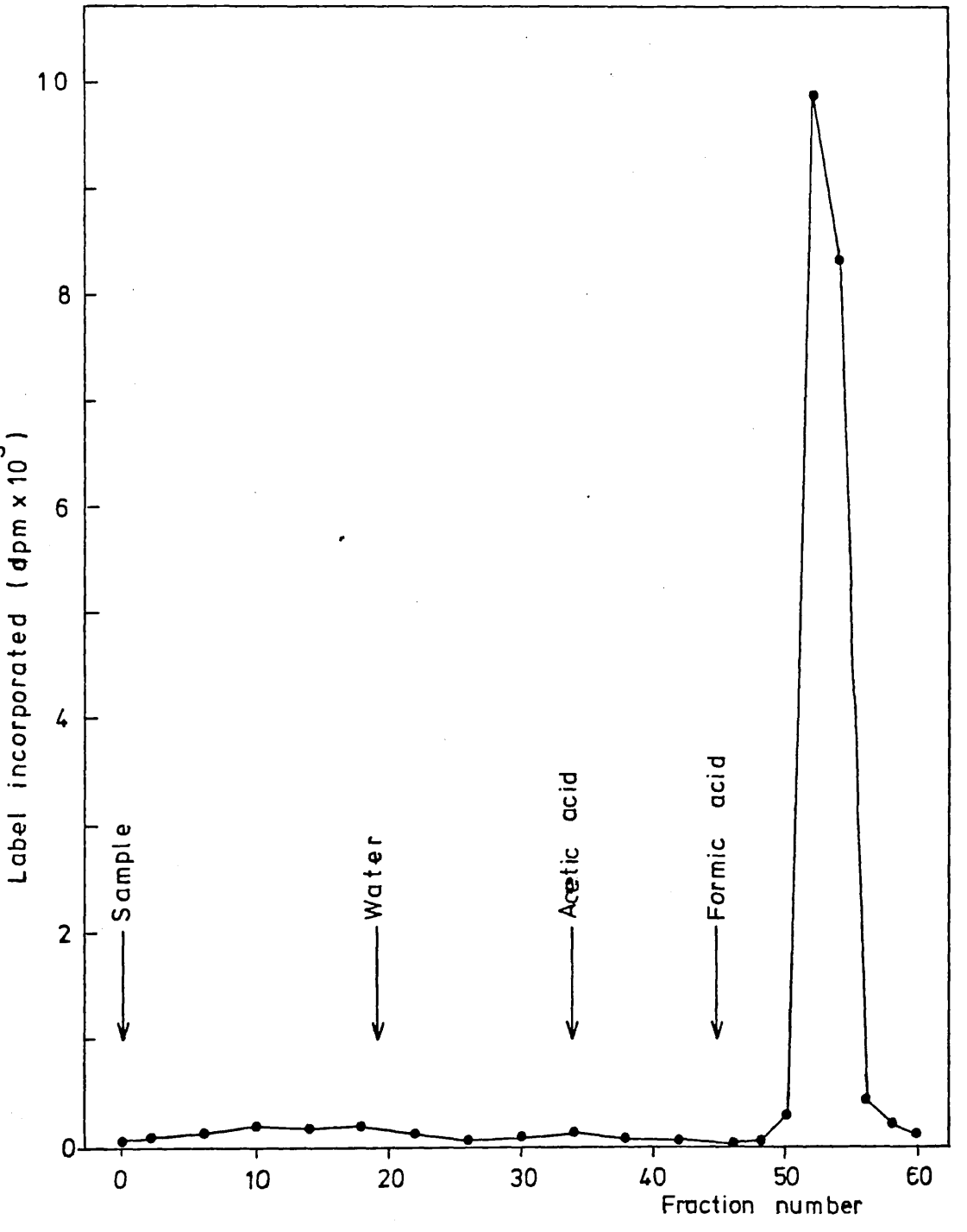


Fig. 24. Dowex 1X8-400 (acetate) chromatography of unknown metabolite.

The unknown metabolite was prepared from isolated hepatocytes as detailed in Figs. 21 and 22 by Dowex 1X8-400 (acetate) and Dowex 1X4-400 (borate) anion-exchange chromatography. The fractions containing the unknown metabolite were pooled following Dowex 1X4-400 (borate) chromatography, successively washed with methanol and rotary-evaporated to remove TEAB, and then reapplied to a Dowex 1X8-400 (acetate) column (Section II.B.8).

columns, does not result in breakdown of the unknown and suggested that the unknown metabolite may be a phosphate ester, a proposal later confirmed by colorimetric analysis (Section III.B.5).

2. Charcoal treatment and barium acetate precipitation

The possibility that the unknown metabolite contained a nucleotide moiety was eliminated by treatment of the hepatocyte extract with activated charcoal (Van Schaftingen et al., 1980b). No absorbtion of $[^{14}C]$ material onto the charcoal was found and the chromatographic properties of the unknown metabolite were unchanged by the treatment (Fig. 21-24).

A classical method for the purification of phosphate esters is their precipitation as the barium salt (Cardini and Leloir, 1957) and thus this technique was used in an attempt to further purify the unknown metabolite. The unknown metabolite, separated by paper chromatography, appeared to be precipitated with Fru-1-P as a water-soluble, ethanol-insoluble barium salt, as judged by Dowex 1X4-400 (borate) or Dowex 1X8-400 (acetate) chromatography following the solubilisation of the precipitate, by either Amberlite IRC-50(H^+) resin (Fig. 25) or 0.5M-H_2SO_4 (results not shown). This was further evidence for the presence of a phosphate group in the unknown metabolite since, in broad terms, P_i and sugar diphosphates form water-insoluble barium salts and sugar monophosphates form water-soluble, ethanol-insoluble salts. Neutral sugars are not precipitated by this procedure (Cardini and Leloir, 1957). Since a substantial proportion of the counts associated with Fru-1-P and the unknown metabolite were not precipitated by barium treatment, the supernatant was applied to a Dowex

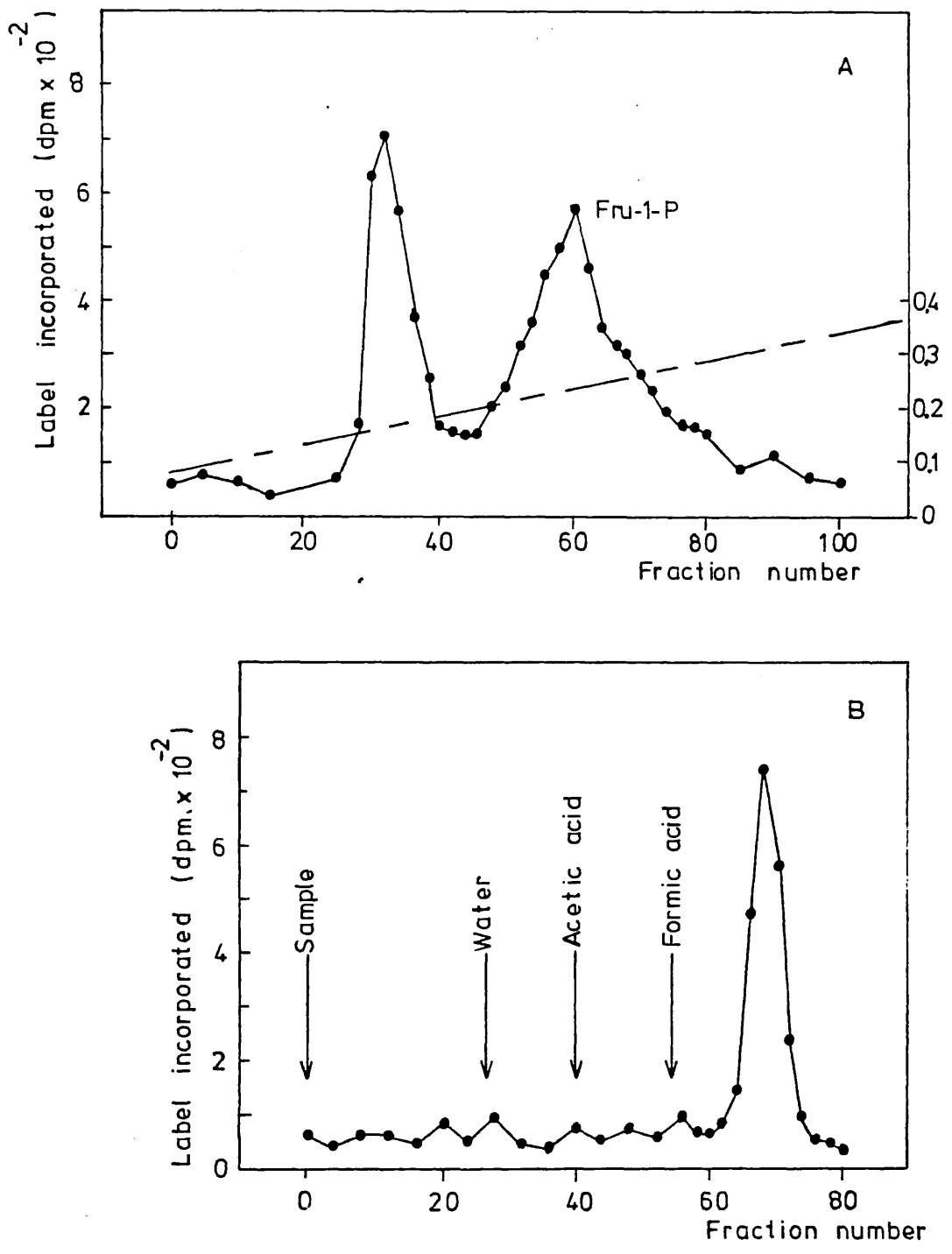


Fig. 25. Anion-exchange chromatography of water-soluble, ethanol-insoluble barium salts of Fru-1-P and the unknown metabolite.

Following incubation of isolated hepatocytes (69mg/ml) with 2mM- [U-^{14}C] fructose for 10 min neutralised perchloric acid extracts (Section II.B. 2.b.iii) were applied to Whatman No. 3. chromatography paper and separated in solvent A (Section II.B.7.a). The region containing Fru-1-P and the unknown metabolite was eluted with water and the eluate treated with barium acetate (Section II.B.4.b). The water-soluble, ethanol-insoluble barium salts were solubilised with Amberlite IRC-50 (H^+) resin and were than applied to A, Dowex 1X4-400 (borate) and B, Dowex 1X8-400 (acetate) columns (Section II.B.8).

1X4-400 (borate) column. A [^{14}C] labelled compound was eluted in the same position as the unknown metabolite (Fig. 26A) indicating that the unknown may not have been fully precipitated. However, Dowex 1X8-400 (acetate) chromatography revealed that the unknown had been broken down during the barium acetate fractionation procedure since no radioactivity was detected in the formic acid eluted fraction but was instead found in the flow-through and acetic acid fractions (Fig. 26B). In several experiments of this type the proportion of [^{14}C] material eluted in the flow-through and in the acetic acid fraction varied as shown by the two profiles drawn in Fig. 26B. The experiments were essentially carried out in the same way and the reason for the differences is not known.

The breakdown of the unknown metabolite was further confirmed when the barium precipitated and supernatant fractions were applied separately to a Bio-Gel P-2 column (Fig. 27). The molecular weight of the precipitated fraction was estimated to be 170 and, when passed down a Dowex 1X8-400 (acetate) column, it eluted with formic acid (results not shown). The radioactive material in the supernatant fraction eluted at a volume corresponding to a molecular weight of between 180 and 300; the shape of the peak suggested possible heterogeneity. This was confirmed when this material was shown to elute as two peaks, in the flow-through and with acetic acid, respectively from a Dowex 1X8-400 (acetate) column (results not shown).

Partial breakdown of the unknown was demonstrated following the removal of P_i and the sugar diphosphates from hepatocyte extracts as the water-insoluble barium salts. Glycogen was removed by adding one volume of 95% (v/v) ethanol to the super-

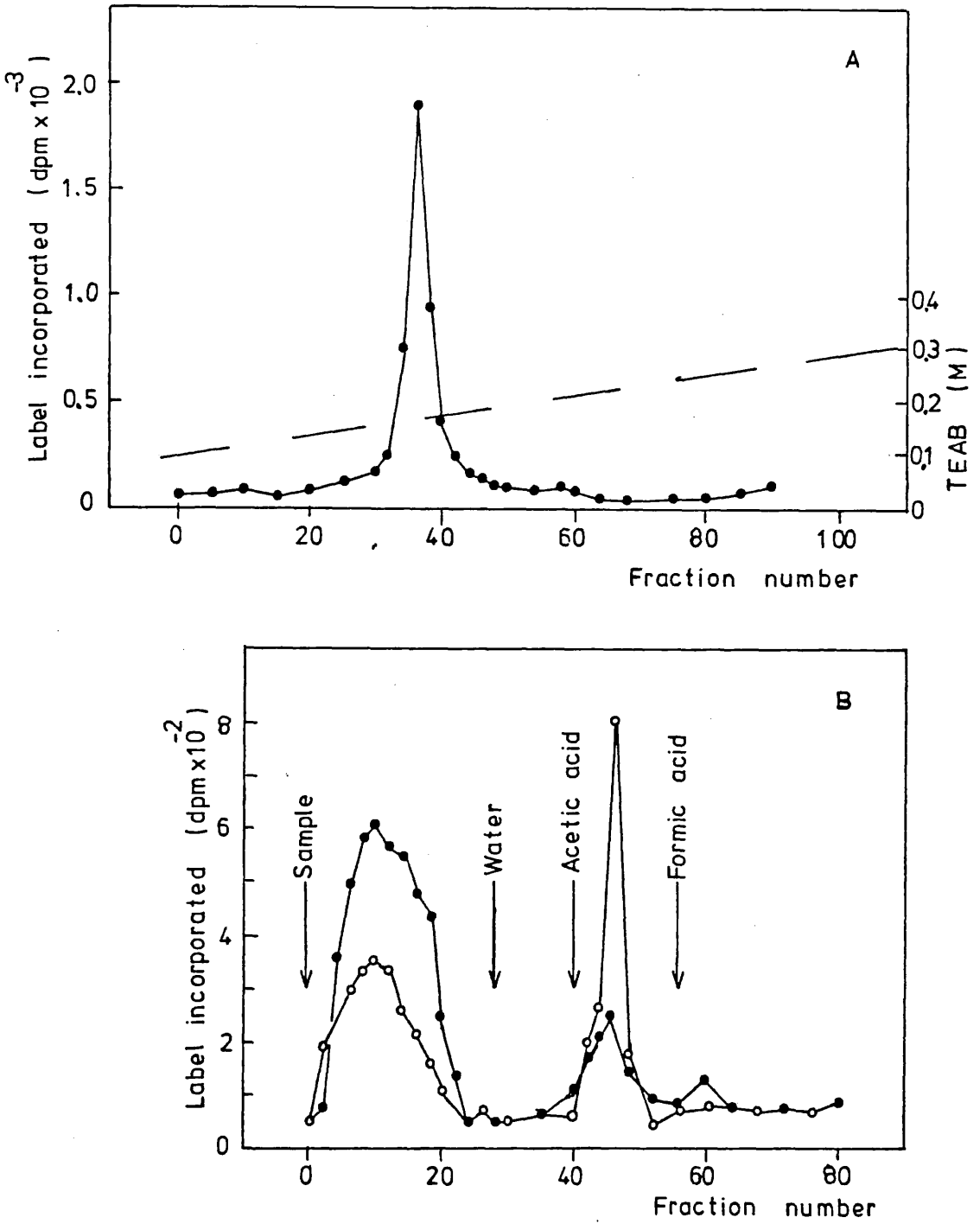


Fig. 26. Anion-exchange chromatography of the soluble radioactive material following barium acetate and ethanol treatment of Fru-1-P and the unknown metabolite.

For full experimental details see Fig. 25.

The supernatant remaining after the precipitation of both water-insoluble and ethanol-insoluble barium salts was rotary evaporated to dryness, dissolved in water and applied to either A, Dowex 1X4-400 (borate) or B, Dowex 1X8-400 (acetate) columns (Section II.B.8).

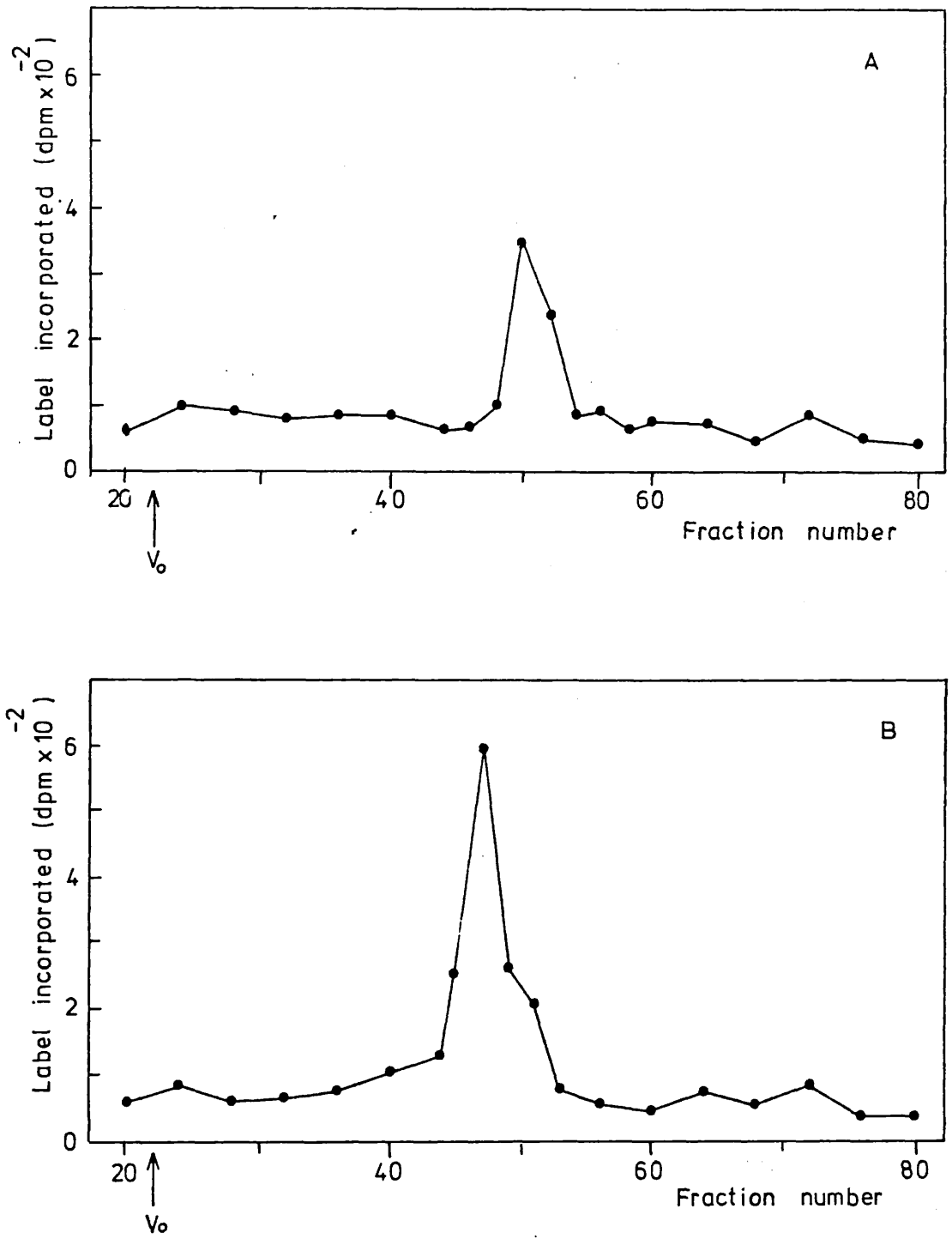


Fig. 27. Bio-Gel P-2 gel-filtration of the products of barium acetate treatment of the unknown metabolite.

The unknown metabolite was purified from hepatocyte extracts by elution from a Dowex 1X8-400 (acetate) column followed by gel-filtration on a Bio-Gel P-2 column as detailed in Figs. 21 and 23. Following barium acetate fractionation (Section II.B.4.b) the water-soluble, ethanol-insoluble barium salts (A) and the water- and ethanol- soluble products (B) were applied to Bio-Gel P-2 columns and eluted with water.

natant which was then applied directly to a Dowex 1X8-400 (acetate) column without adding a further 3 vol. of ethanol (Fig. 28). The pooled peak of radioactivity eluting with formic acid was concentrated and applied to a Bio-Gel P-2 column where it was shown to contain three peaks of radioactivity, corresponding to molecular weights of 600 (unknown metabolite), 260 (Fru-1-P) and 170 (a breakdown product of the unknown metabolite as previously found in Fig. 27A). These results suggest that breakdown of the unknown metabolite also occurs following treatment with barium acetate. In support of this proposal several phosphate esters (e.g. Fru-2-P) have been reported to be susceptible to barium hydrolysis (Pontis and Fischer, 1963).

The elution profiles of the unknown metabolite and its breakdown products, after barium treatment or paper chromatography, from the three column chromatographic procedures used in this study are summarised in Table 7. These results suggest that the unknown metabolite breaks down on either paper chromatography or barium treatment to give three labelled products; a neutral unphosphorylated compound (which elutes in the flow-through from a Dowex 1X8-400 (acetate) column), a charged molecule (probably a monocarboxylic acid, which is eluted by acetic acid) and a phosphorylated ester (possibly a triose phosphate, of molecular weight 170 which is eluted by formic acid).

3. Purification of unknown metabolite

In view of the breakdown of the unknown metabolite following paper chromatography or barium acetate fractionation

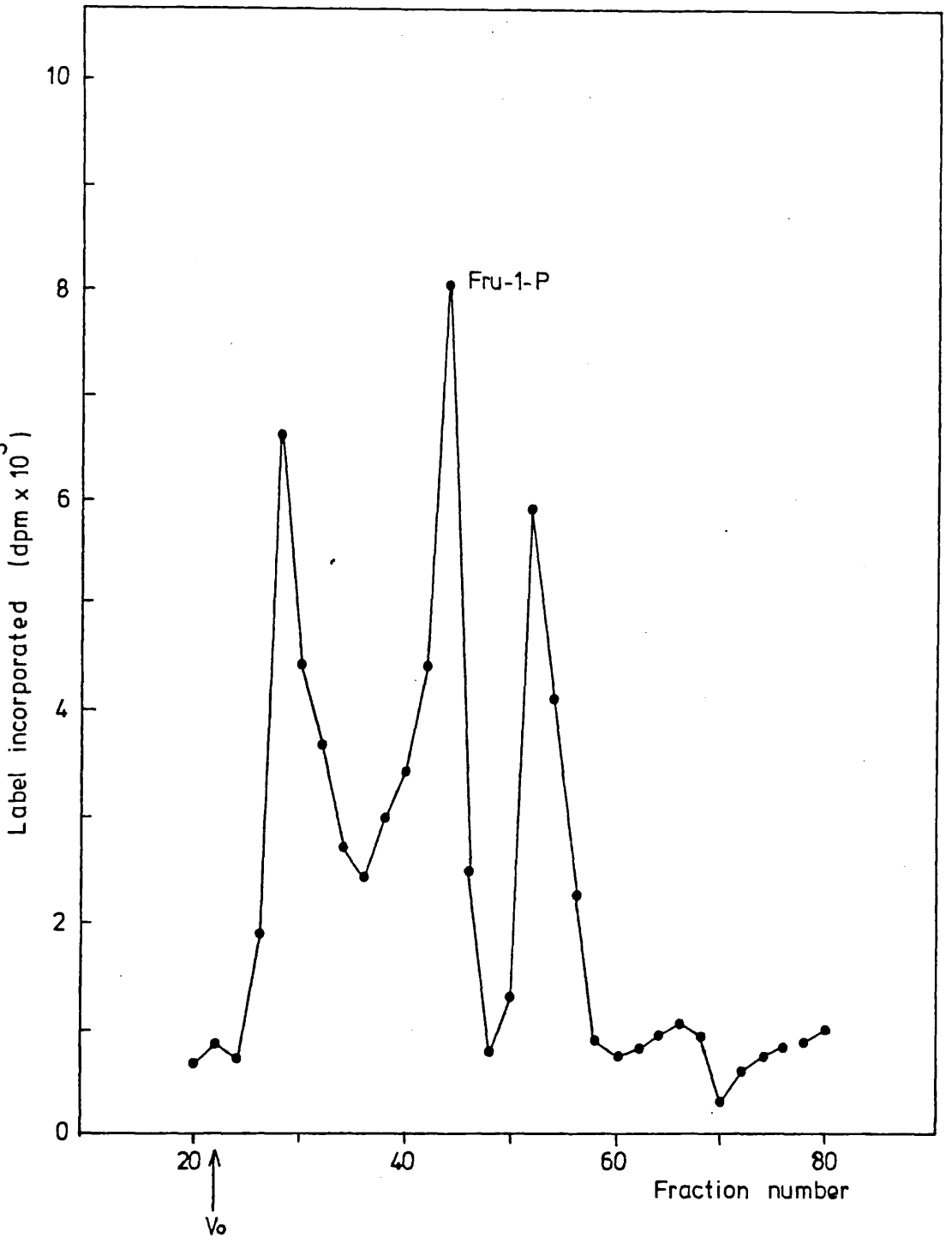


Fig. 28. Bio-Gel P-2 gel-filtration of water-soluble barium salts of fructose metabolites in a perchloric acid extract of hepatocytes.

Following incubation of isolated hepatocytes (68mg/ml) with 2mM-[U-^{14}C] fructose for 10 min, neutralised perchloric acid extracts were treated with barium acetate (Section II.B.4.b). The water-insoluble barium salts were precipitated, 1 vol. 95% (v/v) ethanol was added to precipitate glycogen and the remaining supernatant was applied to a Dowex 1X8-400 (acetate) column. The fractions eluted by formic acid were pooled and applied to a Bio-Gel P-2 column (Section II.B.8.b and 9).

Table 7. Summary of the elution characteristics of the unknown metabolite.

