STUDIES OF THE REGULATION OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE

by

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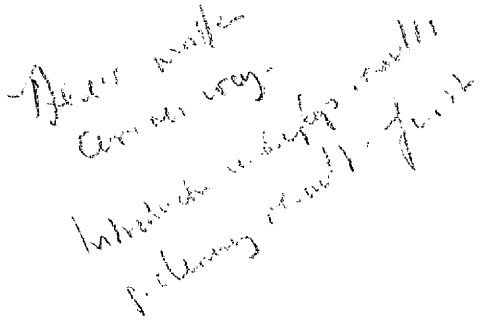
TO MY MOTHER AND FATHER



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ABBREVIATIONS

Abbreviations used are as recommended in the Biochemical Journal Instructions to Authors, 1981, with the following additions :

cAMP or cyclic AMP	adenosine 3',5' cyclic monophosphate
cAMP-PrK	cyclic AMP-dependent protein kinase
DTNB	5,5'-dithio-bis (2-nitrobenzoate)
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetate
$F1,6P_2$	fructose 1,6-bisphosphate
$F1,6P_2$ase	fructose 1,6-bisphosphatase
$F2,6P_2$	fructose 2,6-bisphosphate
$F2,6P_2$ase	fructose 2,6-bisphosphatase
F6P	fructose 6-phosphate
NEM	N-ethylmaleimide
PEP	phosphoenolpyruvate
PFK-1	phosphofructokinase-1
PFK-2	phosphofructokinase-2
P_i	inorganic phosphate
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N' tetramethylethylenediamine
Treacl	triethanolamine hydrochloride

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SUMMARY

Fructose 1,6-bisphosphatase has been isolated from rat liver by a newly developed procedure which includes absorption on a column of Procion Red HE-3B immobilised to cross-linked Sepharose-6B, then specific elution using a step of fructose 1,6-bisphosphate and AMP. A number of criteria indicated that the enzyme was not subjected to any significant degree of proteolytic modification during the purification. Purified fructose 1,6-bisphosphatase was homogeneous as judged by polyacrylamide gel electrophoresis and was composed of identical subunits in the molecular weight range of 39000-42000. Molecular weight values of 158000 and 148000, obtained by gel filtration chromatography and sedimentation equilibrium analysis respectively, indicated that the native enzyme was tetrameric. The amino acid composition of the enzyme was similar to that reported by Tejwani et al (1976); the enzyme contained no tryptophan.

The catalytic subunit of cyclic AMP-dependent protein kinase catalysed incorporation of ^{32}P from [γ-^{32}P]ATP into homogeneous rat liver fructose 1,6-bisphosphatase *in vitro*. Approximately 4 moles of phosphate were incorporated per mole of the tetrameric enzyme, but this phosphorylation was not associated with an increase in enzyme activity using standard assay conditions. Fructose 1,6-bisphosphatases from rabbit muscle and from ox liver could not be phosphorylated by cyclic AMP-dependent protein kinase. The dephosphorylation of phosphorylated rat liver fructose 1,6-bisphosphatase *in vitro* was catalysed by phosphoprotein phosphatases 1, 2A and 2C from rat liver. Phosphatase 2A was the most active of these three against fructose 1,6-bisphosphatase and this finding is in

agreement with the postulate that phosphatase 2A is the major phosphoprotein phosphatase involved in the control of gluconeogenesis (Cohen, 1982). The phosphorylation of fructose 1,6-bisphosphatase *in vivo* was also examined. Isolated rat hepatocytes were incubated with $^{32}P_i$ and subsequently treated with either glucagon (10^{-9}M), insulin (10^{-7}M) or, as a control, saline. Hepatocyte extracts were run on native polyacrylamide gels and fructose 1,6-bisphosphatase was identified by activity staining. The band containing fructose 1,6-bisphosphatase in each gel was then excised and subjected to SDS polyacrylamide gel electrophoresis in a 10% slab gel. Autoradiography of the gel showed that ^{32}P was incorporated into fructose 1,6-bisphosphatase *in vivo* and that the amount of ^{32}P in the enzyme from glucagon treated cells appeared to be greater than in the enzyme from control or insulin-treated cells. "Cleveland mapping" was used to confirm that the phosphorylated band was indeed fructose 1,6-bisphosphatase.

A comparative study of the non-phosphorylated enzyme with fructose 1,6-bisphosphatase which had been phosphorylated *in vitro* by cyclic AMP-dependent protein kinase was carried out. It was found that phosphorylation produced a decrease in the K_m for fructose 1,6-bisphosphate from 2.87 $\pm$ 0.16 μM to 1.94 $\pm$ 0.06 μM. However, no differences between non-phosphorylated and phosphorylated forms of the enzyme in respect of activation by Mg^{2+} ions or inhibition by AMP could be discerned. Fructose 2,6-bisphosphate which was synthesised by the method of Van Schaftingen and Hers (1981a), was found to inhibit fructose 1,6-bisphosphatase with a K_i of approximately 0.3 μM. Inhibition by fructose 2,6-bisphosphate was synergistic with inhibition by AMP. Again, no differences in the sensitivities of

non-phosphorylated and phosphorylated forms of fructose 1,6-bisphosphatase to inhibition by fructose 2,6-bisphosphate could be discerned in the presence or absence of AMP.