Treatment	Chromatography		
	Dowex 1X4 (borate) eluted with TEAB(M)	Dowex 1X8 (acetate) eluted with	Bio-Gel P-2 molecular weight
Untreated hepatocyte extract	0.18	Formic acid	583
Barium acetate precipitated fraction	0.18	Formic acid	170
Barium acetate supernatant fraction	0.18	Flow-through Acetic acid	300-180
Paper chromatography Solvent B	0.18	Flow-through Acetic acid Formic acid	Not determined

and the inability to detect if breakdown was occurring by Dowex 1X4-400 (borate) chromatography, these three purification procedures were omitted in the routine purification of the unknown metabolite. Hepatocytes were incubated with 2mM - [U-^{14}C] fructose for 10 min and reactions stopped with 4.2M-perchloric acid (Section II.B.2.b.iii). Following neutralisation with potassium carbonate, extracts were treated with activated charcoal (5% w/v extract) to remove nucleotides. The charcoal was sedimented by centrifugation (15 min at maximum speed in a bench centrifuge) and the supernatant removed and diluted to 30 ml with water. The diluted sample was applied to a Dowex 1X8-400 (acetate) column and eluted successively with water, 2M-acetic acid and 4M-formic acid (Section II.B.8.b.). The formic acid fractions were pooled and the formic acid removed by rotary-evaporation to dryness under vacuum at 50°C. The samples were washed several times with water and rotary-evaporated to dryness each time before being resuspended in a minimum volume (1 ml) of distilled, deionised water and applied to a Bio-Gel P-2 column (Section II.B.9). The fractions containing the first peak of [^{14}C] material (the unknown metabolite) eluted from the column were pooled and the volume reduced to between 1-2 ml by rotary-evaporation. The unknown obtained by this procedure was stable for several weeks, if stored at -20°C, as judged by its chromatographic properties on Bio-Gel P-2 and Dowex 1X8-400 (acetate) columns.

4. Acid hydrolysis, alkaline phosphatase and liver aldolase treatment of the unknown metabolite.

The behaviour of the unknown metabolite, purified as

described in Section III.B.3, was further studied. A sample was treated with 2M-TFA for 30 min at 100°C, the hydrolysate was rotary-evaporated to dryness (to remove TFA), resuspended in 1 ml of water and applied to a Bio-Gel P-2 column. The elution profile of the radioactive products is shown in Fig. 29A. A major, possibly heterogenous, peak of radioactivity was eluted between fractions 48 and 60 corresponding to a molecular weight range between 120 and 200. When these fractions were pooled and applied to a Dowex 1X8-400 (acetate) column the majority of the radioactivity was found in the flow-through and formic acid fractions in a 2:1 ratio (Fig. 29B). One possibility is that the formic acid eluting fraction may be an acid-stable triose phosphate. The $[^{14}C]$ material eluting at fraction 65, from the Bio-Gel P-2 column, is in a 1:3 ratio with the major peak and since it corresponds to a molecular weight of 90 may signify the presence of a triose. Fig. 30 shows that the material eluting in the flow-through from the Dowex 1X8-400 (acetate) column runs as two peaks on paper chromatography in solvent E and as a single peak of low mobility following t.l.c. in solvent G. In neither solvent system did the radioactive products correspond to either fructose or glucose.

When the unknown metabolite was incubated with alkaline phosphatase (25 units) and 0.01M-Tris/HCl, pH 8.0, for 30 min at 37°C and the products separated on a Bio-Gel P-2 column, a $[^{14}C]$ labelled compound was observed to elute at a volume corresponding to a molecular weight of 470-500 in addition to a major peak at 180 (Fig. 31A). The latter was shown to elute in the flow-through from a Dowex 1X8-400 (acetate) column (Fig. 31B) but the fraction was not homogenous as

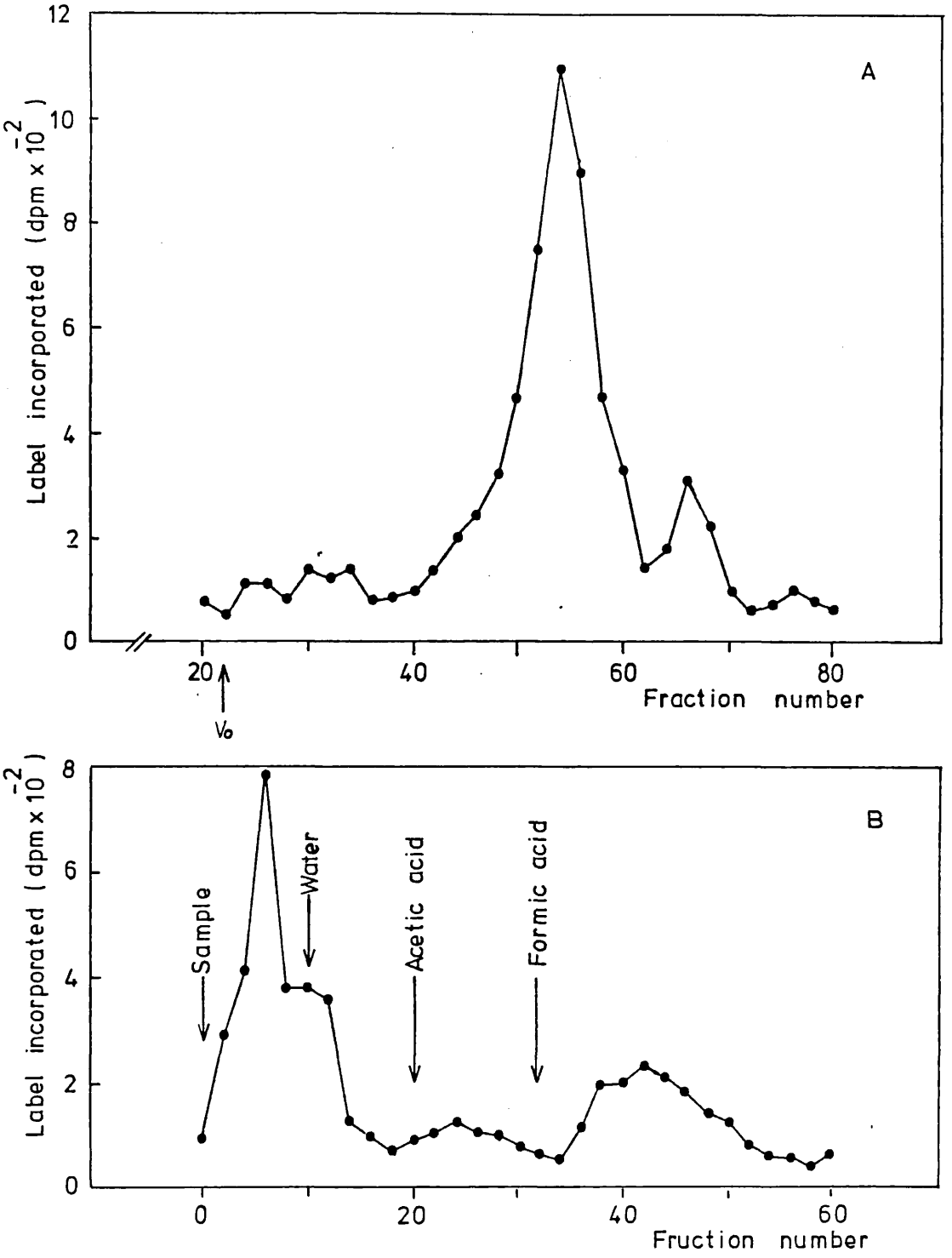


Fig. 29. A, Bio-Gel P-2 chromatography followed by B, Dowex 1X8-400 (acetate) chromatography of the $[^{14}C]$ products of acid hydrolysis of the purified unknown metabolite.

The unknown metabolite was purified as described in Section III.B.3. For experimental details of acid hydrolysis see text. See Figs. 21 and 23 for details of column chromatography.

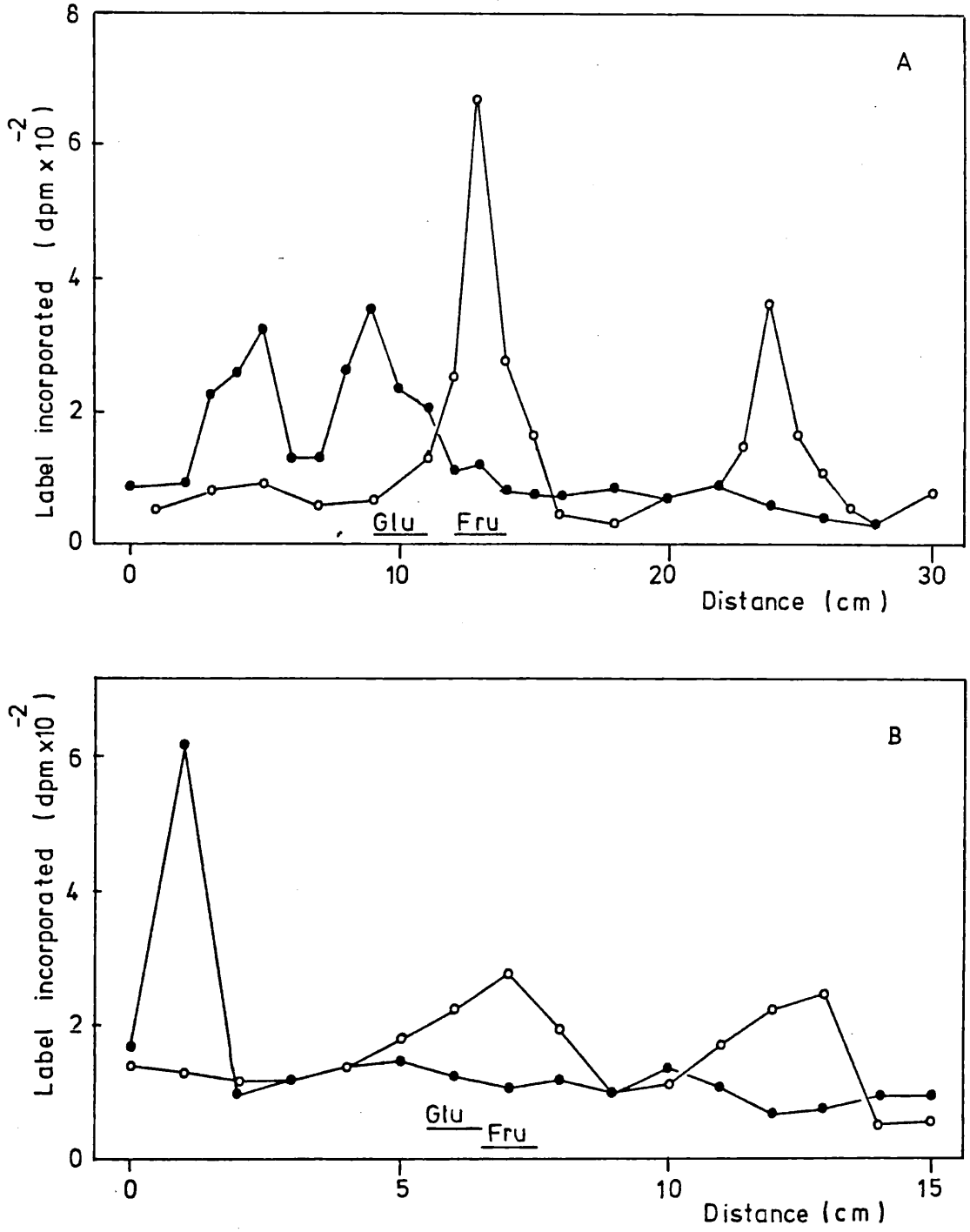


Fig. 30. Paper chromatography (A) or t.l.c. (B) of $[^{14}C]$ products of acid hydrolysis or alkaline phosphatase treatment of the purified unknown metabolite.

The $[^{14}C]$ products of acid hydrolysis (●-●) or alkaline phosphatase (o-o) treatment were separated on Bio-Gel P-2 and Dowex 1X8-400 (acetate) columns (Figs. 29 and 31). The fractions from the major peaks were pooled, concentrated and chromatographed in solvents E (paper chromatography) and G (t.l.c.) as detailed in Sections II.B.7.a and c.

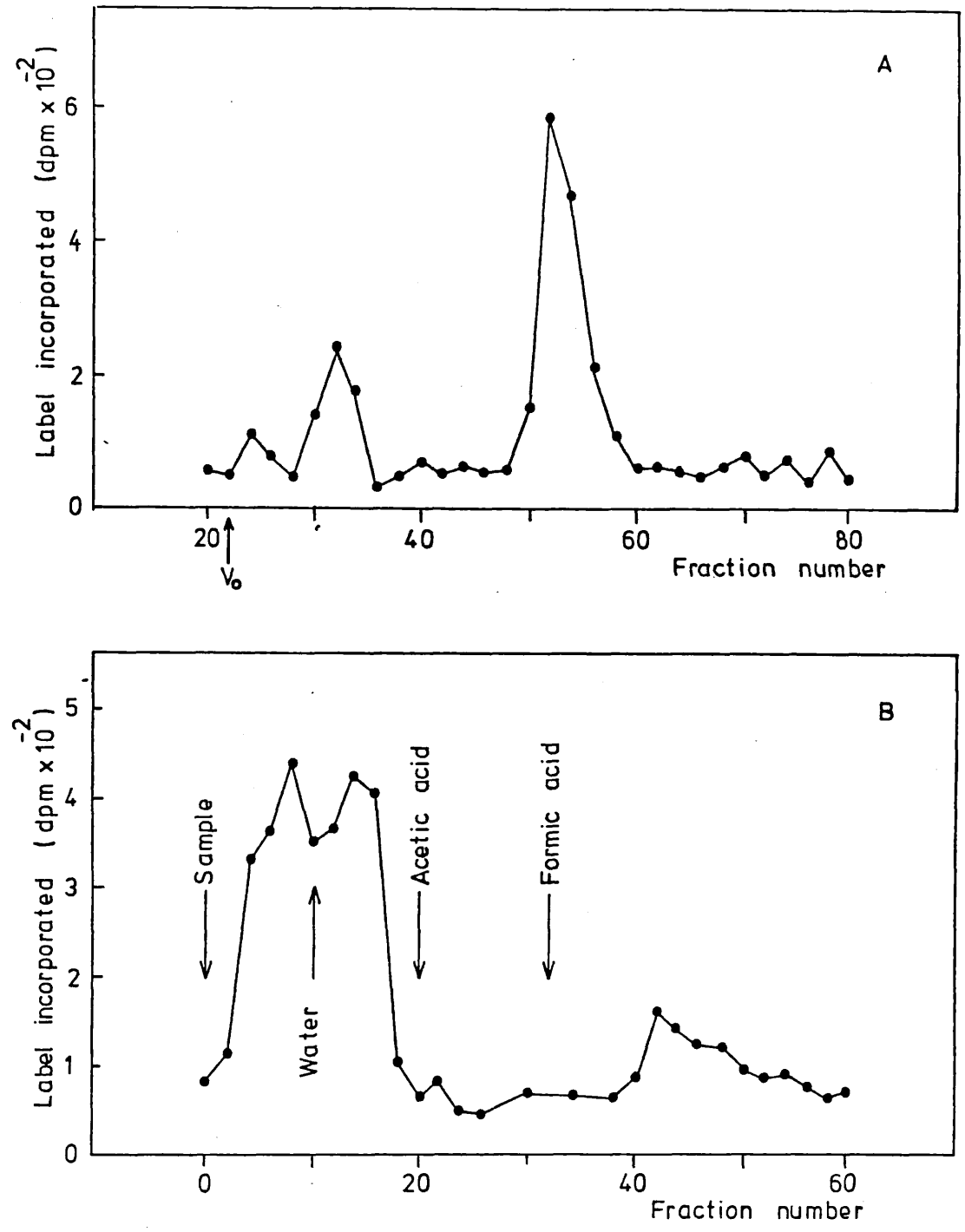


Fig. 31. A, Bio-Gel P-2 chromatography followed by B, Dowex 1X8-400 (acetate) chromatography of the $[^{14}C]$ products of alkaline phosphatase treatment of the purified unknown metabolite.

The unknown metabolite was purified as described in Section III.B.3. For experimental details of alkaline phosphatase treatment see text. See Figs. 21 and 23 for details of column chromatography.

demonstrated by paper chromatography in solvent E or by t.l.c. using solvent G (Fig. 30). Both chromatographic procedures resulted in the fractionation of two compounds but unfortunately neither co-chromatographed with the compounds obtained by acid hydrolysis. However, following alkaline phosphatase treatment, one peak of radioactivity did coincide with a fructose standard.

The effect of the unknown metabolite on liver aldolase activity with Fru-1-P was studied using a preparation which had been purified by Dowex 1X8-400 (acetate), Dowex 1X4-400 (borate) and finally Dowex 1X8-400 (acetate) chromatography. Under these conditions the unknown metabolite inhibited aldolase activity assayed with Fru-1-P by 24% and 80% at concentrations estimated to be 20 and 40 µg of the unknown/ml respectively. However, since the preparation was not treated with barium acetate or passed through a Bio-Gel P-2 column, P_i was present in the extract. Hepatic P_i concentrations are 5mM and Woods et al. (1970) have reported that 1mM-P_i inhibits liver aldolase by up to 30%, Hence, some if not all of,the observed inhibition could be attributed to the presence of P_i. However, it is possible that the accumulation of Fru-1-P, in isolated hepatocytes after a fructose load, may be due to the accumulation of the unknown metabolite. This possibility needs further investigation.

The unknown metabolite, partially purified by Dowex 1X4-400 (borate) chromatography, was shown to react with the liver aldolase preparation used to assay hepatic Fru-1-P concentrations (Section II.B.10.a.ii). Hepatocytes were incubated with 2mM-$\left[U\text{-}^{14}C\right]$ fructose for 10 min and the

perchloric acid extract was subjected to Dowex 1X4-400 (borate) chromatography. The fractions containing Fru-1-P and the unknown metabolite were pooled separately and the Fru-1-P content estimated enzymatically. The yield of radioactive Fru-1-P (0.271 µmol/g hepatocytes) agreed closely with the data from the enzymatic determination (0.268 µmol/g) confirming that the two assay systems gave identical results. When the unknown metabolite was assayed by both these methods the molar yields (0.639 and 0.582 µmol/g respectively) in each case was similar, assuming that fructose is incorporated in an intact form into the unknown and that one molecule of dihydroxyacetone phosphate is generated per molecule of unknown. These results suggested that the unknown is metabolised to dihydroxyacetone phosphate possibly by liver aldolase but, since the aldolase was a relatively crude preparation, confirmation of this requires the use of a purified enzyme preparation. When the unknown metabolite, purified as described in Section III.B.3, was subjected to aldolase treatment and the products applied to a Dowex 1X8-400 (acetate) column a neutral product was shown to elute in the flow-through fraction in addition to a charged compound eluting with formic acid (data not shown).

Further confirmation of the degradation of the unknown metabolite by the crude liver aldolase preparation was shown by an electrophoretic study. The unknown metabolite was shown to separate from Fru-1-P on high-voltage electrophoresis and the products of aldolytic cleavage can be identified (Fig. 32). The aldolytic cleavage of Fru-1-P is known to yield glyceraldehyde and dihydroxyacetone phosphate, the latter is converted in the assay system used to glycerol-3-phosphate. Thus, the peaks running near the origin and just ahead of Fru-1-P in

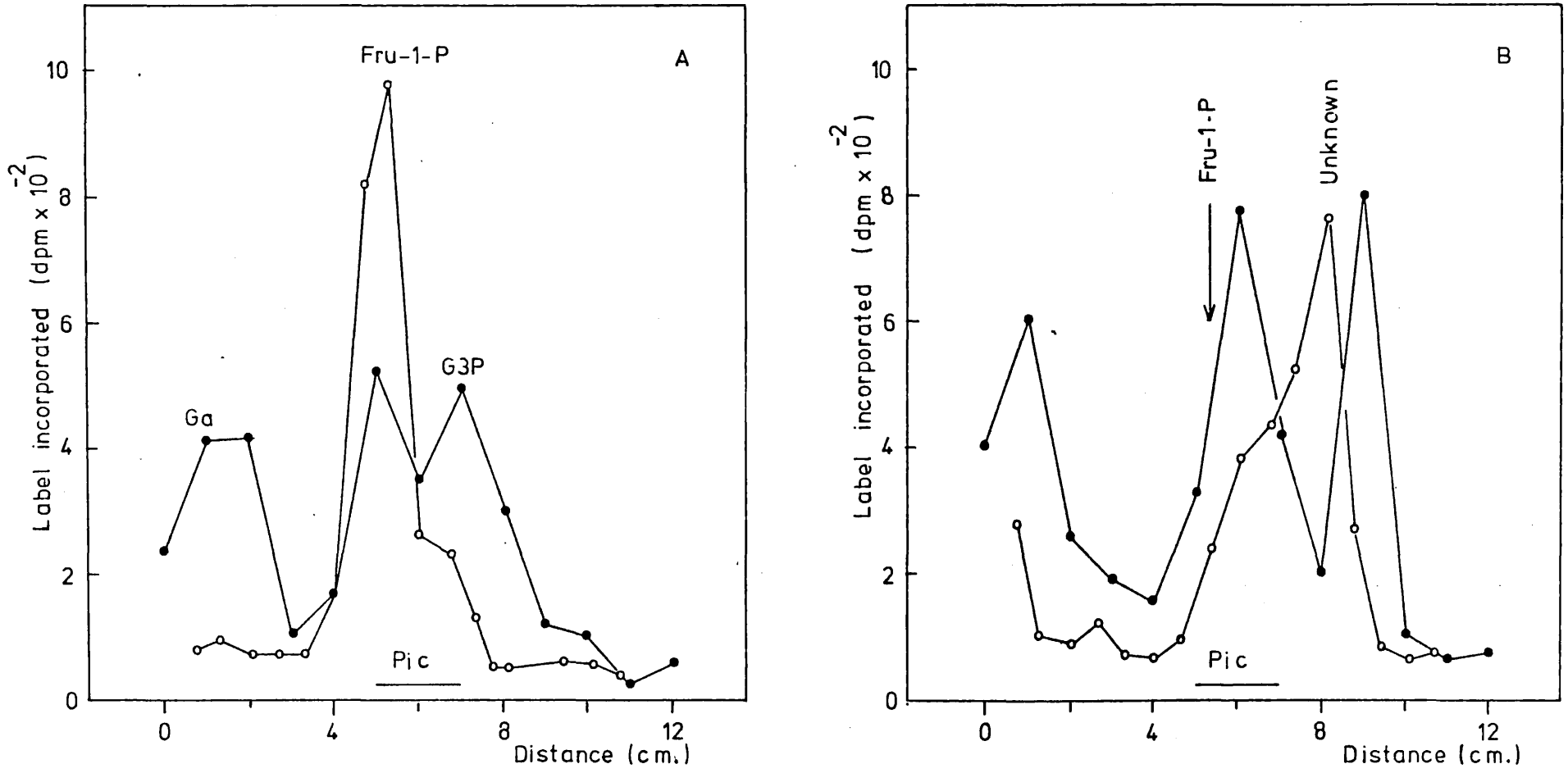


Fig. 32. High-voltage electrophoretic separation of Fru-1-P(A) and the unknown metabolite (B) and the products of their aldolytic cleavage.

The unknown metabolite and Fru-1-P were separated by electrophoresis in solvent H (o-o) as described in Section II.7.d. Following incubation with liver aldolase, glycerol-3-phosphate dehydrogenase and NADH (Section II.B.10.a.ii) the products of aldolytic cleavage (●-●) were similarily separated by electrophoresis.

Fig. 32A can be attributed to glyceraldehyde and glycerol-3-phosphate respectively. The unknown metabolite appears to be broken down to three products; a neutral metabolite possibly corresponding to glyceraldehyde, a second product which appears to run with Fru-1-P and a third product which runs ahead of the unknown metabolite (Fig. 32B). It is difficult to draw conclusive information from the electrophoretic data other than that degradation of the unknown metabolite, similar to that obtained with Fru-1-P, appears to occur in the presence of a crude liver aldolase preparation.

The experimental evidence reported here clearly shows that the unknown metabolite is different from Fru-1-P, whereas Fru-1-P breaks down to fructose following alkaline phosphatase treatment or acid hydrolysis (results not shown), the unknown metabolite appears to be degraded to a range of products by both these treatments. Acid hydrolysis or alkaline phosphatase treatment of the unknown resulted in different products which did not appear to be either fructose or glucose. The only indication that the unknown metabolite bore some structural similarities to Fru-1-P was its apparent reaction with a crude liver aldolase preparation. As discussed earlier (Section III. A.2) the presence of an enzyme in the crude aldolase preparation responsible for converting the unknown metabolite to Fru-1-P cannot be discounted. The characteristic features of the aldolase reaction with Fru-1-P were discussed in Section I.A.3. If liver aldolase does react with the unknown metabolite it would be expected to have a similar structure to Fru-1-P to enable a Schiff's base to form with a lysine group at the enzyme's active site, allowing the subsequent proton loss, gain and exchange to occur (Fig. 4). The Fru-1-P assay

involves both aldolase and glycerol-3-phosphate dehydrogenase in order that the conversion of NADH to NAD^+ may be measured. It, therefore, follows that one of the products of degradation of the unknown must be either dihydroxyacetone phosphate or a closely related metabolite.

5. Liver perfusion

In order to increase the yield and to decrease the preparation time, perfused livers were used as a source of the unknown metabolite.

Isolated rat livers were perfused with 2mM - $[U\text{-}^{14}C]$ fructose for 10 min as described in Section II.B.3. Neutralized perchloric acid extracts were applied to a Dowex 1X4-400 (borate) column and fractions assayed for carbohydrate and $[^{14}C]$ content (results not shown). A peak of $[^{14}C]$ - carbohydrate material was eluted at the same TEAB concentration as that obtained following incubation of isolated hepatocytes with 2mM - $[U\text{-}^{14}C]$ fructose (cf. Fig. 18). The formic acid fraction obtained following Dowex 1X8-400 (acetate) chromatography of a hepatocyte extract, incubated with 2mM - $[U\text{-}^{14}C]$ fructose (Fig. 21), was chromatographed on a Dowex 1X4-400 (borate) column with a perchloric acid extract of liver, perfused with 2mM-fructose for 10 min (Fig. 33). Two of the three carbohydrate-positive peaks co-chromatographed with $[^{14}C]$ labelled compounds, which had previously been characterised as the unknown metabolite and Fru-1-P (Fig. 22). Fractions 53-64 (Fig. 33), containing the unknown metabolite, were pooled and assayed for phosphate, fructose and total carbohydrate (Table 8). The results obtained from the two assays

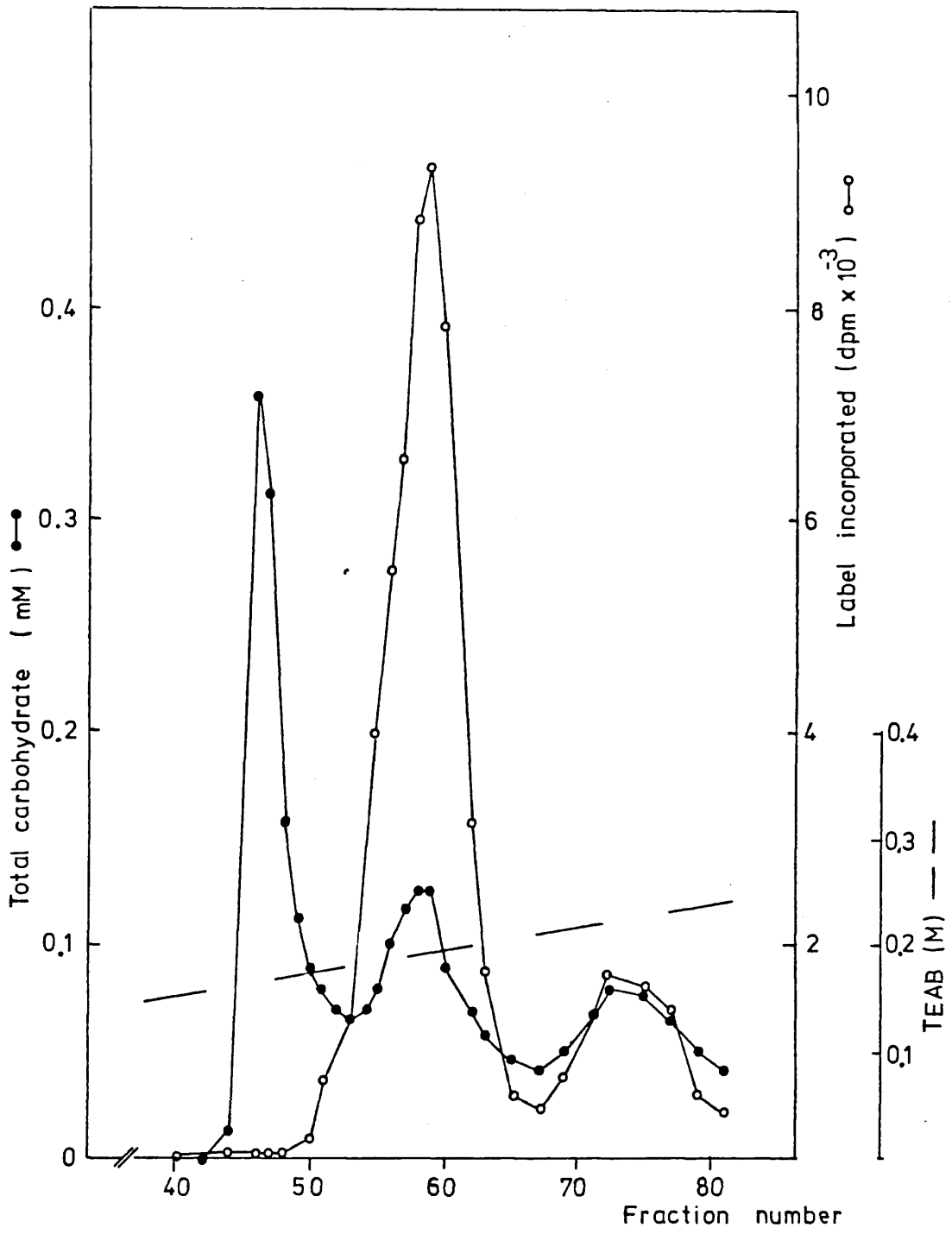


Fig. 33. Dowex 1X4-400 (borate) chromatography of a perchloric acid extract of liver following perfusion with 2mM-fructose.

Isolated rat liver (15g) was perfused for 10 min with 2mM-fructose as described in Section II.B.3. For other experimental details see text. Samples were applied to a Dowex 1X4-400 (borate) column, eluted with a linear gradient of 0.1-0.4M-TEAB and fractions assayed for radioactivity (o-o) and total carbohydrate (●-●) as detailed in Section II.B.10.b.i.

Table 8. Constituents of unknown metabolite extracted from the perfused liver and subjected to Dowex 1X4-400 (borate) chromatography.

For experimental details see text. Constituents were assayed colorimetrically as detailed in Section II.B.10.b. Results are means of results obtained from 3 different liver perfusions with s.e.m. as shown.

Constituent assayed	Concentration µmol/g liver	
Phospnate	0.597	0.069
Fructose (Roe assay)	0.286	0.045
Fructose (cysteine-carbazole assay)	0.075	0.026
Total carbohydrate	1.131	0.153

used for the determination of fructose and the total carbohydrate assay were in agreement for Fru-1-P (results not shown). However, for the unknown metabolite, there appeared to be 4-times as much fructose determined by Roe's resorcinol-HCl assay as that determined by the cysteine-carbazole reaction. Furthermore, there was a 4-fold discrepancy in the total carbohydrate compared to the fructose estimation by Roe's method. It therefore seems unlikely that fructose forms a major part of the carbohydrate moiety within the unknown metabolite. This is confirmation of earlier chromatographic findings which failed to identify fructose as a component of

the unknown metabolite. The specificity of the Roe's resorcinol-HCl assay for ketohexoses is not absolute since dihydroxyacetone and other ketoses interfere with the assay, and are reported to give products which absorb at a similar wavelength, and may also be assayed by Roe's method (Burton, 1957). Hence, dihydroxyacetone could be a component of the unknown metabolite.

The ratio of phosphate to total carbohydrate was 1:2. The concentration of free reducing sugar, determined by Nelson's method (Sectión II.B.10.b.iii), was typically estimated to be similar to that of phosphate; whereas, following acid treatment it approached total carbohydrate values (data not shown). However, when the unknown metabolite was purified, according to the scheme described in Section III.B. 3, the ratio of phosphate to total carbohydrate to acid-revealed reducing sugar was approximately 1:1:1 whilst fructose, determined by the resorcinol-HCl method, was in a 0.5 ratio to the other constituents. The reason for the apparent discrepancy between the material eluted from a Dowex 1X4-400 (borate) column and the purified compound is that the former contained a reducing sugar contaminant, later identified as glucose by the glucose oxidase method (Section II.B.10.a.i).

When perchloric acid extracts of a liver perfused with 2mM-fructose were fractionated into water-soluble, ethanol-insoluble barium salts and then subjected to Dowex 1X4-400 (borate) chromatography two peaks of phosphate containing material were obtained, corresponding to Fru-1-P and material eluting in the same position as the unknown (Fig. 34). The

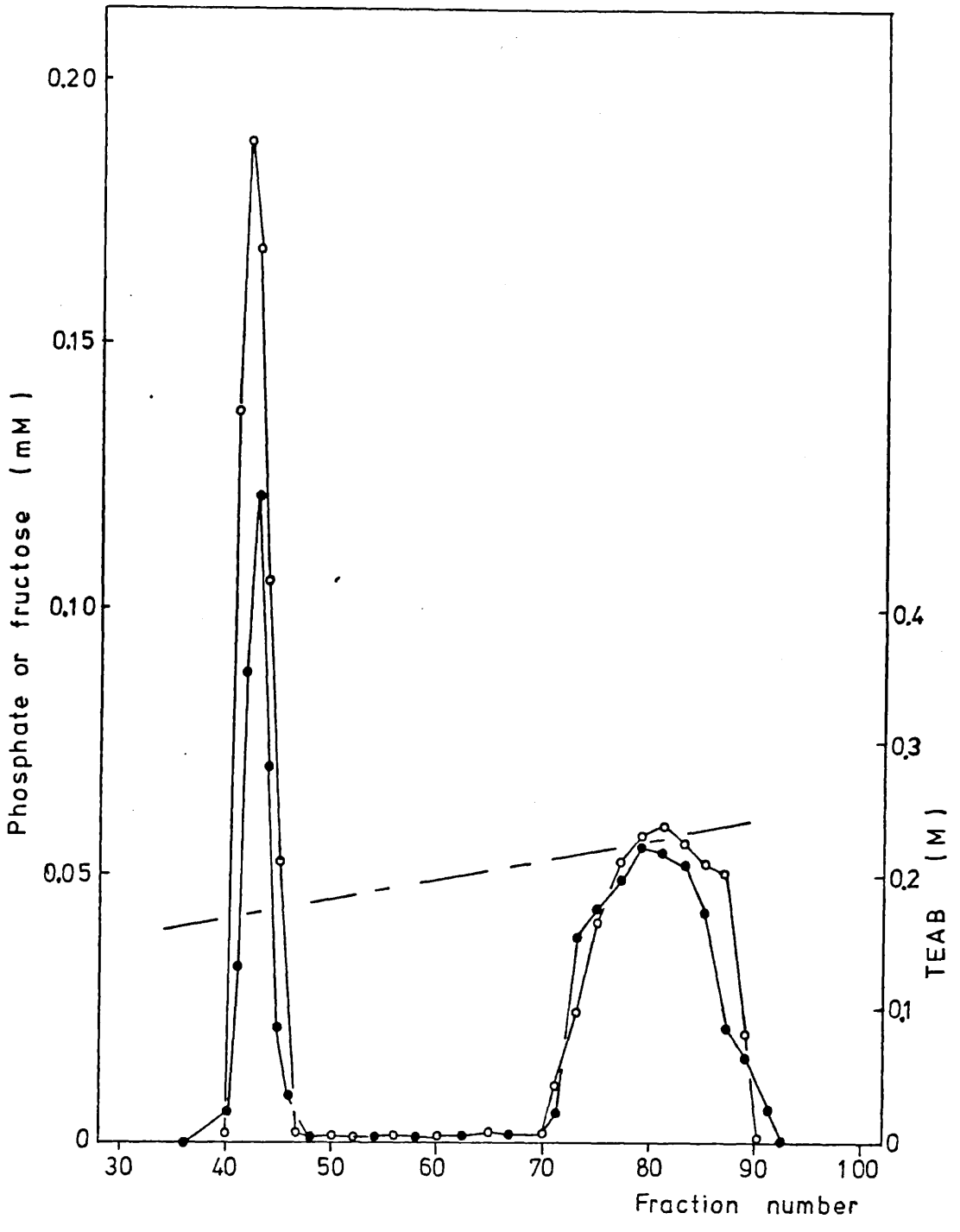


Fig. 34. Dowex 1X4-400 (borate) chromatography of water-soluble, ethanol-insoluble barium salts of a perchloric acid extract of liver, following perfusion with 2mM-fructose.

Isolated rat liver (15g) was perfused for 10 min with 2mM-fructose as described in Section II.B.3. Neutralised perchloric acid extracts were treated with barium acetate. The water-soluble, ethanol-insoluble barium salts were solubilised with 0.5M-H_2SO_4 (Section II.B.4.b.ii) and applied to a Dowex 1X4-400 (borate) column (Section II.B.8.a). Samples were eluted with a linear gradient of 0.1 - 0.4M-TEAB and fractions assayed for phosphate (o-o) and fructose, using Roe's resorcinol-HCl assay (●-●), as detailed in Section II.B.10.b.v. and ii.

ratio of P_i to resorcinol-positive material (estimated by reference to a fructose standard curve) was again shown to be 2:1 despite earlier experiments (Section III.B.2) showing that the unknown was broken down during barium acetate fractionation. This suggests that the part of the molecule responsible for the resorcinol-positive reaction is unaffected by barium acetate treatment. However, there no longer appeared to be any carbohydrate material, as judged by the phenol-sulphuric assay of Dubois et al. (1956). This negative result further confirms that the unknown metabolite has broken down since the assay is specific for simple sugars with free, or potentially free, reducing groups.

Similar results were obtained using livers from 24h-starved, as opposed to fed, rats (results not shown). In addition, there did not appear to be any change in the amount of unknown metabolite synthesised following perfusion of liver with 2mM-fructose under these conditions.

These experiments have shown that perfusion of isolated rat liver with low concentrations of fructose (2mM) results in the synthesis of Fru-1-P and the unknown metabolite, previously identified in extracts of isolated hepatocytes incubated with 2mM-fructose. The unknown metabolite was shown to contain carbohydrate, reducing sugar and phosphate. Following the breakdown of the unknown metabolite by barium acetate fractionation the phosphate ester formed was shown to contain resorcinol-positive material and to have a molecular weight typical of a triose phosphate and is tentatively proposed to be dihydroxyacetone phosphate, although this would require further study.

6. Amino acid analysis and mass spectrometry

The unknown metabolite was purified from the livers of fed rats perfused with 2mM-fructose for 10 min as described in Section II.B.3 except that glycogen was removed by precipitation with one volume 95% (v/v) ethanol before the Dowex 1X8-400 (acetate) chromatography. In order to aid the identification of the unknown metabolite a hepatocyte extract, from cells incubated with 2mM- $\left[U-^{14}C\right]$ fructose, was added to the perfused liver extract and the purification of the unknown metabolite was followed by assaying for radioactivity.

Approximately 200µg of the unknown metabolite was purified by the above procedure and colorimetric analysis revealed the presence of 43µg phosphate (0.45µmol) and 80µg total carbohydrate (0.44µmol) identified by the phenol-sulphuric reaction using fructose as a standard. Thus, there was approximately 80µg of unidentified material in the unknown. The unknown metabolite was hydrolysed with 2M-TFA as described in Section III.B.4 and the products separated on a Bio-Gel P-2 column. Fractions over a molecular weight of 200, between 100-200 and below 100 were pooled separately and each analysed for amino acids and by mass spectrometry.

a. Amino acid analysis

The unknown metabolite and acid hydrolysed fractions were separated on t.l.c. in solvent G and the chromatogram developed with ninhydrin to identify amino acids. No ninhydrin-positive material was detected in the unknown metabolite or in the acid hydrolysed fraction containing material of molecular weight below 100. However, both the higher molecular

weight fractions contained ammonia and some amino acids. In the high molecular weight (> 200) fraction there was ninhydrin-positive material of low mobility which had the same R_f value as a radioactive product in this t.l.c. system (see Fig. 30). Both the higher molecular weight fractions (100-200, > 200) of the hydrolysate contained at least one other amino acid which had an $R_{ammonia}$ value of 0.47 and was unlabelled.

The > 200 and 100-200 molecular weight fractions were also analysed on an amino acid analyser and both were shown to contain an excess of ammonia. Atmospheric ammonia is rapidly taken up by acid solutions and since the fractions were kept for several days at pH2 prior to amino acid analysis the high ammonia concentration obtained is not altogether unexpected. The high molecular weight fraction (> 200) was also shown to contain lysine (0.06μmol) and serine (0.03μmol). In addition, several peaks were obtained prior to the elution of the individual amino acids, these are proposed to be due to unhydrolysed peptides which would not be expected to be totally hydrolysed by the relatively mild acid conditions used here. These peptides, being of high molecular weight, could have co-purified with the unknown metabolite. However, this is unlikely since no ninhydrin-positive material was found prior to acid hydrolysis. Hence, the results support the proposal that the peptides isolated after acid hydrolysis form part of the unknown metabolite molecule. Both lysine (0.08μmol) and serine (0.14μmol) were also present in the 100-200 molecular weight fraction and in addition glycine (0.14μmol) was identified in this fraction.

From these experiments 20µg lysine, 18µg serine and 10µg glycine were estimated to be present in the acid hydrolysate. The amount of unhydrolysed peptide present could not be estimated by this method but probably accounts for the remainder of the unidentified material in the unknown. If all the previously unidentified material is attributed to these amino acids, and they are assumed to be present as a tripeptide, the molar ratio of tripeptide to carbohydrate to phosphate is approximately 1:1:1 and thus, the unknown metabolite could be a phosphoglycopeptide. No reports of the occurrence of natural phosphoglycopeptides could be found in the literature.

Lindqvist et al. (1983) have studied the effects of a fructose load on the concentration of free amino acids in rat liver. Of the three amino acids identified here these workers showed that only the concentration of lysine was significantly affected (increased by 38% over control values). However, the tripeptide is probably already present in the cell and is unlikely to be formed from free amino acids in response to a fructose load.

b. Mass spectrometry

The unknown metabolite was analysed by e.i. and iso-butane c.i. mass spectrometry as detailed in Section II.B.14. Molecular weights are easily determined from iso-butane c.i. spectra since intense peaks are usually obtained at M + 1, where M is the molecular weight of the molecule (Williams and Fleming, 1973; McLafferty, 1980). However, the c.i. spectra of the unknown metabolite were complex suggesting that the molecule was breaking down under the analysis

conditions used, the highest molecular weight ion found was at m/z 470. The presence of Fru-1-P in the unknown metabolite was suggested by reference to the c.i. spectra obtained for standard Fru-1-P under similar conditions. The presence of phosphate was further confirmed by a major peak at m/z 96 $(M + 1)^+$.

The e.i. spectra of the unknown metabolite indicated a molecular weight of 486, which was again significantly lower than that determined by gel-filtration. However, this latter method of molecular weight determination is known to be inaccurate when water is used to elute the sample and thus is not an accurate method for the determination of the molecular weight of the unknown metabolite. Both methods confirm that the unknown metabolite has a molecular weight higher than that expected for a simple hexose phosphate. The presence of carboxylic groups within the molecule is suggested by the appearance of a major peak at m/z 60 (Williams and Fleming, 1973) and this is further evidence to support the proposal that amino acids are present.

The majority of the work reported in the literature is on the trimethylsilyl (TMS) derivatives of carbohydrates since this increases their volatility and thermal stability and enables accurate and reproducible mass spectra to be obtained (Zinbo and Sherman, 1970). Thus, if more time and further structural information had been available, it would have been of interest to prepare a TMS derivative of the unknown metabolite and to investigate its c.i. and e.i. spectra.

Similar analysis of the acid hydrolysate fractions of the unknown metabolite further suggested that amino acids were present in the 100-200 molecular weight fraction. The amino acids were identified from iso-butane c.i. spectra as serine; m/z 106 $(M + 1)^+$ and 61 $(M + H - CO - H_2O)^+$, lysine; m/z 146 $(M + 1)^+$ and glycine; m/z 76 $(M + 1)^+$ and 58 $(M + H - H_2O)^+$ - Vetter, 1980. The presence of carboxylic and phosphate groups was again confirmed by e.i. spectra, with peaks at m/z 60 and 95 respectively. The loss of NH_3 in e.i. spectra is indicative of amino acids (McLafferty, 1980). Hence, the molecular ion at m/z 128 could have arisen from the loss of NH_3 from lysine. The e.i. spectra also confirmed the presence of glycine (m/z 75) in this fraction. The presence of a metastable ion giving a peak at m/z 105 corroborated peaks at m/z 156 and 128 as the parent and daughter respectively. Other major peaks were found at m/z 184, 170, 142, 134, 122, 106, 82, 78 and 64. The ion (m/z 156) may have arisen from the loss of CO from m/z 184, alternatively it may be a homologue of m/z 170. This latter ion would have the correct molecular weight for a triose phosphate, which had earlier been proposed to be present in the acid hydrolysate of the unknown metabolite (Section III.B.4). However, since no data on the mass spectra of underivatised trioses or triose phosphates was available for reference to these proposals could not be confirmed.

When the high molecular weight (> 200) fraction was analysed by e.i. mass spectrometry the highest mass peaks were obtained at m/z 273 and 272, with peaks at m/z 128 $(M - NH_3)^+$ and 100 $(M - CO_2H)^+$ which could be attributed to lysine and at m/z 105 $(M)^+$ to serine. Since earlier

studies had shown that $[^{14}C]$ was not present in this fraction (Section III.B.4) and had indicated the possible presence of a tripeptide, containing lysine, glycine and serine (Section III.B.6.a), the highest mass peaks can probably be attributed to the loss of either OH or NH_3 to give m/z 273 or H_2O to give m/z 272 from the tripeptide (M = 290).

The low molecular weight fraction (< 100) obtained after acid hydrolysis was shown by e.i. to contain phosphate (m/z 95). Experiments with $[U\text{-}^{14}C]$ fructose had suggested the presence of a triose, however, the spectra obtained here did not yield a peak at m/z 90 and attempts to identify the other low mass peaks in the spectra were unsuccessful. However, iso-butane c.i. spectra suggested the presence of a triose (m/z 91) and confirmed the presence of phosphate (m/z 96) and TFA (m/z 114) in this fraction.

To summarise, e.i. and isobutane c.i. spectra of the unknown metabolite have suggested that it has a molecular weight of at least 470 and that amino acids, phosphate and Fru-1-P may be components of the molecule. When the fractions from a Bio-Gel P-2 column, following acid hydrolysis of the unknown metabolite, were pooled according to molecular weight and analysed by e.i. and iso-butance c.i. mass spectrometry the presence of amino acids (glycine, serine and lysine) and P_i was confirmed. Furthermore, the existence of a triose and a triose phosphate in the hydrolysate was suggested, although whether the triose moiety is the same in each case was not clear from the data obtained.

7. Identification of unknown metabolite

The experiments described within this section have clearly shown that the unknown metabolite contains carbohydrate, phosphate and amino acid. There is evidence from both e.i. and isobutane c.i. spectra and amino acid analysis that serine, glycine and lysine are present possibly as a tripeptide which forms part of the molecule. Throughout this study problems were encountered with the unknown metabolite breaking down under different conditions, notably after barium treatment and following elution from a paper chromatograph, and as a result the identification of the unknown was hindered. However, a purification scheme has been worked out in which breakdown was minimised and it was possible to show that the unknown metabolite contained carbohydrate and phosphate in a 1:1 ratio. In addition problems were encountered in determining the nature of the carbohydrate moiety. It is clearly not glucose as evidenced by both its chromatographic behaviour and lack of reaction with glucose oxidase. Although under certain conditions (e.g. following alkaline phosphatase treatment) there was evidence that the carbohydrate co-chromatographed with fructose evidence from colorimetric studies indicated that the fructose could be modified in some way.

Despite the discrepancy in the molecular weight determinations by gel-filtration and e.i. and iso-butane c.i. mass spectrometry all methods suggested that the unknown metabolite had a minimum molecular weight of 470 indicating the presence of a component other than fructose and phosphate in the unknown. This work is supported from the results of studies with high-speed liver supernatants (Section III.A.2) which had suggested that the formation of the unknown metabolite

required the presence, in the liver extract, of a low molecular weight compound, which can be removed by Sephadex G-25 filtration. The evidence presented here suggests that this may be a tripeptide. If the unknown metabolite resulted from the formation of a phosphoglycopeptide between Fru-1-P and the tripeptide it would be expected to have a molecular weight of 532. The complex $[^{14}C]$ metabolite profiles obtained following acid hydrolysis, alkaline phosphatase treatment or barium acetate fractionation suggest that the unknown metabolite does not break down simply to the unlabelled tripeptide and $[^{14}C]$ Fru-1-P.

The interaction of carbohydrates with amino acids is well established particularly with reference to the browning reaction (Mohammad et al., 1949; Lead and Hannan, 1950). Griffiths (1974) studied the biochemical effects of 1-(ε-N-lysyl)-1-deoxy-D-fructose, formed by an Amadori rearrangement of the product resulting from the reaction of D-glucose with the ε-amino group of lysine. He reported that on heating with acid, fructose-amino acid derivatives are decomposed rather than hydrolysed. Thus, the sugar moiety gives decomposition and rearrangement products rather than the free sugar and only low amounts of the free amino acids are obtained. This could be an explanation for the complex products obtained following acid hydrolysis of the unknown metabolite in the experiments described in Sections III.B.4. and 6. Recent experiments by Acharya and Manning (1983) have shown that glycolaldehyde forms a Schiff base with the ε-amino group of lysine in proteins. This was reported to undergo an Amadori rearrangement, in the absence of reducing agents, to form an aldoamine containing a new aldehyde group which

is available for reaction with other ε-amino groups of proteins (Fig. 35). Similar reactions with glucose or glyceraldehyde result in the formation of ketoamines which do not have a free reactive aldehyde group (Acharya et al., 1983).

In this context Fru-1-P has been shown to bind to liver aldolase through the formation of a Schiff base between the ε-amino group of a lysine molecule at the active site of the enzyme and the carbonyl group at C-2 on the fructose moiety (Section I.A.3, Fig. 4). It is therefore possible that the tripeptide could react at the same position via a similar rearrangement.

In summary, although the unknown metabolite was not positively identified by these studies there was evidence that it was formed as a result of an amino acid - carbohydrate interaction, with Fru-1-P linked, possibly at C-2, to the ε-amino group of lysine in a tripeptide containing lysine, serine and glycine.

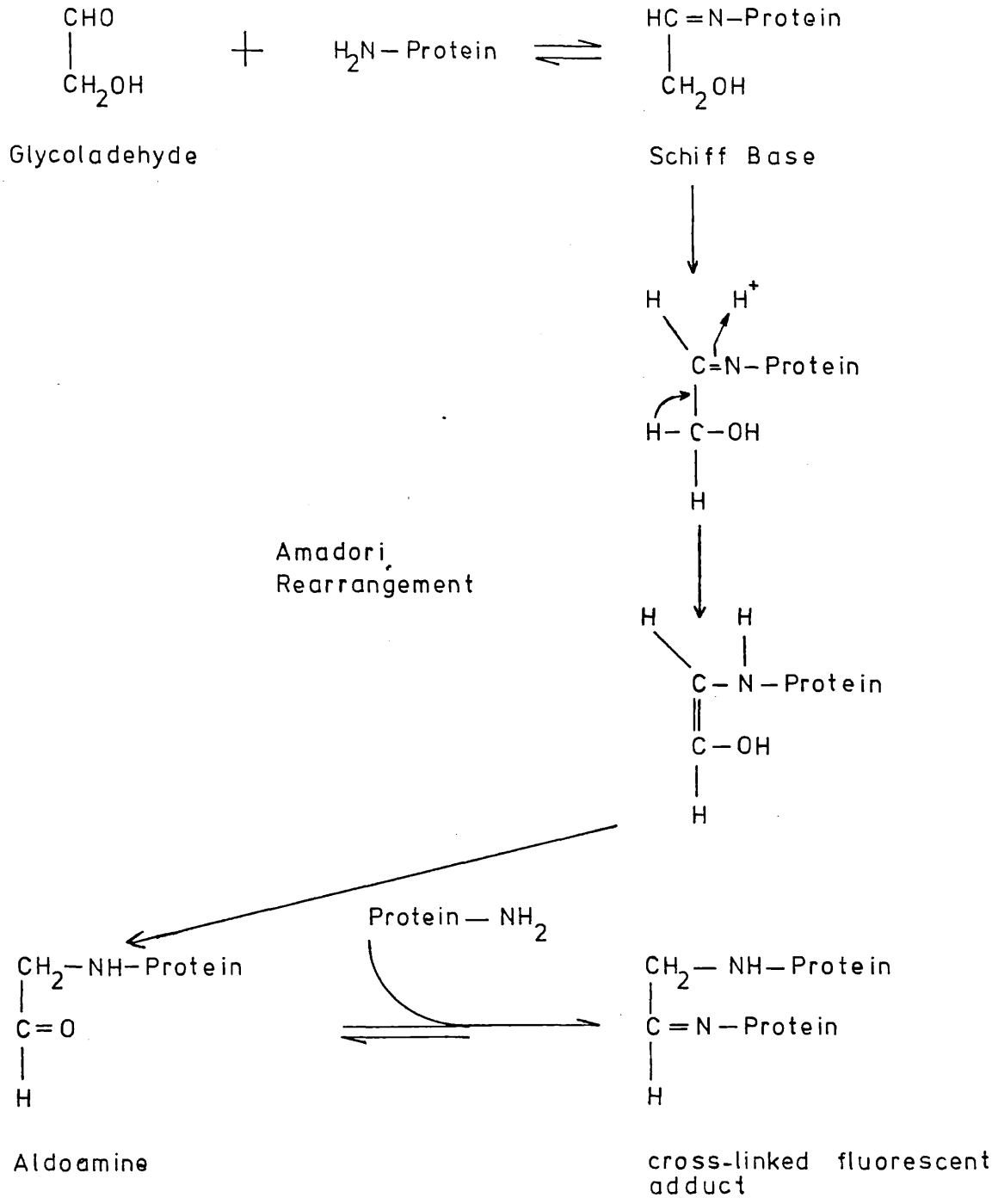


Fig. 35. Cross-linking potential of glycolaldehyde (Acharya and Manning, 1983).

C. Studies on hepatic fructokinase

Crude liver fructokinase was shown to have an activity between 2.47 and 2.79µmol/min/g liver. This agrees well with values of 2.58 and 3.12µmol/min/g liver reported by other workers (Adelman et al., 1967a; Sánchez et al., 1971a). It also correlates well with the initial rate of fructose removal from hepatocytes and the perfused liver determined earlier as 1.31µmol/min/g cells and 1.57µmol/min/g liver respectively (Section III.A.1).

1. Purification of fructokinase

Fructokinase was purified from rat liver according to the method of Sánchez et al. (1971a) as described in Section II.B.5. A typical purification is shown in Table 9. The colorimetric assay described in Section II.B.6.d, following ADP production using pyruvate kinase and reacting the pyruvate formed with 2,4-dinitrophenylhydrazine, was used to determine fructokinase activity during the purification. ATPase and sorbitol dehydrogenase were destroyed during the heat- and acid—precipitation steps. Myokinase however, is heat- and acid—stable and hence co-purified with fructokinase. The inclusion of a gel-filtration step in the purification successfully separated myokinase (mol. wt. 68 000) from fructokinase (mol. wt. 28 000).

The $(NH_4)_2SO_4$ precipitated enzyme was either chromatographed on DEAE-cellulose (Fig. 36) and Sephadex G-100 (121-fold purification) or applied directly to the gel-filtration column as shown in Fig. 37 (75-fold purification). The increased enzyme purification achieved, when the DEAE-cellulose step was included, was accompanied by a considerable

Table 9. Purification of rat liver fructokinase

Step	Volume ml	Activity units/ml	Protein mg/ml	Specific activity units/mg	Purification	Yield
Homogenate	144	0.434	26	0.017	1	100
20 000g supernatant	110	0.544	14	0.039	2.3	96
pH 5 supernatant	98	0.581	12	0.048	2.9	91
Heat supernatant	85	0.572	6	0.095	5.7	78
0-45% $(NH_4)_2 SO_4$	4.4	6.742	29	0.233	13.9	47
DEAE-cellulose	20	1.086	2.8	0.388	22.8	35
Sephadex G-100	8	0.824	0.4	2.060	121.2	10
Sephadex G-100*	68	0.284	0.2	1.420	75.0	25

*Results from a separate experiment.

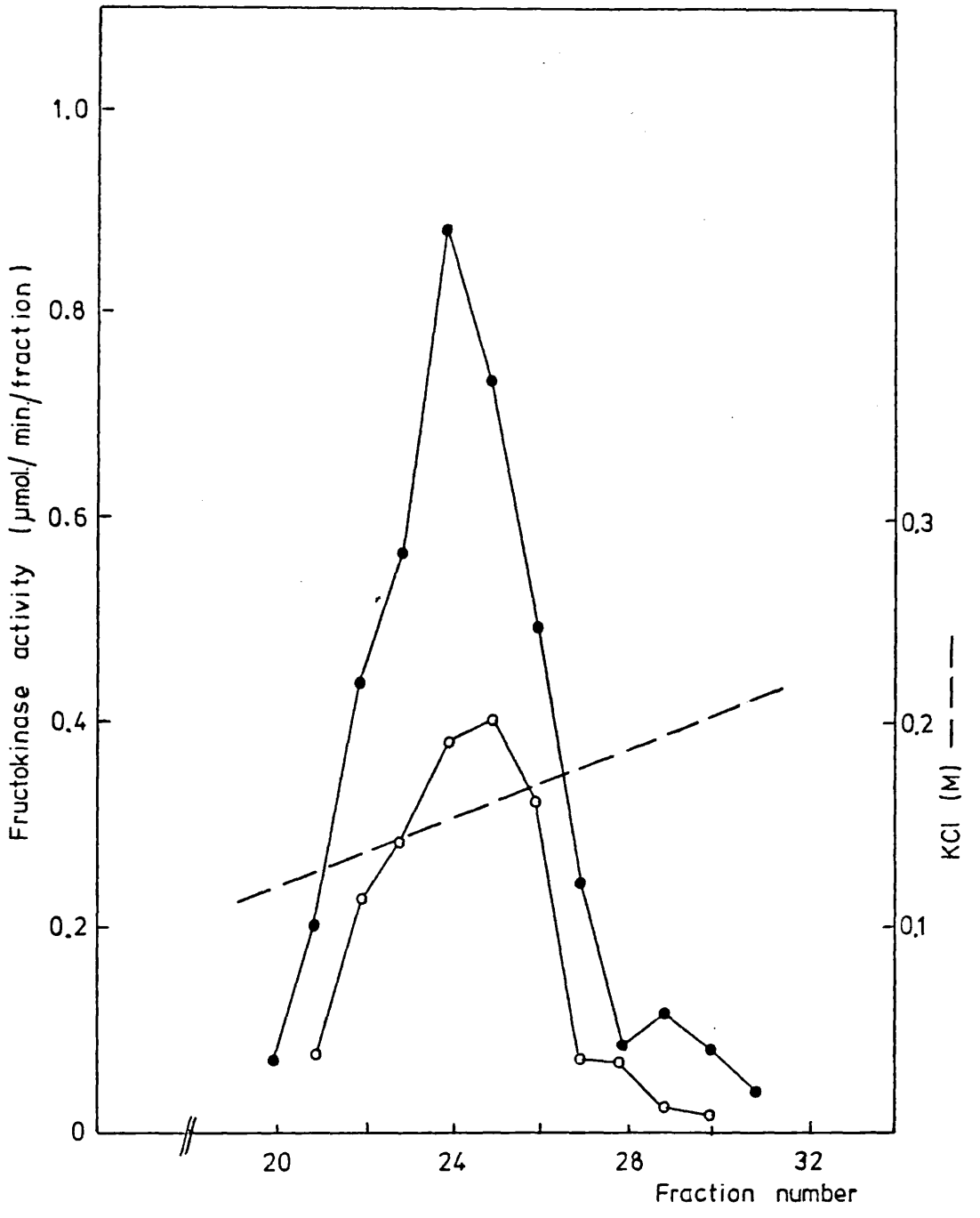


Fig. 36. DEAE-cellulose chromatography of ATP (●—●) and GTP (o—o) dependent fructokinase activities.

The 0-45% $(NH_4)_2SO_4$ fraction was diluted in 0.01M-Tris/HCl, pH 7.5, and applied to a DEAE-cellulose column. Fructokinase was eluted with a linear gradient of 0-1M-KCl in the same buffer (total vol. 360 ml). Fractions (2 ml) were collected and ATP-dependent activity determined with 5mM-fructose and 6mM-MgATP using the colorimetric assay (Section II.B.6.d). GTP-dependent fructokinase was assayed using 2mM-[U-^{14}C] fructose and 6mM-MgGTP (Section II.B.6.e.ii).

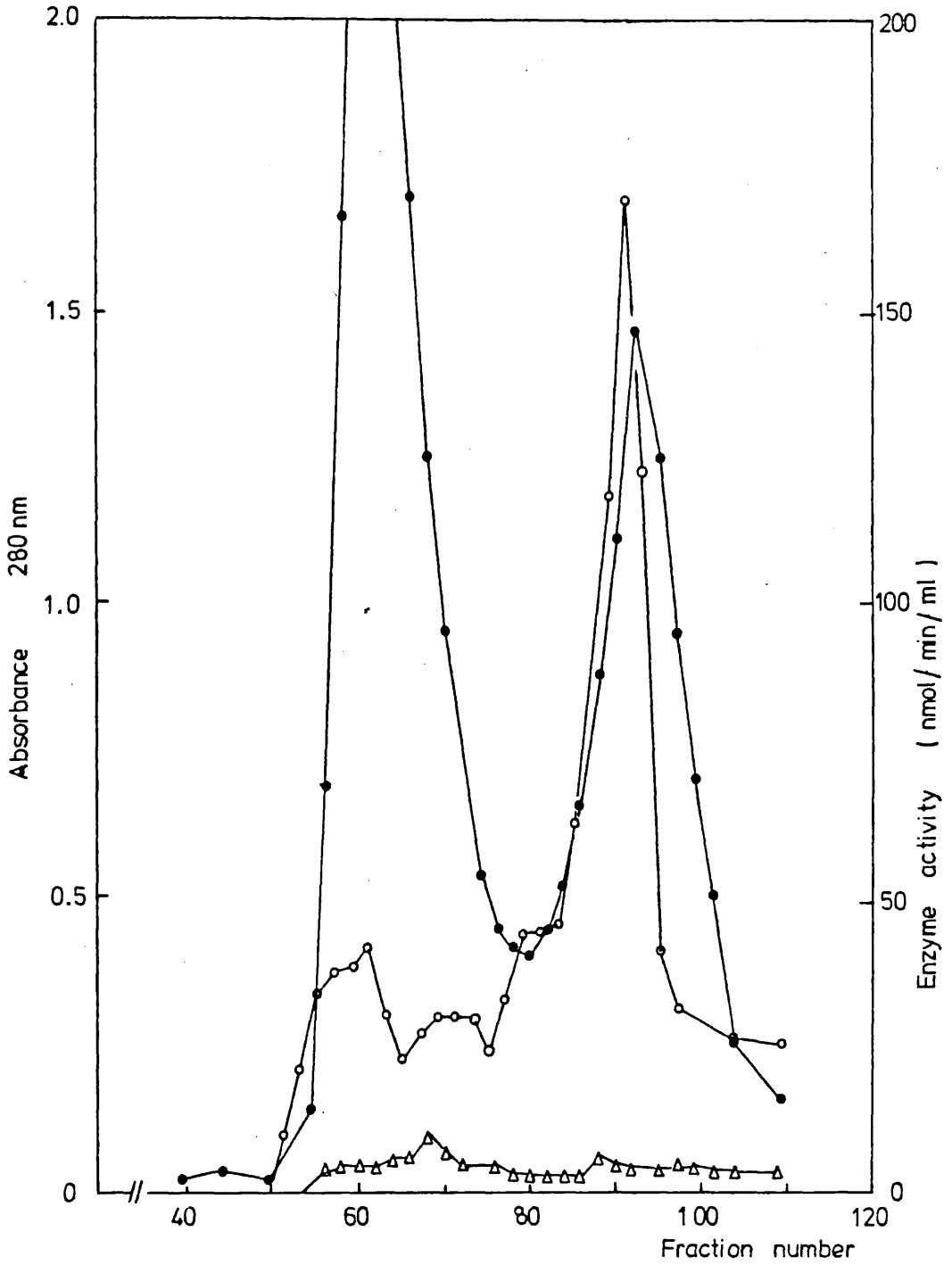


Fig. 37. Purification of fructokinase by Sephadex G-100 chromatography.

The 0-45% $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-100 column and eluted with 0.01M-Tris/HCl buffer, pH 7.5. Fractions (2 ml) were collected and the absorbance at 280 nm (●—●), fructokinase - with 2mM- [U-^{14}C] fructose and 6mM-MgATP (o—o) - and myokinase (Δ—Δ) activities determined. Enzymes were assayed as detailed in Section II.B.6.c. and e.ii.

decrease in overall enzyme recovery. It was therefore decided not to include the DEAE-cellulose chromatography step in further purification procedures.

The purified enzyme preparation was stored at -18°C in 0.01M-Tris/HCl buffer, pH 7.4, for several months without any significant loss of activity.

2. Comparison of the colorimetric and radiochemical assays for fructokinase

The radiochemical assay, using [U-^{14}C] fructose lacked an ATP-regenerating system and hence ADP, a known inhibitor of fructokinase activity, accumulated during the assay. It was therefore important to ensure that enzyme activities were determined from initial reaction rates, before sufficient ADP could accumulate to inhibit the enzyme. Reaction rates were only found to be linear with low enzyme concentrations (less than 10µg/ml protein) as shown in Fig. 38. The colorimetric assay included an ATP-regenerating step but there was a good correlation between the two methods providing low concentrations of enzyme were used for the assays (Table 10).

3. Properties of fructokinase

The experiments described in Section III.A.2 have already shown that purified rat liver fructokinase can utilise either ATP or GTP, but not CTP or UTP, as a phosphate donor. With both ATP and GTP the sole labelled product was found to be Fru-1-P. Other workers have shown ATP to be the only nucleotide triphosphate to act as phosphate donor in the fructokinase reaction (Parks et al., 1957; Adelman et al.,

Fig. 38. A, Time-course of Fru-1-P formation and B, effect of protein concentration on fructokinase activity.

The 0-45% $(NH_4)_2SO_4$ fraction was assayed with 2mM-[U-^{14}C] fructose and 6mM-MgATP at 37°C as detailed in Section II.B.6.e.ii.

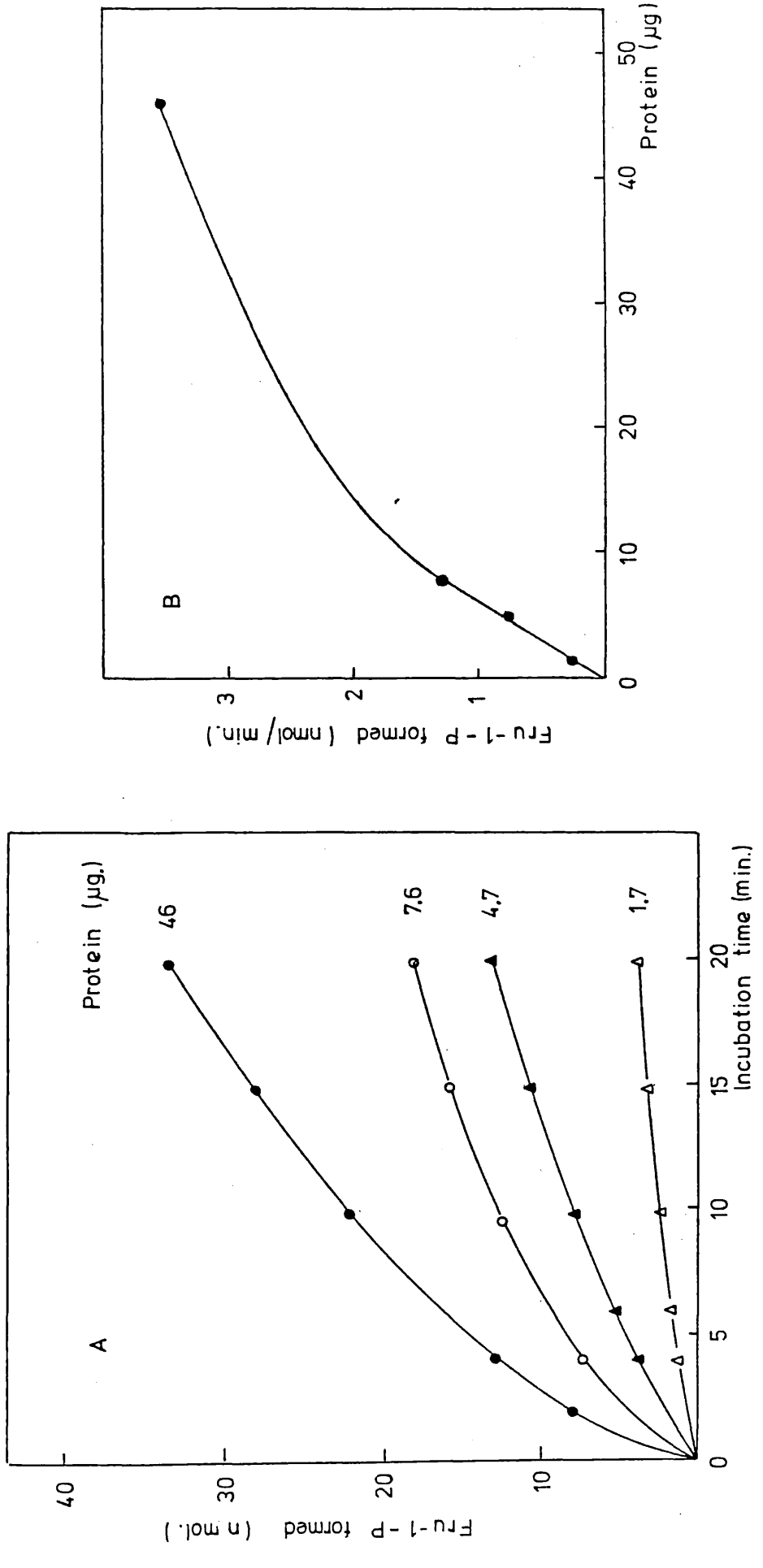


Fig 38. A, Time-course of Fru-1-P formation and B, effect of protein concentration on fructokinase activity.

Table 10. Comparison of fructokinase activity determined using the colorimetric and radiochemical assays.

The 0-45% $(NH_4)_2SO_4$ precipitated enzyme was incubated for 15 min with 5mM-fructose, 0.5M-KCl and 6mM-MgATP. For full experimental details see Section II.B.6.d and e.i. Results are means ± s.e.m. of results with four different enzyme preparations.

Assay Method	Fructokinase activity µmol/min/mg protein
Colorimetric	0.215 ± 0.061 (n = 4)
Radiochemical	0.198 ± 0.039 (n = 4)

1967a). It was therefore decided to investigate further the kinetics of GTP utilisation and to compare these to the parameters obtained with ATP in order to assess their physiological significance.

a. Nucleotide specificity

Sánchez et al. (1971b) have shown that if Mg^{2+} or ATP are not present at the same concentration an excess of either will inhibit fructokinase activity. All incubations therefore contained equimolar concentrations of Mg^{2+} and ATP or Mg^{2+} and GTP. Adelman et al. (1967a) were unable to show any reaction with up to 25mM-GTP. Their assay was essentially similar to that used here except that it included an ATP regenerating system and had a nucleotide-Mg^{2+} ratio of 0.75 : 1.00. According to Sánchez et al. (1971b) an ATP-Mg ratio

of 0.75 : 1.00 would result in a 10% loss of enzyme activity assayed in the presence of 0.1M-KCl. The effect of Mg^{2+} concentration on the GTP reaction has not been studied. However, if excess Mg^{2+} were found to also inhibit the GTP reaction this could explain the negative results of Adelman et al. (1967a).

Fru-1-P was demonstrated in Section III.A.2 to be formed by incubation of a Sephadex G-25 filtrate, of a 100 000g supernatant of a liver homogenate, with 2mM- [U-^{14}C] fructose, 0.5M-KCl and 6mM-ATP or GTP. In the absence of K^+ a very low yield of Fru-1-P was found with 6mM-GTP and reduced levels with 6mM-ATP (Table 11). The GTP-dependent reaction was

Table 11. Incubation of Sephadex G-25 filtrate with 6mM-GTP or ATP in the presence or absence of KCl for 15 min.

Incubations were at 37°C and included 2mM- [U-^{14}C] fructose as described in Section II.B.6.e.i. Results are mean of three experiments with s.e.m. as shown.

Nucleotide triphosphate	Fru-1-P produced µmol/min/g liver		
	- KCl	+ 0.1M-KCl	+ 0.5M-KCl
6mM-GTP	0.139 ± 0.016	0.472 ± 0.016	0.837 ± 0.098
6mM-ATP	0.712 ± 0.030	1.456 ± 0.056	1.873 ± 0.081
ATP/GTP ratio	5.12	3.08	2.24

affected by KCl to a greater extent than the ATP reaction,as reflected by the ratio of reaction rates. Inclusion of 0.5M-KCl in the assay medium resulted in a 6-fold increase in the production of Fru-1-P from GTP compared to only a 2-fold increase from ATP. The stimulation by K^+ of the ATP-dependent fructokinase activity is consistent with the data of other workers (Hers, 1952a; Parks et al., 1957; Sánchez et al., 1971b). The preparation showed enzyme activity in the absence of K^+ in contrast to similar incubations with the purified enzyme (Sephadex G-100 filtrate) - results not shown. These results are in agreement with the findings of other workers. Parks et al. (1957) and Sánchez et al (1971b) have shown the purified enzyme (400-fold) to have an absolute requirement for K^+. Hers (1952a),however, has shown enzyme activity on incubation of the partially purified enzyme (20-fold) from beef liver in the absence of K^+. This apparent difference in K^+ requirement of the crude and purified enzyme has not been studied further.

Before the nature of ATP and GTP utilisation by rat liver fructokinase could be studied further it was necessary to establish that two isoenzymes of fructokinase, with differing specificities towards ATP and GTP, were not present in the enzyme preparation. This seems unlikely since an established purification procedure was used and the enzyme activity, determined with both ATP and GTP, was shown to co-chromatograph on DEAE-cellulose (Fig. 36) and on Sephadex G-100 (data not shown). Furthermore, the ratio of Fru-1-P production from GTP and ATP was shown to be constant throughout the purification procedure (Fig. 39 and Table 12). If

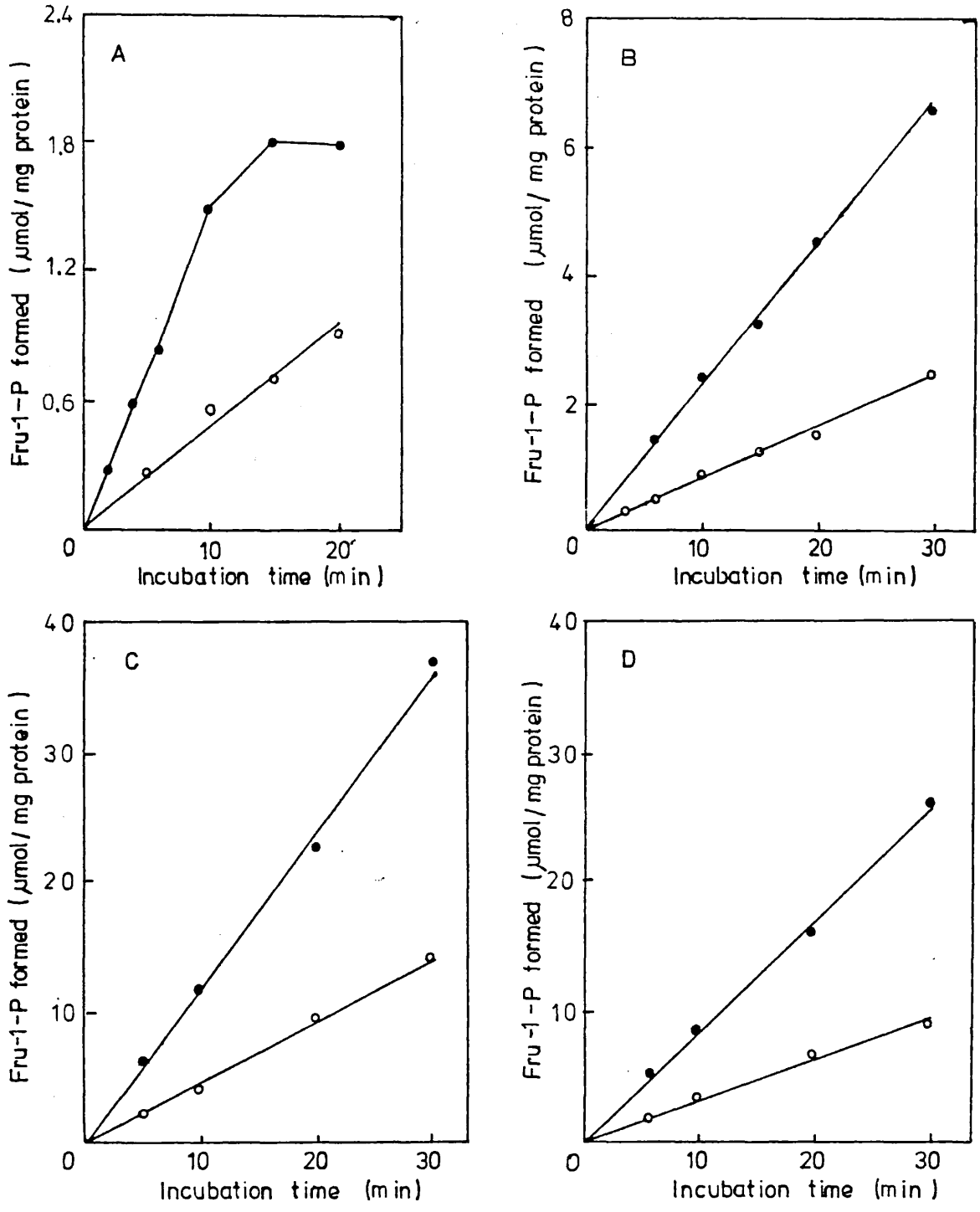


Fig. 39. The effect of incubation time on the ATP(●—●) and GTP(o—o) dependent formation of Fru-1-P by fractions obtained during the purification of fructokinase.

A, 0-45% $(NH_4)_2SO_4$ fraction; B, enzyme purified by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography; C, as B followed by Sephadex G-100 chromatography; D, enzyme purified by $(NH_4)_2SO_4$ fractionation and Sephadex G-100 chromatography. All assays included 2mM-[U-^{14}C] fructose and 6mM-MgATP or 6mM-MgGTP as detailed in Section II.B.6.e.ii.

Table 12. Fru-1-P production from 2mM-fructose and 6mM-ATP or GTP at different stages of enzyme purification.

Fructokinase was assayed with 2mM-[U-^{14}C] fructose and 0.1M-KCl. See Fig. 39 for other details.

Purification step	Fru-1-P produced µmol/min/mg protein		ATP/GTP ratio
	6mM-ATP	6mM-GTP	
Sephadex G-25	0.019	0.006	3.1 : 1.0
$(NH_4)_2SO_4$ (A)	0.142	0.048	3.0 : 1.0
DEAE-cellulose (B)	0.232	0.078	3.0 : 1.0
Sephadex G-100 (C)	1.185	0.430	2.8 : 1.0
Sephadex G-100 (D)	0.855	0.297	2.9 : 1.0

two enzymes were to exist one would expect the ratio of activities to change during the purification.

The purity of the GTP was checked by t.l.c. using cellulose acetate plates developed in 2M-formic acid : 2M-LiCl, 1:1 v/v(Randerath and Randerath, 1968). The R_f value of the U.V. absorbing material was consistent with the value reported for GTP by Randerath and Randerath (1968) - results not shown. Furthermore, there was a clear separation between GTP and ATP and there was no observable contamination of the GTP by ATP. It seems clear, therefore, that fructokinase does utilise GTP as a phosphate donor contrary to the statement by Adelman et al. (1967a).

b. Enzyme kinetics

The kinetics of the GTP- and ATP— dependent reactions were investigated. Michaelis constants of 0.83mM and 1.43mM were obtained for fructose and ATP respectively, at 0.1M-KCl with ATP as substrate (Figs. 40 and 41). These values are in good agreement with those determined by other workers. Sánchez et al. (1971a) have reported a K_m for fructose of 0.80mM and for ATP of 1.33mM at a similar KCl concentration. They have also shown that by increasing the K^+ concentration to 0.4M the K_m for fructose is decreased to 0.46mM without any significant effect on the K_m for ATP (1.56mM). Other workers, using 0.5M-KCl have reported K_m values for ATP of 1.5mM (Hers, 1952b, Adelman et al., 1967a) and for fructose of 0.4-0.5mM (Hers, 1952a; Parks et al., 1957; Adelman et al., 1967a). Parks et al (1957) reported a K_m for ATP of 0.2mM which is 10 times lower than anyone else has reported. With GTP as phosphate donor the K_m values were found to be 0.56mM for fructose and 1.65mM for GTP (Figs. 40 and 42 respectively).

ADP has been shown, by Parks et al. (1957), to be a strong non-competitive inhibitor of the ATP reaction. However, Sánchez et al. (1971b) have shown ADP to be a non-competitive inhibitor towards fructose and a competive inhibitor with respect to ATP. The K_i for ADP, with respect to ATP, is reported to be either 1.3mM (Parks et al., 1957) or 1.7mM (Sánchez et al., 1971b). With the Sephadex G-100 enzyme, the reaction with GTP and fructose in the absence and presence of 2mM-ADP was linear with time (Fig. 43). This confirmed the absence of myokinase from the enzyme preparation.

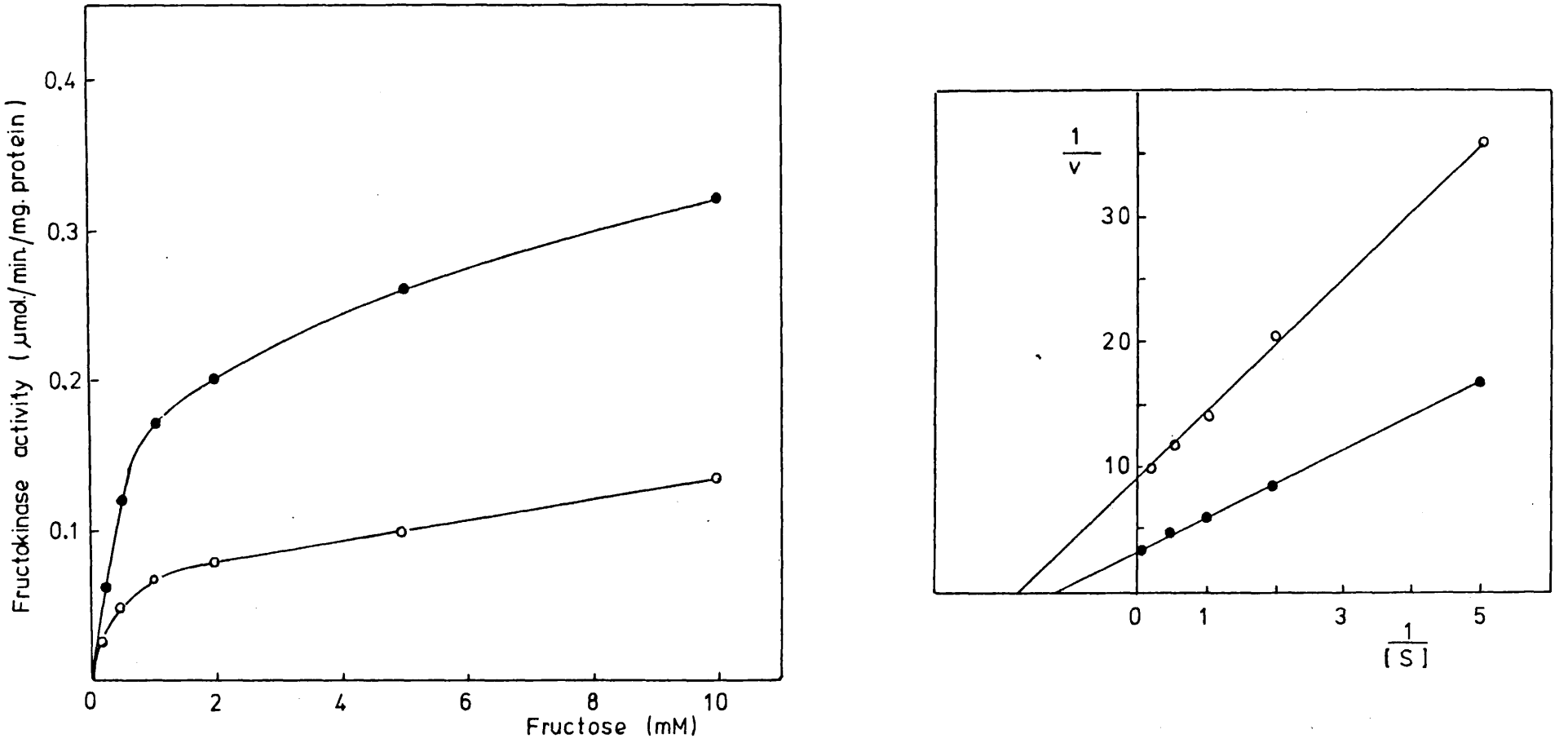


Fig. 40. The effect of fructose concentration on ATP (●—●) and GTP (o—o) dependent fructokinase activity.

Incubations were with the DEAE-cellulose fraction and 6mM-MgATP or 6mM-MgGTP for 30 min at 37°C as detailed in Section II.B.6.e.ii.

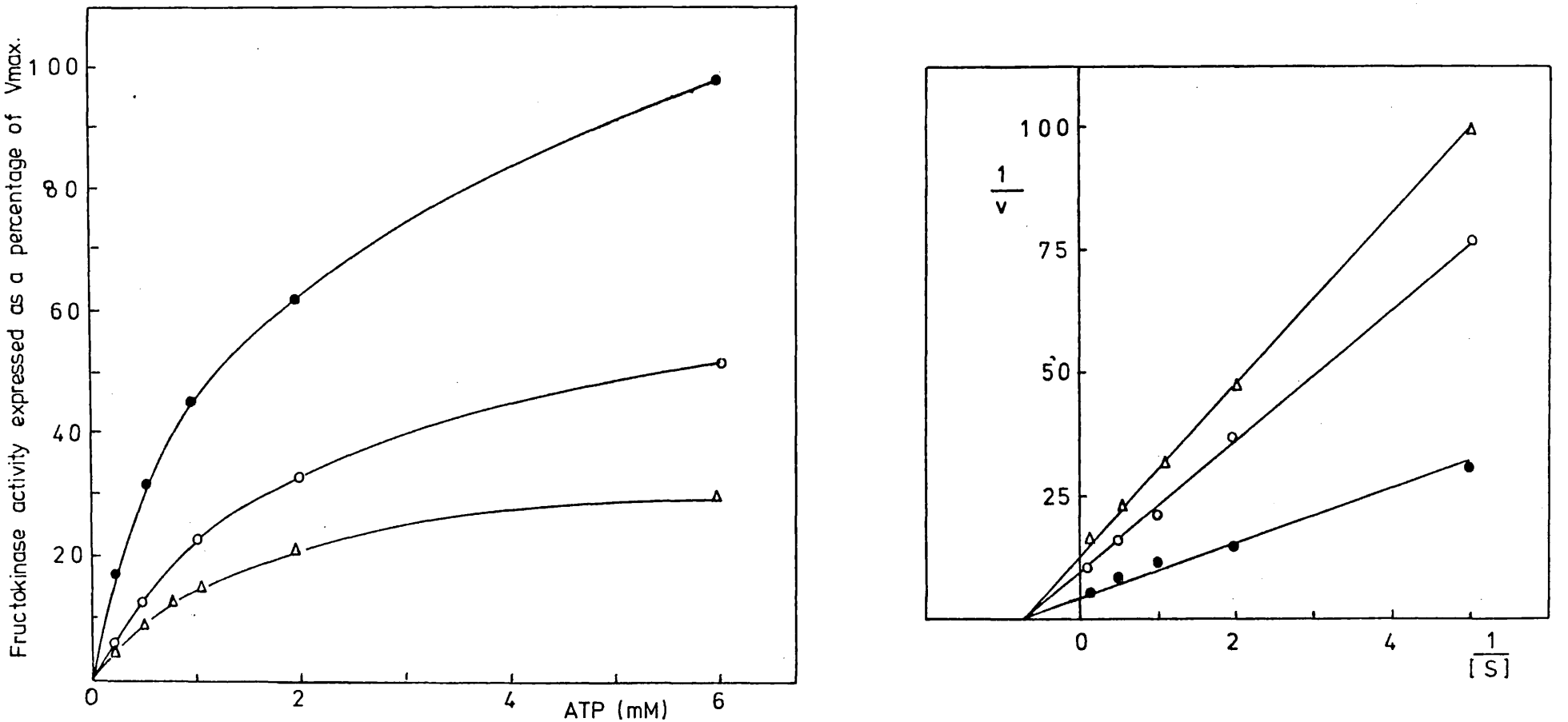


Fig. 41. The effect of ADP on ATP-dependent fructokinase activity.

The DEAE-cellulose fraction was incubated with 2mM-[U-^{14}C] fructose and MgATP, in the absence of ADP (●—●), and in the presence of 1mM (o—o) or 2mM - MgADP (△—△) for 30 min at 37°C as detailed in section II.B.6.e.ii.

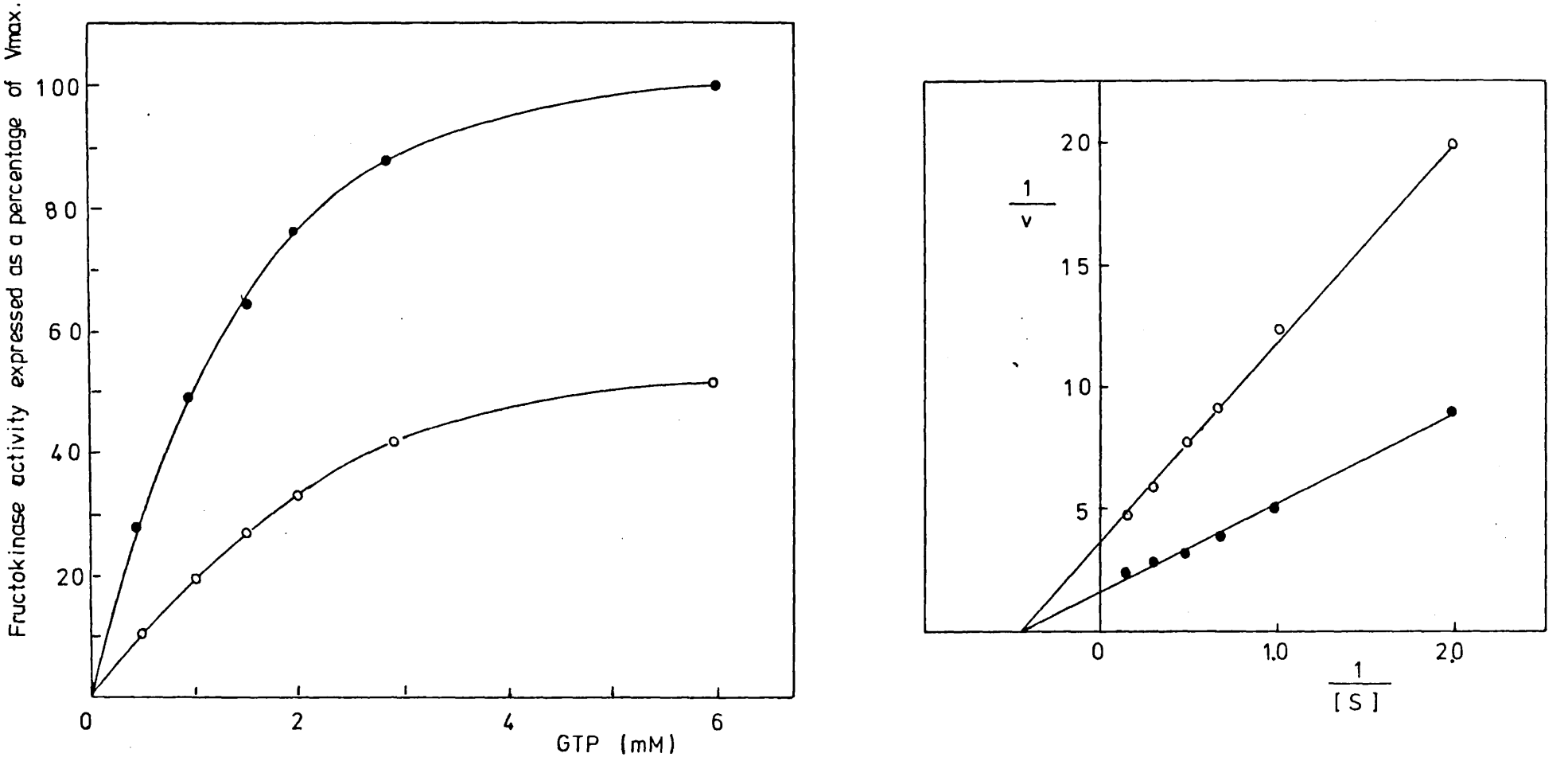


Fig. 42. The effect of ADP on GTP-dependent fructokinase activity.

The Sephadex G-100 fraction was incubated with 2mM-[U-^{14}C] fructose and MgGTP in the absence of ADP (●—●) and in the presence of 2mM-MgADP (o—o) for 20 min at 37°C as detailed in Section II.B.6.e.ii.

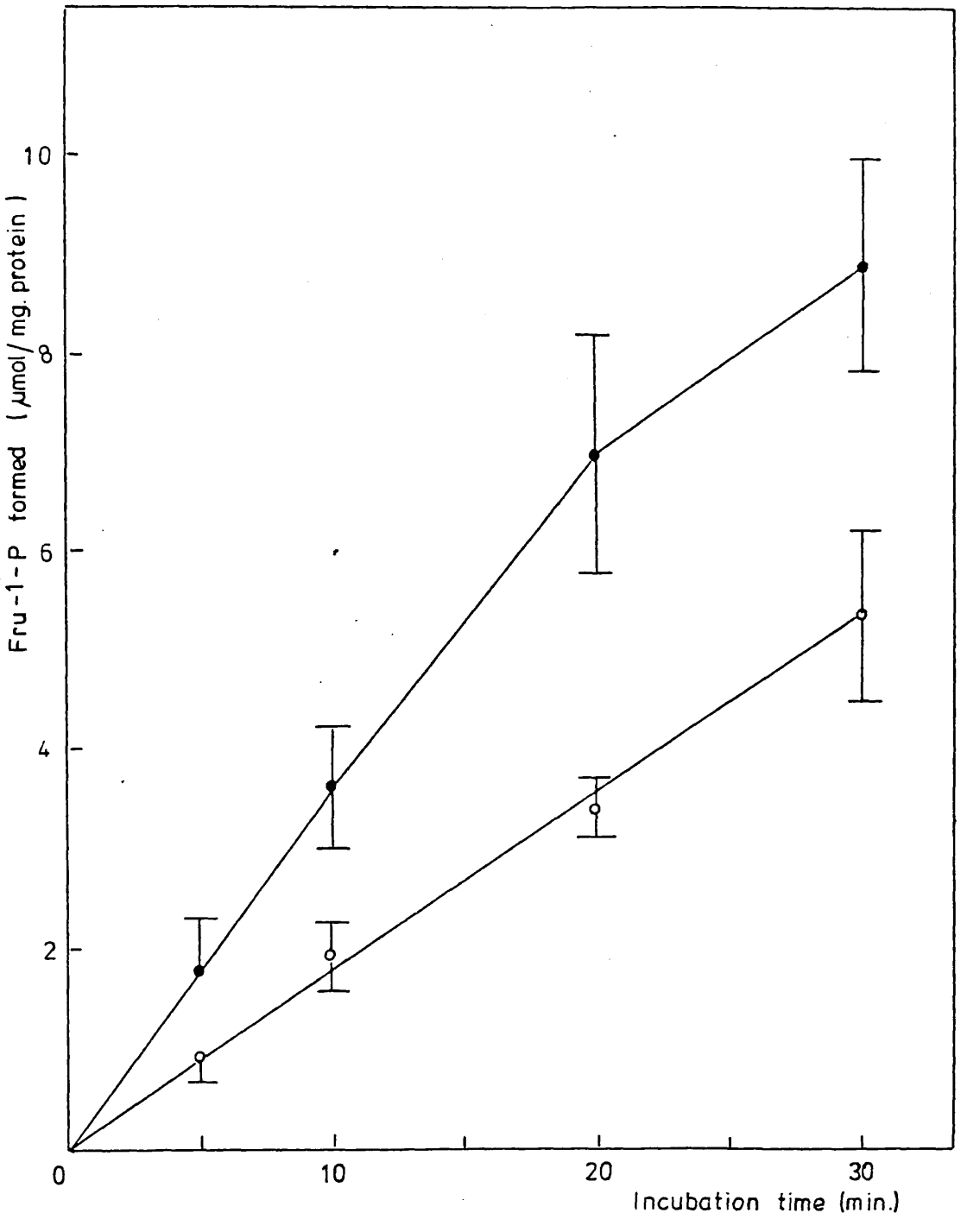


Fig. 43. Time-course of GTP-dependent Fru-1-P formation in the absence (●—●) and presence of 2mM-MgADP (o—o).

The Sephadex G-100 fraction was incubated with 6mM-MgGTP and 2mM-[U-^{14}C] fructose for 20 min at 37^{o}C as detailed in Section II.B.6.e.ii.

The reaction with GTP was also shown to be inhibited by ADP (Fig. 43). Both the ATP and GTP reactions were non-competitively inhibited by ADP as shown in Figs. 41 and 42. ADP inhibition constants of 0.55mM and 1.30mM were obtained from Fig. 44 for the GTP and ATP-dependent reactions respectively.

In the absence of ADP the GTP-dependent reaction was inhibited after 20 min incubation as shown in Fig. 43. The most likely cause was accumulation of GDP and hence the effect of GDP concentration on the ATP and GTP reactions was studied. GDP, like ADP, was found to inhibit both reactions and a common inhibition constant of 1.20mM was obtained (Fig. 45). Similar concentrations of other nucleotide diphosphates; CDP and UDP, were without effect on enzyme activity.

Sánchez et al. (1971b) have shown that K^+ partially overcomes ADP inhibition of the enzyme. This has led them to propose two binding sites on the enzyme; one for K^+ and fructose, the other for ATP and ADP. This would be the minimum number of sites possible if GTP and GDP bind to the same site as ATP and ADP. The results obtained in the present study have shown ADP to be a non-competitive inhibitor with respect to ATP. This suggests that ATP and ADP bind to different sites on the enzyme. Since the GTP reaction was similarily inhibited by ADP, and both reactions were inhibited by GDP, a minimum of three sites must exist - with separate sites for the nucleotide triphosphates and diphosphates.

Since ATP and GTP are present in the cytosol at

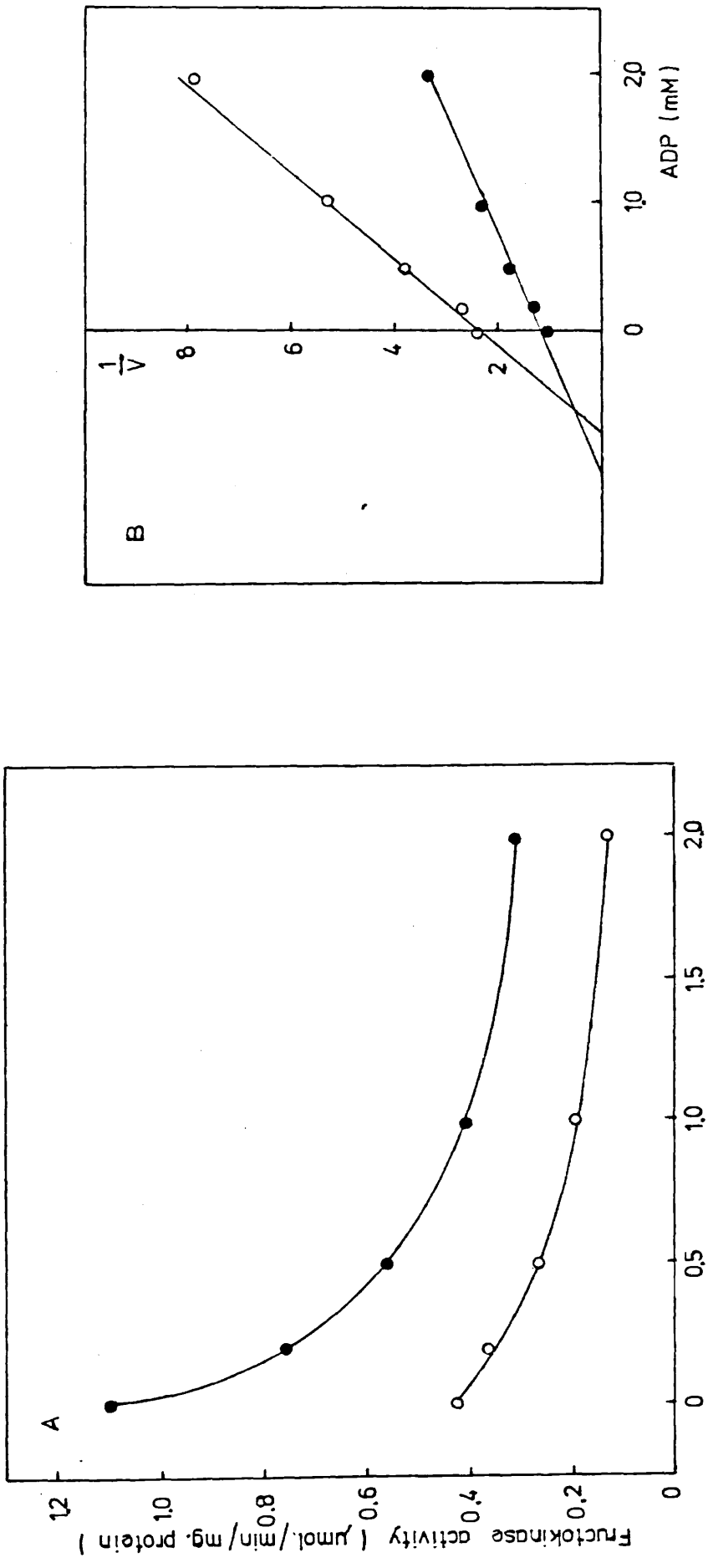


Fig. 44. A, The effect of ADP concentration on ATP (●—●) and GTP (o—o) dependent fructokinase activity and B, Dixon plots of data.

The Sephadex G-100 fraction was incubated with 2mM-[U-^{14}C]fructose and 6mM-MgATP or 6mM - MgGTP for 20 min at 37^{o}C as detailed in Section II,B.6.e.ii.

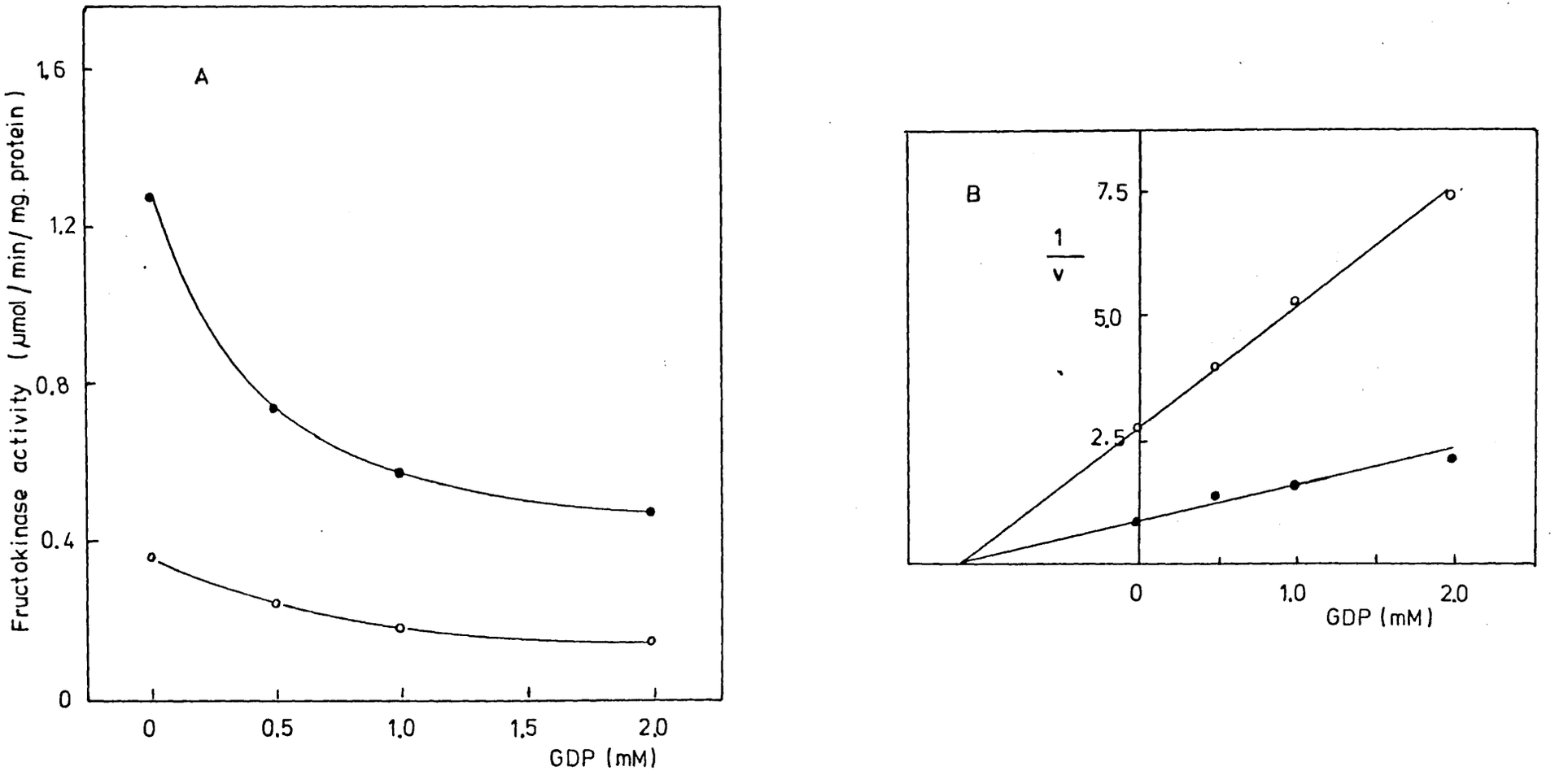


Fig. 45.A, The effect of GDP concentration on ATP (●—●) and GTP (o—o) dependent fructokinase activity and B, Dixon plots of data.

The Sephadex G-100 fraction was incubated with 2mM-[U-^{14}C] fructose and 6mM-MgATP or 6mM-MgGTP for 20 min at 37^{o}C as detailed in Section II.B.6.e.ii.

physiological concentrations of approximately 3mM and 1mM respectively it was of interest to examine the interaction of the two nucleotides with respect to fructokinase activity at these concentrations. Fig. 46 shows the activity with GTP alone (K_m 1.40mM), ATP only (K_m 1.43mM) and the activity when ATP concentrations are varied in the presence of 1mM-GTP (K_m 0.45mM). The data confirms the earlier observation that the enzyme has a similar affinity for GTP and ATP. When the two nucleotides were combined the activity found was greater than that predicted suggesting that there may be a positive co-operative interaction between the two nucleotides. However, confirmation of the latter hypothesis will require further study with a purified enzyme preparation.

4. Physiological significance of GTP utilisation

As discussed in Section I.B.1.b hepatic GTP levels have repeatedly been shown to fall following a fructose load. The failure of previous workers to demonstrate any fructokinase activity in the presence of GTP (Parks et al., 1957; Adelman et al., 1967a) led Frandsen and Grunnet (1971) to propose that GTP concentrations fall as a consequence of its utilisation in the triokinase reaction. However, triokinase activity with GTP is only 10% of that obtained with ATP as nucleotide triphosphate donor and it therefore seems likely that some other enzyme reaction is utilising GTP. This proposal was supported when GTP was shown to be a phosphate donor in the fructokinase reaction. Furthermore, from the kinetic data obtained (summarised in Table 13) the enzyme

Fig. 46. Interaction of GTP and ATP with fructokinase

The DEAE-cellulose fraction was incubated for 30 min at 37°C with 2mM - [U-^{14}C] fructose and varying concentrations of (A) MgATP (●—●) or MgGTP (o—o) and (B) MgATP with 1mM-MgGTP (▲—▲) as detailed in Section II.B.6.e.ii. The calculated activity of the latter was obtained by adding the activity obtained with 1mM - MgGTP to the individual results with MgATP (△—△).

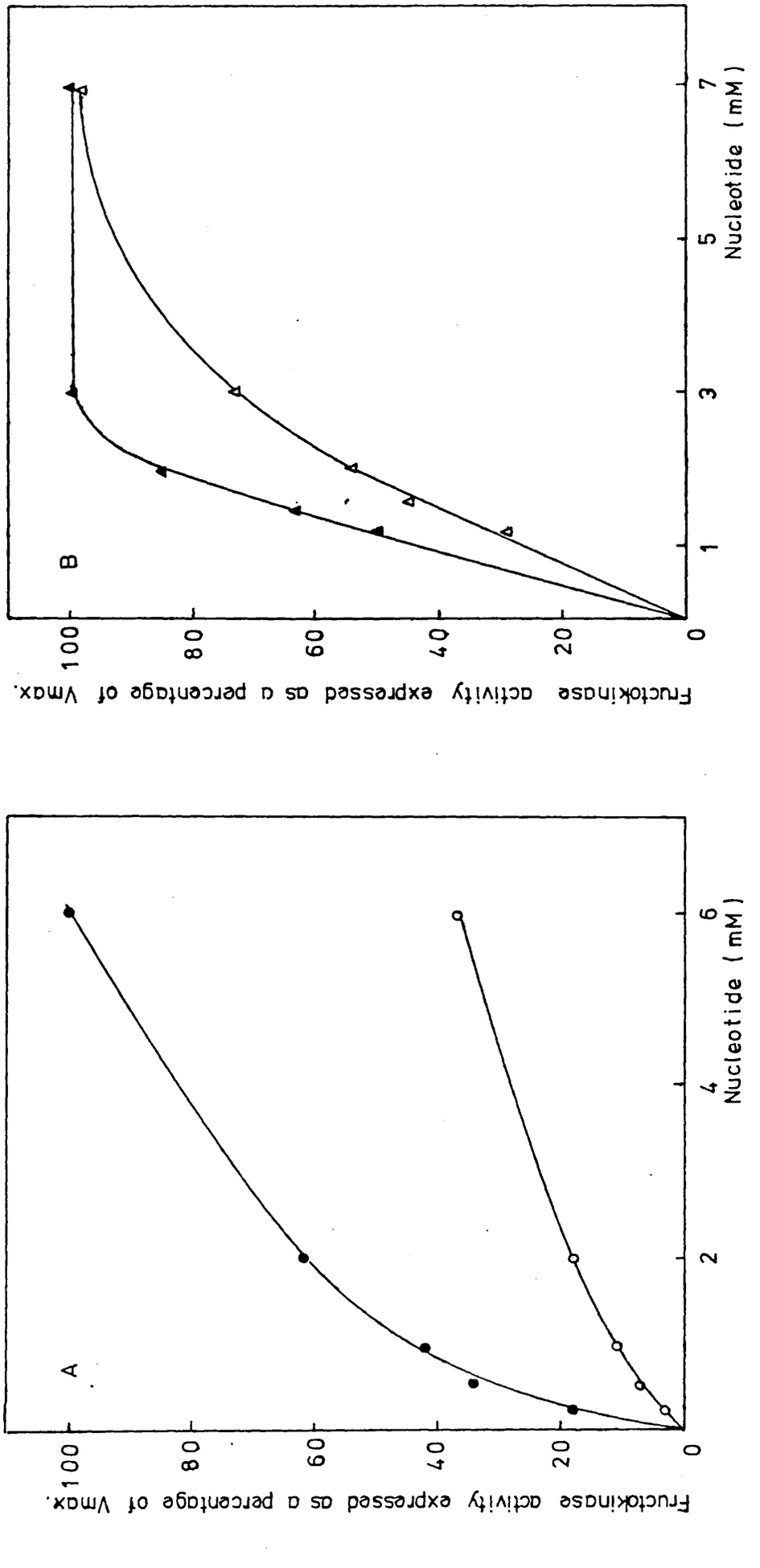


Fig. 46. Interaction of GTP and ATP with fructokinase.

Table 13. Kinetics of purified rat liver fructokinase.

Purification details are as in the text. All values are mM.

Constants	Nucleotide triphosphate	
	ATP	GTP
K_m nucleotide	1.43	1.40-1.65
K_m fructose	0.83	0.56
K_i ADP	1.30	0.55
K_i GDP	1.20	1.20

would appear to have a similar affinity for GTP. Whether GTP is utilised *in vivo* as a phosphate donor in the fructokinase reaction is hard to assess in view of the different physiological concentrations of ATP, 2-3mM (Woods *et al.*, 1970; Van den Berghe *et al.*, 1977a) and GTP, 0.5 - 1.0mM (Van den Berghe *et al.*, 1977a, 1980; Söling, 1982) and the effects on each induced by a fructose load.

ATP would be expected to be the preferred substrate for fructokinase. However, ATP levels have been shown to fall dramatically from 2.4µmol/g liver to less than 1µmol/g liver within 2 min of a fructose load to mice (Van den Berghe *et al.*, 1977a) and are less than 0.5µmol/g liver after a 10 min fructose perfusion of rat liver (Woods *et al.*, 1970). Van den Berghe *et al.* (1977a) reported that GTP concentrations

fall by only 30%, compared to 60% for ATP, within 2 min of fructose loading. These results suggest that ATP is initially used as phosphate donor in the fructokinase reaction, however, as the ATP concentration falls below its K_m the enzyme may switch to using GTP as the major source of phosphate. The GDP concentration, reported by Söling (1982) as 0.04 µmol/g liver (0.08mM), is increased following a fructose load maintaining the sum of guanine nucleotides (GTP + GDP) unchanged (Van den Berghe et al., 1977a). Since GTP levels are already less than 1mM, the GDP concentration will never increase enough to have a significant inhibitory effect on the fructokinase reaction. Hepatic ADP concentrations have been shown to remain fairly constant, at 0.7mM, during the first 10 min of a fructose load (Woods et al., 1970). This ADP concentration is below the K_i for the ATP reaction but well above that for the GTP reaction which would therefore initially be inhibited. However, as the ADP concentration falls (0.2mM after 40 min) the inhibition will be released and GTP more readily used.

From the work of Van den Berghe et al. (1977a) initial rates of ATP and GTP utilisation were calculated as 1.2 and 0.15µmol/min/g liver respectively so that whilst the utilisation of GTP is significant it is nonetheless considerably less (only 13%) than that of ATP. As discussed earlier GTP may also interact with ATP at the binding site and stimulate enzyme activity. However, as the enzyme preparation used for this latter study was only partially purified the effects of contamination by myokinase, nucleoside diphosphate kinase or nucleoside monophosphate kinases cannot be discounted.

D. Regulation of the Fru-6-P/Fru-1,6-P_2 substrate cycle in relation to fructose metabolism

As discussed earlier (Section I.C) PFK and Fru-1,6-P_2ase activities regulate the Fru-6-P/Fru-1,6-P_2 substrate cycle and thereby direct carbon in the direction of glycolysis or gluconeogenesis. Fructose carbon is metabolised via triose phosphate to either lactate or glucose and under these conditions PFK would be expected to be inhibited and Fru-1,6-P_2ase activated. The effects of various fructose loads on hepatic PFK and Fru-1,6-P_2ase activities were studied using isolated hepatocytes. Fru-2,6-P_2, a major regulator of glycolysis and gluconeogenesis had not been identified at the time this work was carried out.

1. Studies on Fru-1,6-P_2ase

a. Effect of substrate concentration

Hepatocyte Fru-1,6-P_2ase was shown to be inhibited by Fru-1,6-P_2 concentrations above 0.1mM, with up to 16% inhibition of enzyme activity occurring in the presence of 0.2mM-Fru-1,6-P_2; a K_m value for Fru-1,6-P_2 of 4.2μM was obtained (Fig. 47). These results were consistent with those obtained by Taketa and Pogell (1965), using a partially purified enzyme preparation and, more recently, by Van Schaftingen and Hers (1981) with a crude liver extract. Since the physiological concentration of Fru-1,6-P_2 is between 0.03 and 0.07μmol/g, under normal conditions Fru-1,6-P_2 would not be expected to cause inhibition of enzyme activity. Van Schaftingen and Hers (1981) showed that the hyperbolic substrate saturation curve and low K_m previously obtained

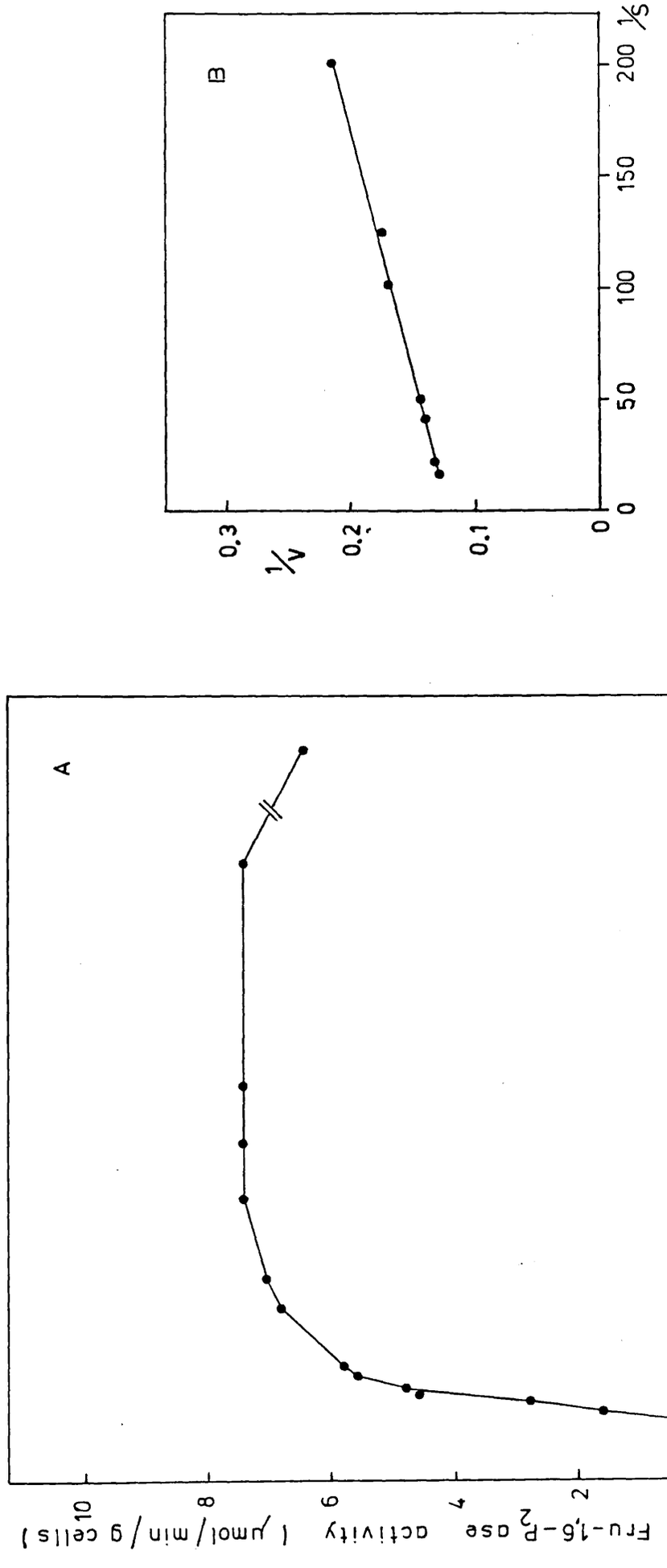


Fig. 47. A, Effect of substrate concentration on Fru-1,6-P_2ase and B, Lineweaver-Burke plot of data.

Fru-1,6-P2ase was extracted from hepatocytes as detailed in Section II.B.2.a and enzyme activity assayed as described in Section II.B.6.a.

could be attributed to the absence of both Fru-2,6-P_2 and AMP from the assay medium. Physiological concentrations of Fru-2,6-P_2 change the saturation curve to a sigmoidal form and thus Fru-1,6-P_2ase is proposed to have very low activity in vitro. In the absence of Fru-2,6-P_2 or when Fru-2,6-P_2 concentrations are depleted, following administration of glucagon or during starvation, the curve was shown to return to the hyperbolic form with a correspondingly lower K_m, and under these conditions Fru-1,6-P_2ase activity will be stimulated by low concentrations of Fru-1,6-P_2.

b. Effect of fructose

Fru-1,6-P_2ase activity, assayed at 0.06mM and 0.20mM-Fru-1,6-P_2, was unchanged following incubation of isolated hepatocytes with 2mM or 10mM-fructose for up to 60 min (data not shown). Incubations with similar concentrations of glucose were also without effect on enzyme activity. As described above, in the absence of Fru-2,6-P_2 and AMP, Fru-1,6-P_2ase is fully active and therefore in order to study the effect of positive effectors it is necessary to assay the enzyme activity under suboptimal conditions - in the presence of AMP and Fru-2,6-P_2. The regulation of Fru-1,6-P_2ase by Fru-2,6-P_2 had not been discovered at this stage but the lack of a fructose effect on enzyme activity may be attributed to a number of different factors. Firstly, the assay conditions used would not be conducive to showing inhibition by Fru-2,6-P_2 and furthermore the enzyme, and thus the intracellular metabolites, had to be diluted considerably in order to determine its activity.

2. Studies on PFK

Hepatic PFK was assayed routinely at two concentrations of Fru-6-P, 0.2mM and 4.0mM, in order to obtain enzyme activities at suboptimal and optimal conditions (Section II. B.6.b). However, Reinhart and Lardy (1980a) have shown that when Fru-1,6-P_2, AMP and P_i are absent from the assay medium there is a very high K_m for Fru-6-P indicating that under certain conditions 4mM-substrate may not be sufficient to measure V_{max}. Since the effects obtained with 0.2mM-substrate were identical to those obtained with 4mM-Fru-6-P all results quoted are for the latter concentration.

a. Effect of fructose

Enzyme activity was not significantly changed following incubation of isolated hepatocytes without added substrates (results not shown) or with 2mM-glucose (Fig. 48) for up to 30 min. However, incubation with 2mM-fructose resulted in a 2-fold increase in PFK activity over basal levels (Fig. 48). Since maximum stimulation was obtained by 20 min, this incubation time was chosen for future experiments. Fig. 49 shows the concentration-dependency of the fructose-induced activation of PFK activity. Maximum activation occurred following incubation with 2mM-fructose; higher fructose concentrations stimulated the enzyme to a lesser extent. Glucose, at concentrations up to 8mM, was shown to be without significant effect on enzyme activity.

Incubation of hepatocytes with low concentrations of fructose (1-10mM) has been reported to cause an accumulation of Fru-1-P and Fru-1,6-P_2, a depletion of ATP and an activation

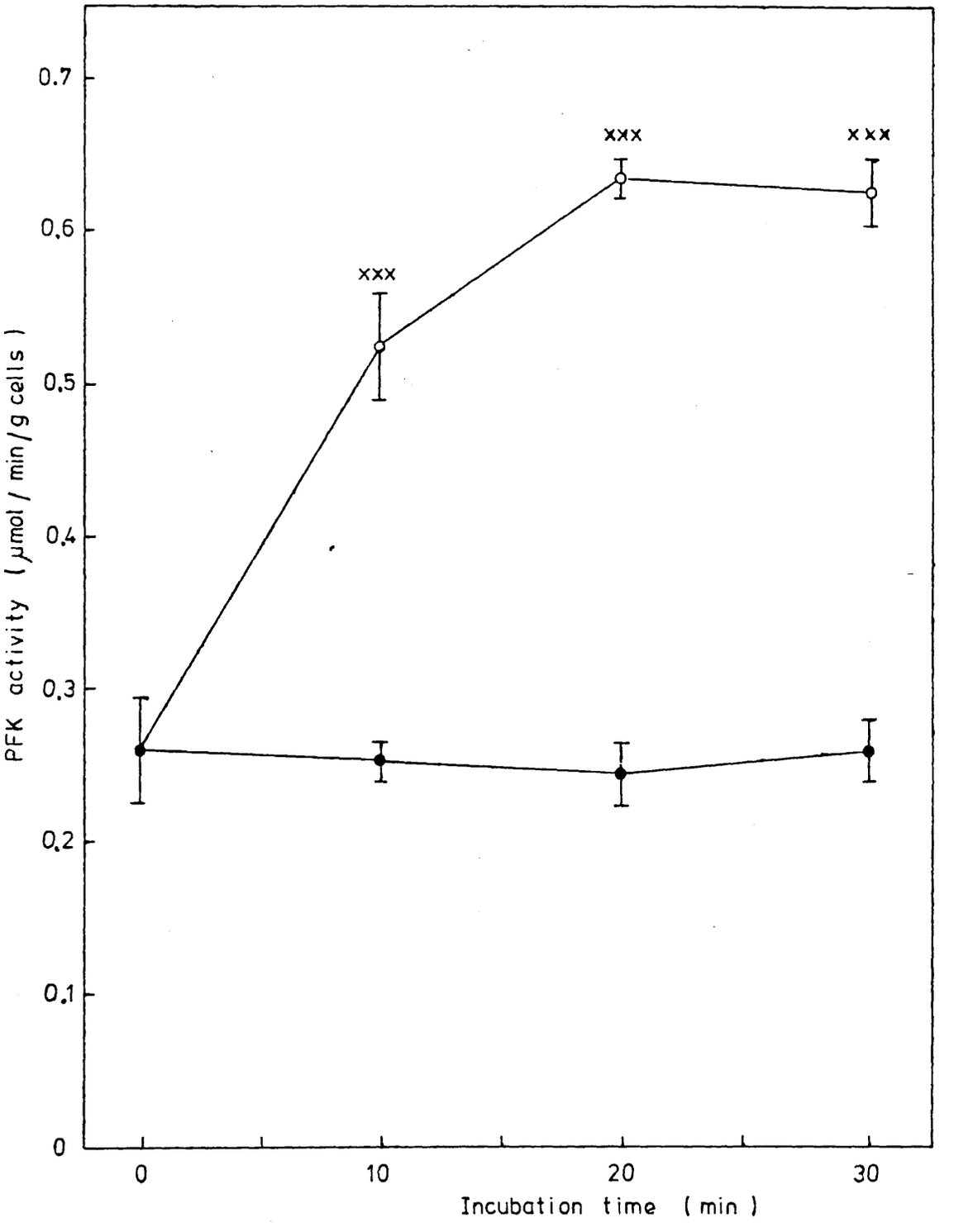


Fig. 48. Time course of the effect of incubating hepatocytes with 2mM-fructose (o-o) or 2mM-glucose (●-●) on PFK activity.

Isolated hepatocytes (74mg/ml) were incubated, with glucose or fructose, and reactions stopped by homogenisation at 4^0C as detailed in Section II. B.2.a. PFK activity was assayed with 4mM-Fru-6-P and 1mM-ATP (Section II. B.6.b). The data plotted are a mean of results from 3 different cell preparations with s.e.m. as shown by the bars. Statistical significance was determined as described in Section II.B.12, with probability values represented by x = < 0.05, xx = < 0.01, or xxx = < 0.001.

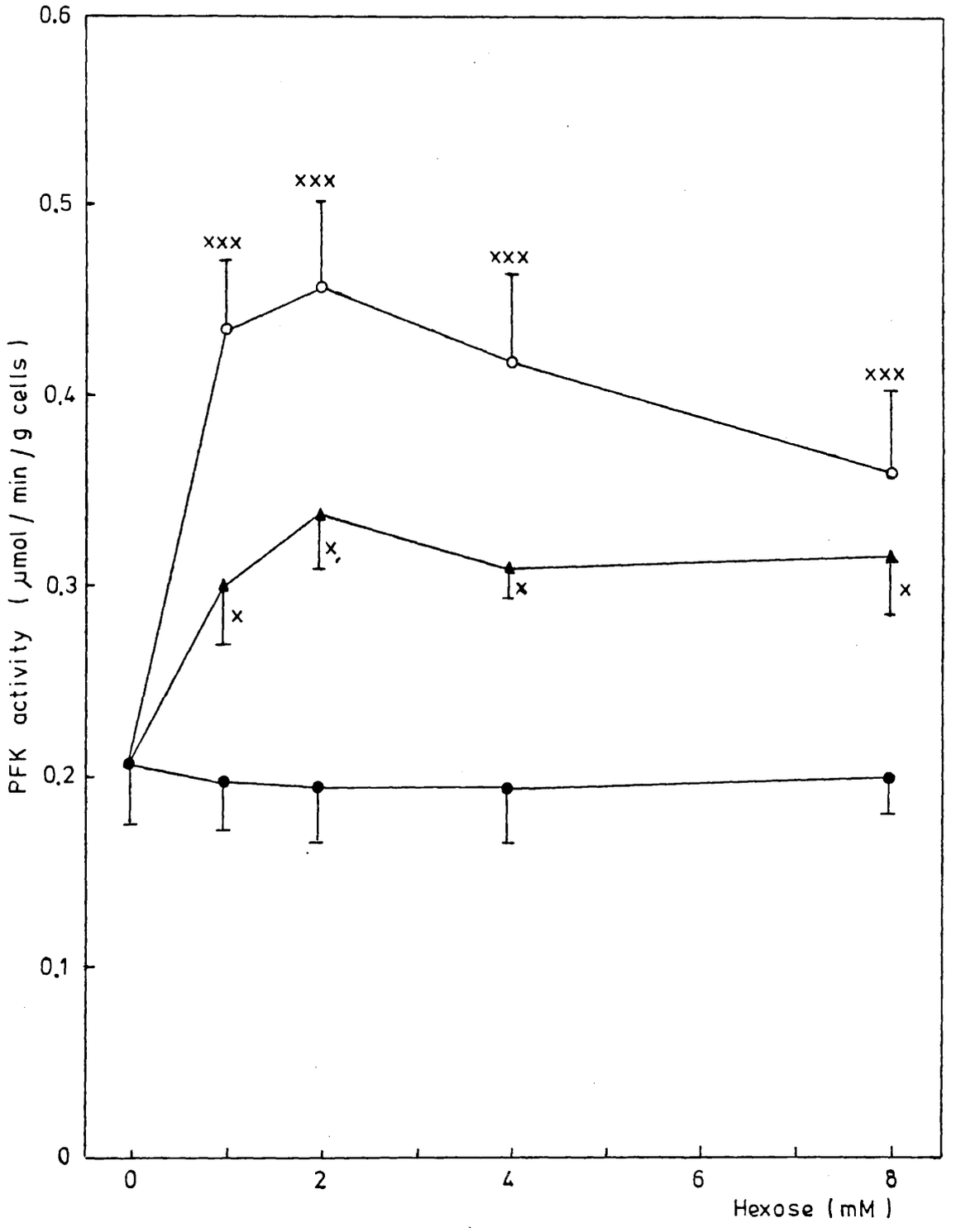


Fig. 49. Effect of incubation of hepatocytes with glucose, fructose or galactose on PFK activity.

Isolated hepatocytes were incubated with glucose (●-●), fructose (o-o) or galactose (▲-▲) for 20 min. For other experimental details see Fig. 48. Results plotted are mean of 7 different cell preparations for glucose and fructose and of 3 preparations for galactose. The statistical analysis is as described in Fig. 48. The mean hepatocyte concentration was 70mg/ml for the glucose and fructose-incubated cells and 67 mg/ml for the galactose.

of pyruvate kinase (Mapungwana, 1982). Fru-1-P and Fru-1,6-P_2 have both been shown to activate pyruvate kinase *in vitro* (Mapungwana, 1982). However, studies on the effect of Fru-1-P on PFK activity *in vitro* revealed that Fru-1-P, at concentrations up to 8mM, inhibited hepatic enzyme activity determined at 4mM-Fru-6-P (results not shown). Under these conditions 4mM-Fru-1-P inhibited PFK activity by 70%. This effect of Fru-1-P on hepatic PFK had previously been reported for the yeast enzyme by Kreuzberg (1978) although he additionally showed that low concentrations of Fru-1-P (< 1mM) were activating in contrast to the results obtained in the present study. Fru-1,6-P_2 is unlikely to be responsible for the enzyme activation since, being the product of the reaction, it would be utilised in the preincubation step, by the coupling enzymes in the assay mixture, prior to the addition of Fru-6-P and the determination of PFK activity. Since activation of PFK was obtained despite a preincubation step, the activator cannot be Fru-1,6-P_2.

ATP, as well as being a substrate, is also an inhibitor of PFK activity, so the depletion of ATP as a result of fructose metabolism could be responsible for the observed activation. However, PFK activity was stimulated most at low fructose concentrations whilst ATP levels are depleted to a greater extent by high concentrations of the sugar. Also, since the concentration of ATP in the assay medium is 1mM, a decrease in ATP concentration in the cell from 3µmol/g to 1µmol/g (Woods *et al.*, 1970) would result in a change of less than 0.03mM in the ATP concentration in the assay. Therefore, a fall in ATP concentration is unlikely to have an effect on PFK activity

which would be observed under the assay conditions used here.

Following the completion of this section of the work Van Schaftingen et al. (1981b) have found that Fru-2,6-P_2 is a potent activator of PFK and Hue et al. (1982), have briefly reported that fructose at 2.5mM increases Fru-2,6-P_2 concentrations 2-fold in hepatocytes from fed rats, whereas 5mM-fructose was without effect. Thus Fru-2,6-P_2 accumulation, at low fructose concentrations, could be responsible for the stimulation reported here.

High glucose (20-50mM) concentrations have been reported to cause a 2-fold increase in hepatic Fru-2,6-P_2 concentrations (Hue et al., 1982) and to stimulate PFK activity (Van Schaftingen et al., 1980a,b). In support of these findings incubation of isolated hepatocytes with 20mM-glucose was shown to increase PFK activity by 50%. Incubation with 20mM-fructose resulted in an inhibition (26%) of PFK (Table 14). These effects can be attributed to the change in hepatocyte Fru-2, 6-P_2 levels reported by Hue et al. (1982).

At high fructose concentrations inhibition of the enzyme by Fru-1-P, whose levels rapidly increase following a large fructose load, will also occur. The effects of 1mM and 20mM-glucose or fructose on the substrate saturation curve for PFK are shown in Figs. 50 and 51 with v/V_{max} values as represented in Table 14.

In order to investigate whether or not the fructose-induced activation of PFK was due to a stable conformational change or to changes in the level of an effector, hepatocyte extracts were desalted on a Sephadex G-25 column and the

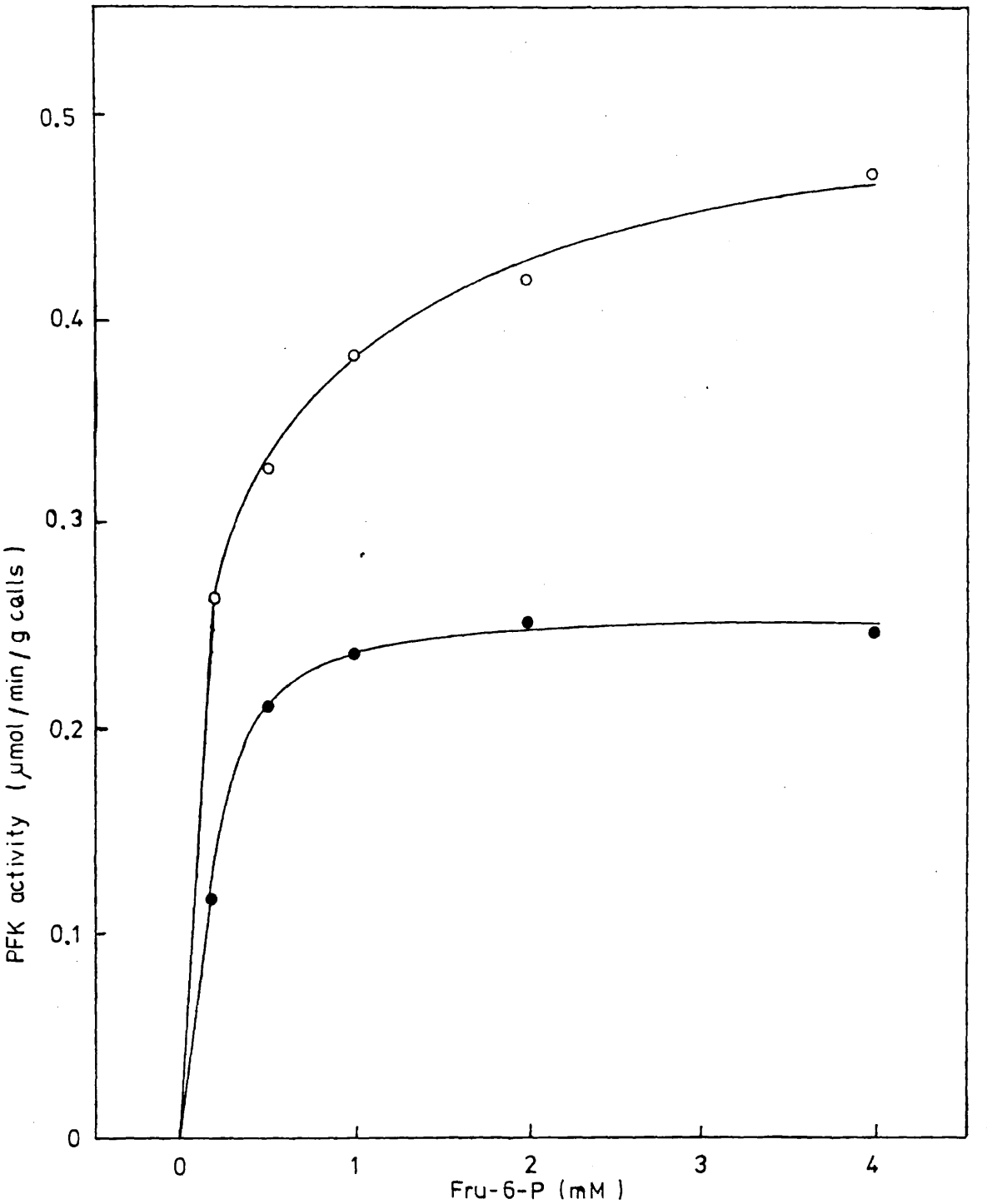


Fig. 50. Effect of Fru-6-P concentration on PFK activity following incubation of hepatocytes with 1mM-glucose (●-●) or 1mM-fructose (o-o) for 20 min.

For full experimental details see Fig. 48.

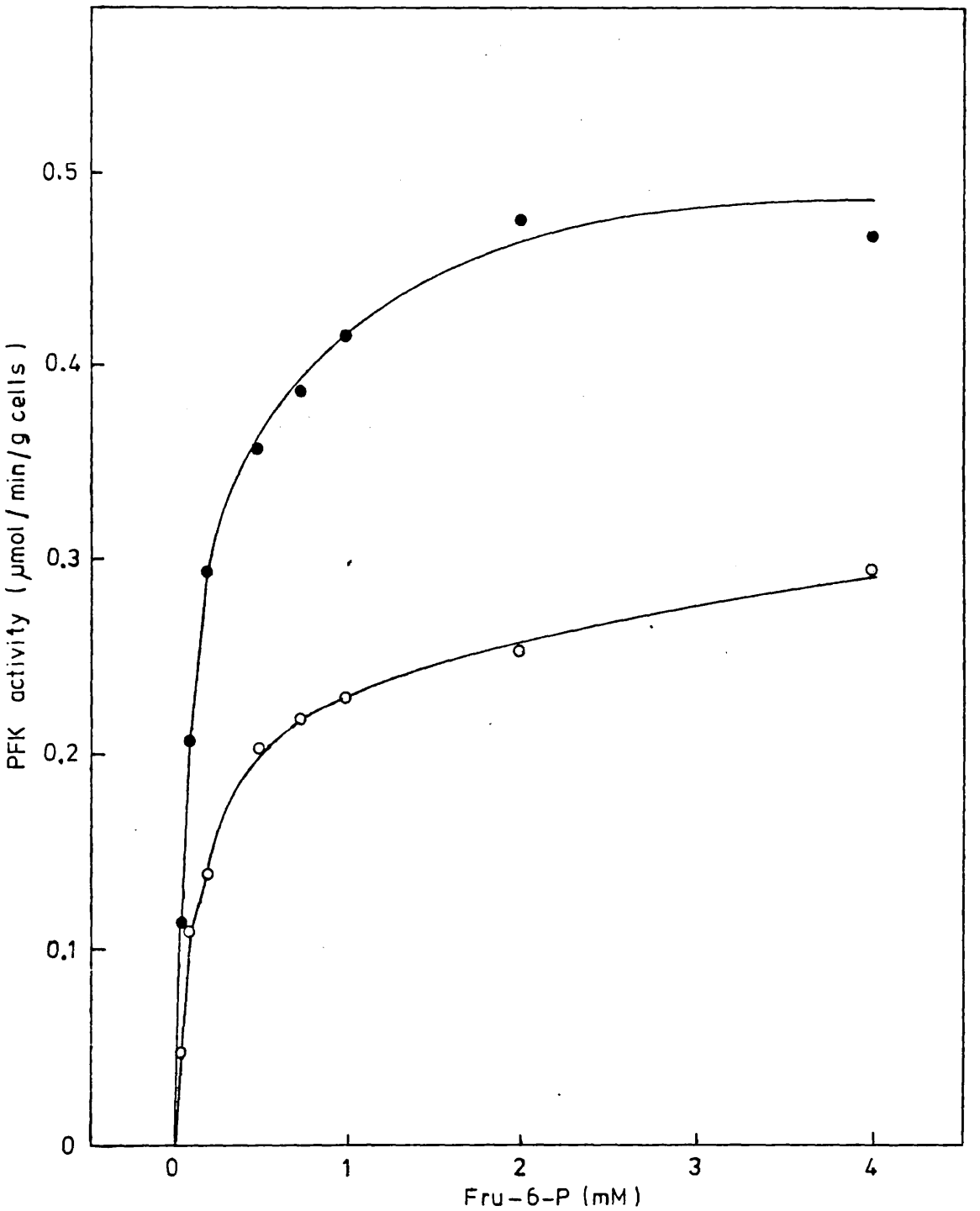


Fig. 51. Effect of Fru-6-P concentration on PFK activity following incubation of hepatocytes with 20mM-glucose (●-●) or 20mM-fructose (o-o) for 20 min.

For full experimental details see Fig. 48.

Table 14. PFK activity in isolated hepatocytes.

For full experimental details see Figs. 50 and 51. The ratio of suboptimal enzyme activity, v, determined at 0.2mM-Fru-6-P, to maximum enzyme activity, V_{max}, was calculated in each instance and is reported as v/V_{max}.

Addition	PFK activity v/V_{max}
None	0.443
Fructose, 20mM	0.328
Fructose, 1mM	0.559
Glucose, 20mM	0.670
Glucose, 1mM	0.248

enzyme eluted with buffer (50mM-Tris/HCl, pH 7.6, 2.5mM-DTT and 0.1mM-EDTA). As the results show (Fig. 52) the activation caused by fructose was lost on passing the extract down a Sephadex G-25 column, thereby suggesting that a low molecular-weight effector was causing the observed activation. However, the activity in the glucose-treated cells increased on passing the extract through the column, thus suggesting that an inhibitor of PFK is also present in these cells. Since both positive and negative effectors of enzyme activity are removed by gel-filtration, the fact that glucose- and fructose-treated cells have the same enzyme activity after such treatment suggests that no stable conformational change has occurred.

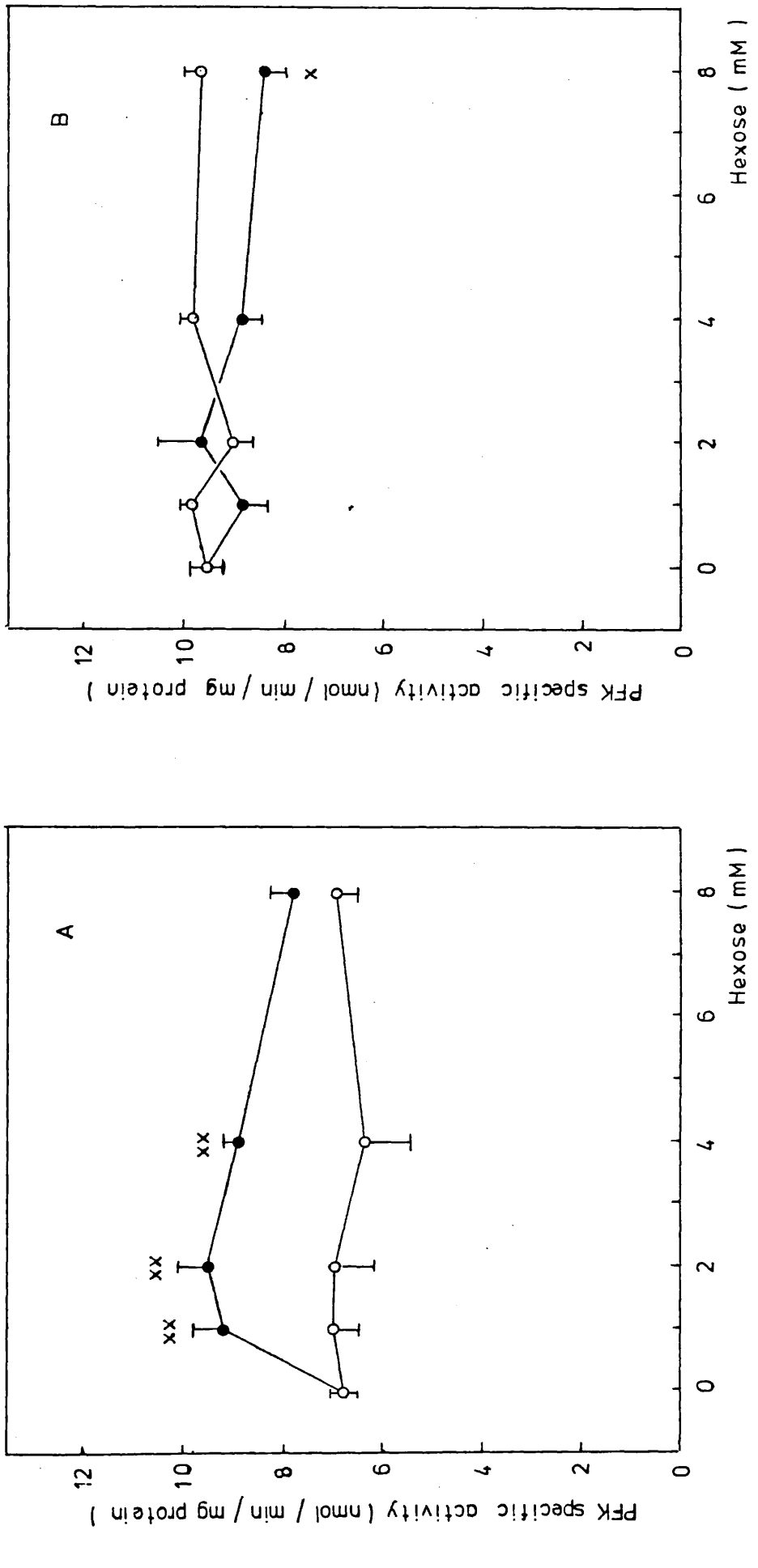


Fig. 52. The effect of Sephadex G-25 gel-filtration of the hepatocyte extracts on the fructose-induced stimulation of PFK.

Hepatocytes (66mg/ml) were incubated with glucose (o-o) or fructose (●-●) for 20 min as described in Section II.B.2.a. Extracts were assayed for PFK activity, with 4mM-Fru-6-P and 1mM-ATP, and protein (A) before and (B) after Sephadex G-25 chromatography. Results are means from 3 different cell preparations with s.e.m. as shown by the bars and significance levels as described in Fig. 48.

In summary, low concentrations of fructose result in stimulation of PFK activity which can probably be attributed to Fru-2,6-P_2, whereas high concentrations of fructose (> 10mM) cause an inhibition of enzyme activity through decreasing Fru-2,6-P_2 and increasing Fru-1-P concentrations.

b. Effects of galactose, dihydroxyacetone and glycerol.

Hepatocytes were incubated for 20 min in the presence of 1-8mM-galactose (Fig. 49), 1-8mM-dihydroxyacetone or 2-8mM-glycerol (Fig. 53). In each case parallel incubations were carried out with up to 8mM-glucose. PFK activity was significantly increased following incubation with either dihydroxyacetone or galactose whereas glycerol was without effect.

Galactose enters the glycolytic pathway via Glc-6-P and thereby bypasses the first regulatory step of glycolysis, between glucose and Glc-6-P. The hexose phosphate pool could well be increased following galactose perfusion and this in turn would result in the increased synthesis of Fru-2,6-P_2 resulting in a stimulation of PFK activity. However, Hue et al.(1982) failed to show any significant effects on Fru-2,6-P_2 concentration following incubation of hepatocytes with 20mM-galactose.

Glycerol and dihydroxyacetone are both rapidly phosphorylated in the perfused liver (Woods and Krebs, 1973), isolated hepatocytes (Mapungwana, 1982) and following intraperitoneal injection (Burch et al., 1970). In isolated hepatocytes glycerol, like fructose, causes a rapid depletion

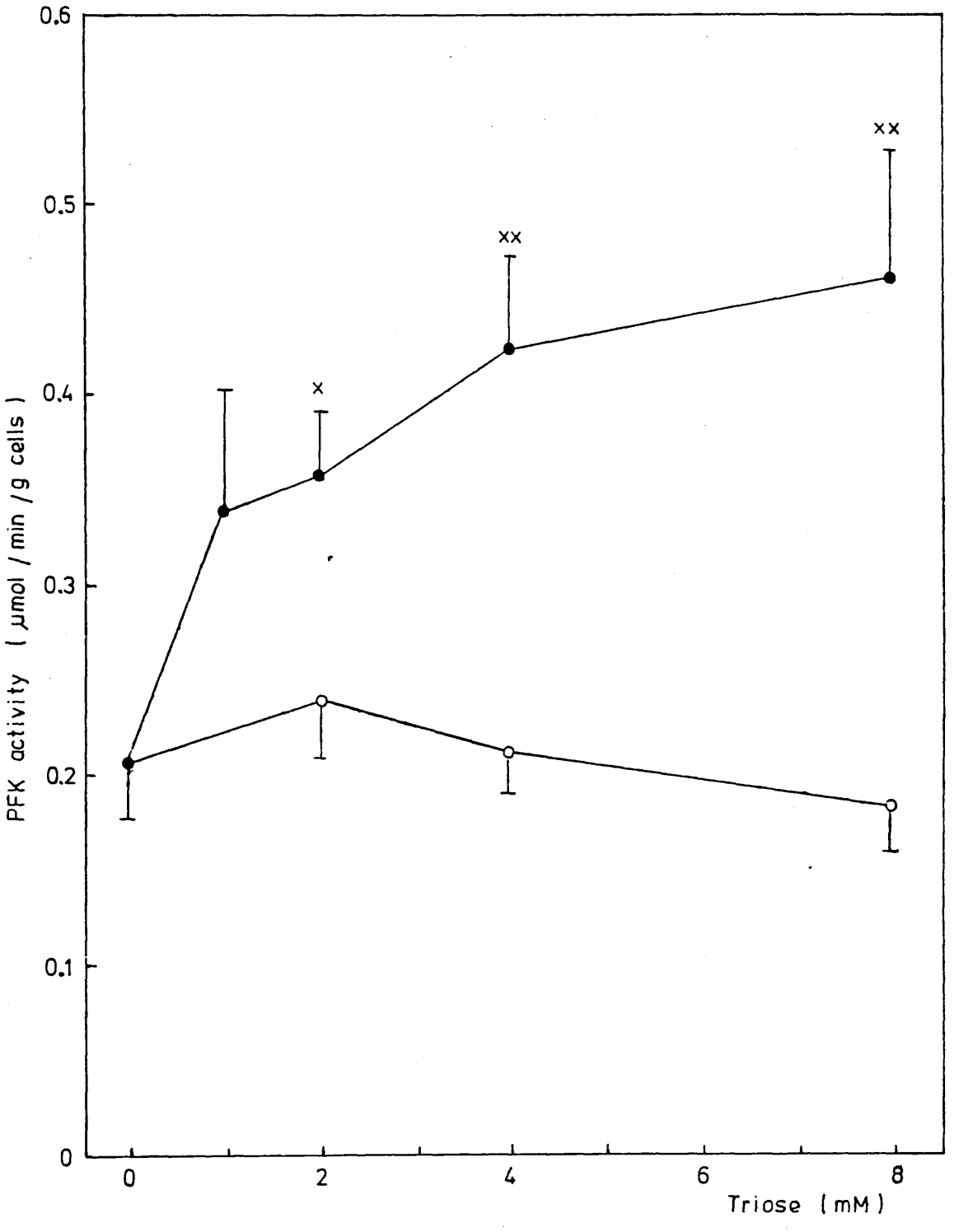


Fig. 53. Effect of incubation of hepatocytes with glycerol of dihydroxyacetone on PFK activity.

Isolated hepatocytes (70mg/ml) were incubated with glycerol (o-o) or dihydroxyacetone (●-●) for 20 min. Other experimental details are as detailed in Fig. 48.

of adenine nucleotides and the accumulation of a phosphorylated intermediate, in this case glycerol-3-phosphate (Mapungwana, 1982; Des Rosiers et al., 1982). The lactate / pyruvate ratio is significantly increased following liver perfusion with 10mM-glycerol due to the utilisation of NAD^+ in the glycerol-3-phosphate dehydrogenase reaction,which is responsible for the conversion of glycerol-3-phosphate to dihydroxyacetone phosphate (Woods and Krebs, 1973). Hence one of the factors responsible for controlling the rate of glycerol metabolism is the rate of reoxidation of NADH to NAD^+ and when this cannot keep up with the rate of utilisation of NAD^+, glycerol-3-phosphate will accumulate. Since glycerol metabolism did not significantly affect PFK activity this is further evidence that the depletion of the adenine nucleotides is not responsible for the fructose-induced activation. Hue et al. (1982) reported that 20mM-glycerol depleted Fru-2,6-P_2 levels in isolated hepatocytes from fed rats. Claus et al. (1982) showed that 10mM-glycerol inhibited PFK activity by 40% in hepatocytes from 24h-starved rats whereas, under similar conditions, Hue and Bartrons (1984) failed to show any effect on Fru-2,6-P_2 levels with up to 20mM-glycerol. The experiments in the present study using hepatocytes from fed rats failed to show a significant effect on PFK activity with glycerol concentrations up to 8mM. However, incubation with 16mM-glycerol resulted in a 20% inhibition of enzyme activity compared to control values which, whilst not as large, was consistent with the findings of other workers (Hue et al., 1982; Claus et al., 1982).

Dihydroxyacetone loading does not lead to a depletion of adenine nucleotides or to the accumulation of a phosphorylated intermediate (Woods and Krebs, 1973; Mapungwana, 1982) however, Fru-1,6-P_2 levels have been shown to increase rapidly (Mapungwana, 1982). As in the case of fructose, Fru-1,6-P_2 is unlikely to be the activator of PFK in dihydroxyacetone incubated cells. Since the concentration of Fru-1,6-P_2 required to activate PFK is 1000-fold greater than that of Fru-2,6-P_2 (Van Schaftingen et al., 1981b) it seems most likely that dihydroxyacetone also increases Fru-2,6-P_2 concentrations. Hue et al. (1982) reported that dihydroxyacetone was without effect on hepatic Fru-2,6-P_2 concentrations however, these workers only incubated hepatocytes with 20mM-dihydroxyacetone. More recently, however, Hue and Bartrons (1984) have found an increased level of the bisphosphate in isolated hepatocytes from starved rats incubated with up to 20mM-dihydroxyacetone.

c. Effect of 24h-starvation on fructose induced stimulation of PFK.

PFK activity was assayed in hepatocytes prepared from 24h-starved rats incubated for 20 min with different concentrations of glucose or fructose (Fig. 54). As in the fed rat, low concentrations of fructose increased PFK activity. However, in this case similar concentrations of glucose also stimulated enzyme activity. Claus et al. (1982) had reported that PFK was maximally active following incubation with 10mM-fructose or 30mM-glucose. The experiments described here indicate that fructose at concentrations as low as 4mM will result in maximum stimulation of enzyme activity.

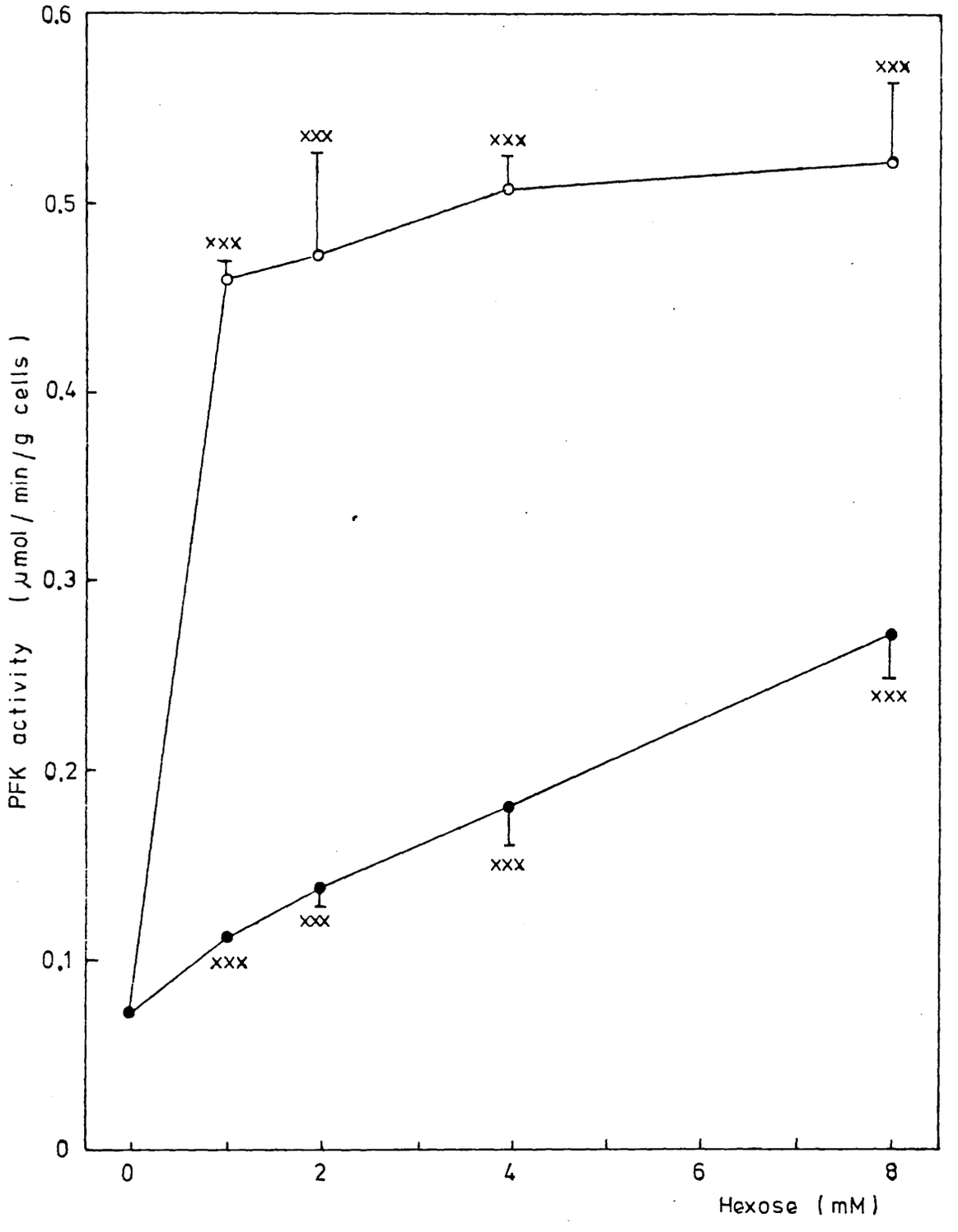


Fig. 54. Effect of glucose and fructose concentrations on hepatocyte PFK activity from 24h-starved rats.

Isolated hepatocytes (112mg/ml) were incubated with glucose (●-●) or fructose (o-o) for 20 min. Other experimental details are as in Fig. 48. Results plotted are means for 4 different cell preparations with s.e.m. represented by the bars. When error bars are not shown they fall within the points. Statistical significance was tested as described in Fig. 48.

Following incubation with glucose or fructose, hepatocytes were treated with perchloric acid and neutralised extracts assayed for Fru-6-P and Glc-6-P content. As explained in Section II.B.10.a.iii, Fru-2,6-P_2 concentrations can be estimated from deviations in the 3:1 ratio of Glc-6-P : Fru-6-P. Fig. 55 shows the variation of Glc-6-P, Fru-6-P and Fru-2,6-P_2 levels following incubation with varying concentrations of glucose or fructose. Both glucose and fructose increased Fru-2,6-P_2 concentrations and hence the results strongly suggest that fructose stimulates PFK activity through increases in the level of hepatic Fru-2,6-P_2. In support of this Riquelme et al. (1983) reported that incubation of isolated hepatocytes from fasted rats with 2.5mM-fructose or 20mM-glucose increased Fru-2,6-P_2 concentrations from 0.3 to 10.3 nmol/g and 14.6 nmol/g respectively. Similar incubations with 2mM-dihydroxyacetone were also shown to increase Fru-2,6-P_2 levels to 9.7 nmol/g. Hue and Bartrons (1984) have recently reported that hepatocyte Fru-2,6-P_2 concentrations are increased following incubation with 1-10mM-fructose. Fructose (1mM) resulted in maximum Fru-2,6-P_2 formation with levels decreasing substantially at higher fructose concentrations.

3. Physiological Significance

The experiments described here have shown that incubation of isolated hepatocytes, from both fed and 24h-starved rats, with low concentrations of fructose (< 4mM) results in maximum stimulation of PFK activity assayed with 4mM-Fru-6-P. There was evidence, from Sephadex G-25 chromatography of hepatocyte extracts, for this stimulation being due to the presence of a positive effector. Furthermore, hexose phosphate analysis

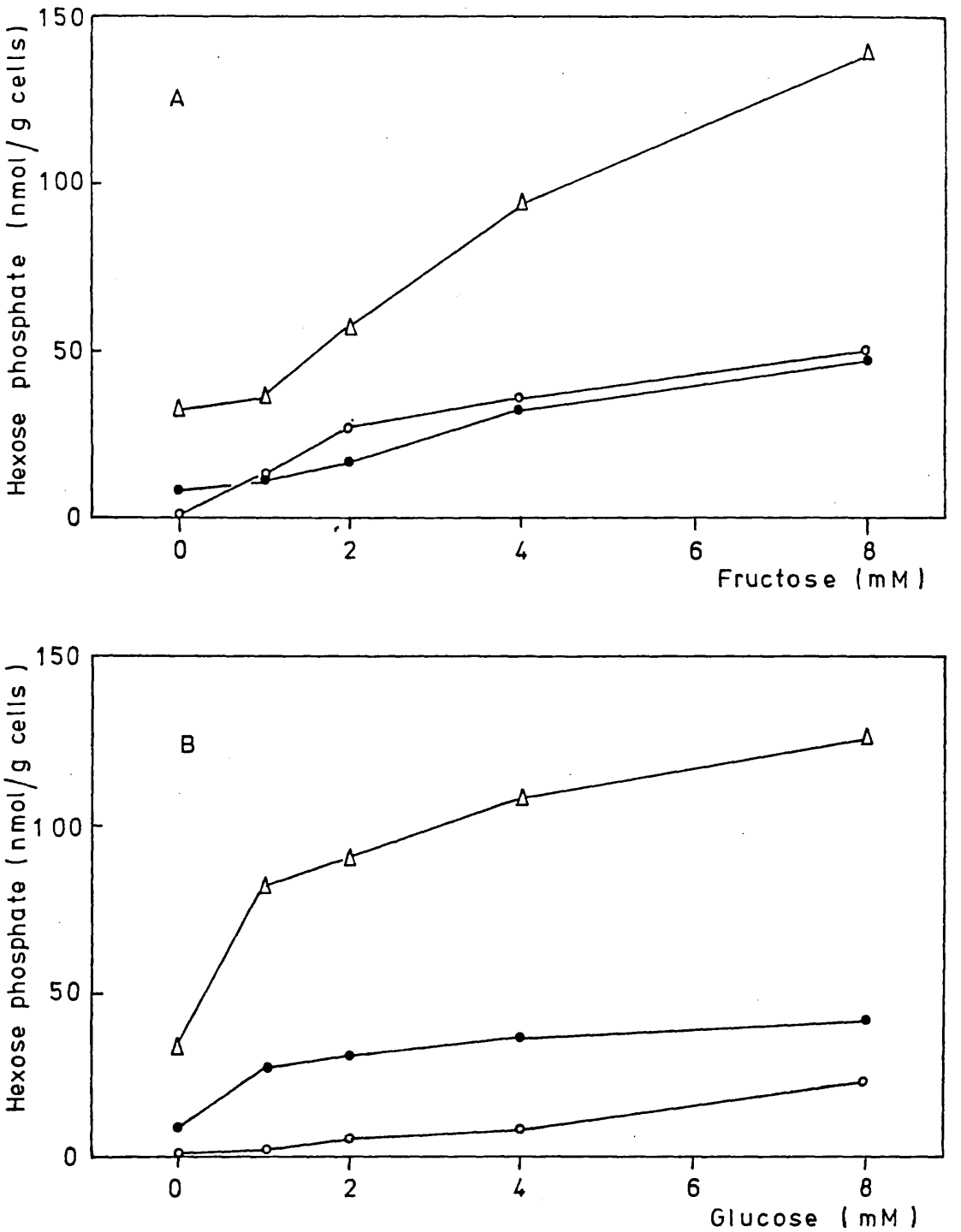


Fig. 55. Effect of A, fructose and B, glucose on hexose phosphate concentrations in hepatocytes from fasted rats.

Hepatocytes from 24h-starved rats were incubated with various concentrations of glucose or fructose for 20 min. See Fig. 54 for other details. Hexose phosphate levels were determined as described in Section II.B.10.a.iii. Hexose phosphates are represented as follows, Fru-6-P (●-●), Glc-6-P (Δ-Δ) and Fru-2,6-P_2 (o-o). Results plotted are mean of results from 2 different cell populations. Mean hepatocyte concentration was 108mg/ml.

of hepatocyte extracts from 24h-starved rats indicated that at low fructose concentrations Fru-2,6-P_2 levels were increased. Recent work by Hue et al. (1982), Claus et al. (1982) and Hue and Bartrons (1984) has confirmed that low concentrations of fructose (< 5mM) significantly increase Fru-2,6-P_2 concentrations. Fru-2,6-P_2 has been shown to be a potent activator of PFK and an inhibitor of Fru-1,6-P_2ase and thus the experimental results support the theory that stimulation of PFK following incubation with low concentrations can be attributed to an increase in Fru-2,6-P_2 concentration. Since incubation with either dihydroxyacetone or galactose, at concentrations up to 8mM, was also shown to result in a stimulation of PFK activity these substrates were also proposed to increase Fru-2,6-P_2 levels in the fed rat. Hue and Bartrons (1984) have reported that dihydroxyacetone (1-20mM) increases Fru-2,6-P_2 levels in hepatocytes from 24h-starved rats but as yet the effect of galactose on Fru-2,6-P_2 concentrations has not been studied by other workers.

Fructose and dihydroxyacetone are gluconeogenic precursors and, as such, would be expected to inhibit glycolysis, through inhibition of PFK and pyruvate kinase, and to stimulate gluconeogenesis, through activation of Fru-1,6-P_2ase. However, dihydroxyacetone and low concentrations of fructose are reported to stimulate PFK (present study) and pyruvate kinase (Mapungwana, 1982) activities. Under these conditions glycolysis would be expected to be stimulated. In support of this proposal dihydroxyacetone (Rognstad and Katz, 1976) and low concentrations of fructose (Clark et al., 1979) have been shown to increase flux through PFK, as determined by the production of ^3H_2O from [3-^{3}H] glucose. In order to decrease

the amount of substrate cycling Fru-1,6-P_2ase would be expected to be inhibited by the increase in Fru-2,6-P_2 which occurs under these conditions and lactate levels should increase. However, Hue and Bartrons (1984) have shown that, in hepatocytes from fasted rats, very little lactate is formed from 1mM-fructose, despite a rapid increase in Fru-2,6-P_2 levels, indicating that Fru-1,6-P_2ase is active and must therefore be less sensitive to changes in Fru-2,6-P_2 than PFK. These workers also showed that at low fructose concentrations glucagon decreased lactate output and Fru-2,6-P_2 levels and thus stimulated gluconeogenesis.

The results described in the present study have also shown that galactose (1-8mM) stimulates PFK activity and under these conditions Fru-2,6-P_2 concentrations were proposed to be increased. Such a proposal is not unrealistic when one considers that high glucose concentrations have repeatedly been shown to increase Fru-2,6-P_2 levels (Hers and Van Schaftingen, 1982) and that galactose metabolism bypasses the first regulatory step of glycolysis, entering the glycolytic pathway as Glc-6-P (Fig. 1).

PFK was shown to be inhibited following incubation with high concentrations of fructose (20mM). The inhibition was proposed to be due to Fru-1-P since Fru-1-P concentrations have been reported to increase following a fructose load (Mapungwana, 1982) and Fru-1-P has also been shown to be an inhibitor of hepatic PFK activity *in vitro* (present study). However, Hue *et al.* (1982) have shown that Fru-2,6-P_2 concentrations are rapidly depleted following incubation of isolated hepatocytes with 10-20mM-fructose. Thus the inhibition of

PFK by high concentrations of fructose is proposed to be due to both an increase in inhibitor (Fru-1-P) and a decrease in activator (Fru-2,6-P_2) concentration. This mechanism may serve to protect the animal against high rates of glycolysis and consequent lactic acidosis which may occur after a fructose load.

Glucagon has been reported to be without effect on gluconeogenesis in the presence of high fructose concentrations (Veneziale, 1972). Since Fru-2,6-P_2 concentrations are already depleted (Hue et al., 1982) and PFK activity has been shown to be inhibited (present study) under these conditions Fru-1, 6-P_2ase would be expected to be maximally active and thus glucagon can have no further effect on the rate of gluconeogenesis from fructose.

From the experiments described in this section the effects of fructose on PFK activity, and hence flux through the Fru-6-P/Fru-1,6-P_2 substrate cycle, appear to be heavily dependent on the fructose concentration. Physiological fructose concentrations (2mM) stimulate flux through the cycle through increasing the concentration of Fru-2,6-P_2, a positive effector of PFK and a negative effector of Fru-1,6-P_2ase. The degree of Fru-1,6-P_2ase inhibition is less than PFK activation due to the enzymes' differing sensitivities towards Fru-2,6-P_2 and thus gluconeogenesis will still occur at low fructose concentrations despite the stimulation of glycolysis. The physiological significance of the increased substrate cycling caused by low fructose concentration remains to be established.

E. Conclusions

The experiments described in this thesis have examined various aspects of hepatic fructose metabolism. As mentioned earlier (Section I.D) the majority of the work reported in the literature has been carried out, following liver perfusion or incubation of isolated hepatocytes, with high concentrations (> 10mM) of fructose. However, it is unlikely that liver cells are ever exposed to such high concentrations since, following ingestion of the ketose, the concentration of fructose in the hepatic portal vein does not exceed 2.5mM (Topping and Mayes, 1971).

The results reported here have shown a differential response to high and low fructose concentrations in common with the findings of other workers studying different biochemical parameters. For example, gluconeogenesis is stimulated by glucagon at low concentrations of fructose (Hue and Bartrons, 1984) but the hormone is without significant effect at high concentrations of the sugar (Veneziale, 1972). Similarly, unexpected changes in the concentration of Fru-2,6-P_2 have been reported following incubation of isolated hepatocytes with fructose. Low concentrations of the ketose increase the level of the bisphosphate, whereas high fructose concentrations deplete hepatic Fru-2,6-P_2 (Hue and Bartrons, 1984) and elevate Fru-1-P levels (Woods et al., 1970). In addition, glycogen synthesis in isolated hepatocytes has been shown to be stimulated by low concentrations (< 5mM) of fructose, whereas, at higher concentrations glycogen metabolism appears to be favoured (Wood et al., 1981).

A differential response to fructose has also been demonstrated for lipid metabolism. Following liver perfusion with low concentrations of fructose (1.5mM) lipogenesis is stimulated whereas, high concentrations of the ketose (8.9mM) inhibit lipogenesis and stimulate β-oxidation (Laker and Mayes, 1979). Hepatic pyruvate dehydrogenase activity appears to be unaffected by physiological fructose concentrations, however, high concentrations have been reported to stimulate enzyme activity (Topping and Mayes, 1977).

Finally, perfusion of liver (Woods et al., 1970), or incubation of isolated hepatocytes (Seglen, 1974; Mapungwana, 1982), with high concentrations of fructose have been reported to increase lactate levels and the administration of fructose to humans can cause lactic acidosis (Woods and Alberti, 1972; Solyst et al., 1980). Recently, Hue and Bartrons (1984) have shown that low fructose concentrations are without effect on lactate levels in isolated hepatocytes (Section III.D.2).

From these examples the hepatic response to a physiological or unphysiological fructose load is clearly different. There are some unexplained or poorly explained phenomena which arise as a result of fructose loading. For example the accumulation of Fru-1-P, the depletion of GTP and the increased lactate and glycerol-3-phosphate production. These phenomena have been reinvestigated in the present study using physiological concentrations of fructose.

Fru-1-P accumulation, following incubation with fructose, or a fructose load, was proposed to be due to inhibition of liver aldolase activity by IMP (Woods et al., 1970). However, Van den Berghe et al. (1977a) later proposed, from a

consideration of the rates of formation of Fru-1-P and IMP, that fructose is metabolised faster by fructokinase and aldolase than the pathways converting the triose phosphates to glucose or lactate (Section I.B.2.a). The observation in the present study that, in addition to Fru-1-P, another metabolite is formed following incubation of isolated hepatocytes with fructose or perfusion of liver with the ketose (Section III.A.2) may provide a better explanation for the accumulation of Fru-1-P. The results showed that the unknown metabolite was the major initial metabolite at low fructose concentrations. However, at higher concentrations of the ketose (> 4mM) Fru-1-P was the predominant product. This finding together with other evidence suggested that the unknown metabolite was formed from Fru-1-P. The concentration of unknown metabolite reached a maximum of 1.2μmol/g cells with 4mM-fructose, whereas Fru-1-P levels were shown to be substantially increased only at higher fructose concentrations. Whether this differential Fru-1-P accumulation is due to an inhibition of liver aldolase activity, possibly by the unknown metabolite, and/or a saturation of the enzyme responsible for the synthesis of the unknown metabolite is not clear from the results obtained in this study.

The unknown metabolite was shown to be synthesised following incubation, of isolated hepatocytes and the perfused liver prepared from both fed and 24h-starved rats, with 2mM-fructose and a purification procedure was established during which breakdown did not appear to occur. The behaviour of the purified unknown metabolite under different conditions was characterised (Section III.B.4). Electron impact and isobutane c.i. mass spectrometry gave a molecular weight of at

least 470 (Section III.B.6.b) whereas, gel-filtration experiments indicated that the molecular weight could be as high as 580 (Section III.B.1). The experimental evidence suggested that the unknown metabolite arose from an interaction between Fru-1-P and a tripeptide, proposed to contain lysine, glycine and serine, present in the cell (Section III.B.7).

GTP levels have been reported to fall following a fructose load (Van den Berghe et al., 1977a). Since fructokinase was reported to be specific for ATP as phosphate donor (Adelman et al., 1967a) whereas, triokinase can also use ITP or GTP (Frandsen and Grunnet, 1971), GTP levels have been proposed to fall as a result of the utilisation of GTP in the triokinase reaction (Section I.B.1.b). However, when fructokinase was purified in this study, GTP was shown to be an alternative substrate for the enzyme. The kinetics of ATP and GTP utilisation by fructokinase were investigated and both diphosphates were shown to inhibit enzyme activity. From a consideration of the physiological concentrations of ATP and GTP, the similar enzyme affinity of both triphosphates and the slower rate of GTP depletion, the utilisation of GTP by fructokinase provides a more satisfactory explanation for the depletion of GTP following a fructose load.

The effect of fructose on the activities of the enzymes controlling the first regulatory step encountered by fructose carbon in the direction of gluconeogenesis was also studied. At subsaturating substrate concentrations the activity of Fru-1,6-P_2ase appeared to be unaffected by incubation with fructose. However, PFK activity was increased following incubation with low fructose (< 8mM) concentrations and

inhibited following incubation with high (20mM) concentrations of the ketose. Low concentrations (< 8mM) of galactose or dihydroxyacetone were also shown to stimulate PFK activity, whereas similar concentrations of glucose or glycerol were without significant effect. The stimulation was proposed to be due to an increase in Fru-2,6-P_2, a potent activator of PFK, at low concentrations of fructose, dihydroxyacetone or galactose (Section III.D.2.a and b). In support of this Hue et al. (1982) have confirmed that incubation of isolated hepatocytes with 2.5mM-fructose or dihydroxyacetone significantly increases hepatic Fru-2,6-P_2 levels. In this study, when hepatocytes were prepared from the livers of 24h-starved rats, low concentrations of glucose and fructose were reported to stimulate PFK activity and Fru-2,6-P_2 levels were shown to increase rapidly under these conditions (Section III.D.2.c).

The physiological significance of the metabolism of fructose on the Fru-6-P/Fru-1,6-P_2 substrate cycle is discussed in Section III.D.3. Briefly, low concentrations of fructose increase substrate cycling by stimulating PFK activity to a greater extent than inhibiting Fru-1,6-P_2ase but, despite this, lactate levels are not significantly increased by physiological fructose concentrations. The explanation for this anomaly remains to be established. Incubation of hepatocytes with galactose, dihydroxyacetone or high glucose concentrations would also be expected to result in a stimulation of glycolysis since PFK activity was shown to be increased by these substrates. Later work, by Hue and Bartrons (1984), has confirmed these findings with the latter two substrates and additionally shown the accumulation of Fru-2,6-P_2 under these conditions.

High fructose concentrations stimulate gluconeogenesis through a depletion of Fru-2,6-P_2 (Hue et al., 1982) and accumulation of Fru-1-P, leading to inhibition of PFK and thus a decrease in substrate cycling. Under these conditions pyruvate kinase activity is reported to be activated by the increase in Fru-1-P concentrations (Mapungwana, 1982) and thus fructose carbon will be directed towards both glucose and lactate and, in the fed animal, lactate levels would be expected to increase.

Finally, glycerol-3-phosphate concentrations have been reported to increase, without any compensating increase in dihydroxyacetone phosphate levels, following liver perfusion (Woods et al., 1970; Sestoft, 1974) or incubation of isolated hepatocytes (Mapungwana, 1982) with fructose. It would be of interest to re-examine this phenomenon in view of the recent discovery of the unknown metabolite.

In conclusion, the evidence presented in this thesis has provided better explanations, than those previously proposed, for the accumulation of Fru-1-P and the utilisation of GTP following a fructose load.Additionally , substrate cycling at Fru-6-P/Fru-1,6-P_2 was proposed to be increased through stimulation of PFK, by Fru-2,6-P_2, at low concentrations of fructose. Furthermore, the differential accumulation of Fru-1-P and the unknown metabolite at high and low fructose concentrations may account for other concentration-dependent effects of fructose.

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