Initial studies indicated that the inhibition of fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate might be partly competitive with respect to fructose 1,6-bisphosphate. However, the synergistic effects with AMP and the findings that fructose 2,6-bisphosphate changed the substrate saturation curve from hyperbolic to sigmoidal and gave upward curving Dixon plots suggested that the inhibition was allosteric in nature. This was supported by the findings that fructose 2,6-bisphosphate reduced the Hill coefficients to 1, both for the activation by Mg^{2+} ions and for the inhibition by AMP. Inhibition by AMP was uncompetitive with respect to fructose 1,6-bisphosphate but became non-competitive in the presence of fructose 2,6-bisphosphate; in agreement with this, direct binding studies showed that AMP could bind to fructose 1,6-bisphosphatase only in the presence of fructose 1,6-bisphosphate or fructose 2,6-bisphosphate. The inhibition of fructose 1,6-bisphosphatase by high concentrations of substrate is known to be mediated through an allosteric site which can be blocked by thiol group reagents (Nimmo and Tipton, 1975b). Reaction of the enzyme with 5,5'-dithio-bis(2-nitrobenzoate) showed the presence of 1 mole/*cule* of reactive thiol groups per subunit. Modification of this thiol group with N-ethylmaleimide could be prevented by low concentrations of fructose 2,6-bisphosphate or partially prevented by high concentrations of fructose 1,6-bisphosphate. Moreover, the modified enzyme, which was considerably less sensitive to inhibition by high levels of substrate, was inhibited by fructose 2,6-bisphosphate in a simple competitive manner with respect to

fructose 1,6-bisphosphate. Thus, the inhibition of fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate appears to be mediated through partly by interaction at the catalytic site and partly by an allosteric effect.

The allosteric properties of rat liver fructose 1,6-bisphosphatase were generally consistent with the concerted transition two-state model proposed by Nimmo and Tipton (1975c) for the allosteric transitions of the ox liver enzyme. The exceptions were that the binding of Mg^{2+} ions was fairly non-exclusive, and the inhibition by AMP was uncompetitive with respect to fructose 1,6-bisphosphate. However, the profound effects of fructose 2,6-bisphosphate on the rat liver enzyme made it necessary to postulate a THREE-state model in which the third conformational state, X, has an allosteric site for fructose 2,6-bisphosphate. This model is satisfactory to explain the existing data but further data from binding studies will be required in order to test the model.

The role of the enzyme in the hormonal control of gluconeogenesis and the possible contribution of cyclic AMP-dependent phosphorylation are discussed.

CHAPTER 1

INTRODUCTION.

1.1 Early Studies of Fructose 1,6-Bisphosphatase

Fructose 1,6-Bisphosphatase (D-fructose 1,6-bisphosphate 1 - phosphohydrolase, EC3.1.3.11, hereafter F1,6P_2ase) catalyses the hydrolysis of D-fructose 1,6-bisphosphate to give fructose 6-phosphate (F6P) and inorganic phosphate (P_i). The enzyme was discovered by Gomori (1943) who demonstrated that a phosphatase with a pH optimum of 9.7 and a substrate specificity for hexosediphosphate was present in extracts of tissues from several mammalian sources. The phosphatase was strongly dependent on Mg^{2+} ions for activity and could be separated from other "non-specific" phosphatases in the extracts. Pogell and McGilvery (1954), who were able to purify the enzyme partially from rabbit liver, established that it was the 1-phosphate of F1,6P_2 which was removed by hydrolysis. They also demonstrated that the enzyme could also be stimulated by Mn^{2+} ions.

Taketa and Pogell (1965) found that F1,6P_2ase in freshly prepared extracts of rat liver had a pH optimum of 7.3. In two independent studies (Taketa and Pogell, 1965; Underwood and Newsholme, 1965) it was demonstrated that the K_m for F1,6P_2 was in the range 1-3 μM and that the enzyme was inhibited by substrate concentrations greater than 0.1 mM. F1,6P_2ase was inhibited by low concentrations of adenosine 5' monophosphate (AMP) in a manner which was non-competitive with respect to F1,6P_2. The response of the enzyme to AMP was sigmoidal. These results, and the finding that papain abolished the inhibition by AMP without altering the catalytic activity at pH 7.5, supported the hypothesis that the binding of AMP occurred at an allosteric site. Inhibition by AMP was not influenced by any other common metabolite apart from Mg^{2+} ions which reduced the inhibition. Underwood and Newsholme (1965) were also able to

show that F1,6P_2ase was inhibited by heavy metal ions such as Fe^{2+}, Fe^{3+} and Zn^{2+} but only the inhibition by Zn^{2+} could be reversed by treatment with EDTA.

F1,6P_2ase from rabbit liver was purified to homogeneity by Pontremoli et al (1965a). The purified enzyme had a molecular weight of 130000 and appeared to be composed of two types of subunit, of molecular weights 29000-31000 and 35000 - 39000 (Sia et al 1969). The pH optimum of the purified enzyme was in the range 9.0 - 9.5 (Pontremoli et al 1965b). Direct binding studies suggested that free F1,6P_2 and Mn^{2+} could interact directly with the enzyme, and that 4 binding sites for F1,6P_2 were available on the enzyme at pH 7.5 and at pH9.2 (Pontremoli et al 1968a). The binding of F1,6P_2 appeared to be cooperative at pH 7.2 and a value of 1.71 was calculated for the Hill coefficient. Further studies demonstrated that F1,6P_2ase contained 4 binding sites for AMP and a value of 50 μM was reported for the dissociation constant of the F1,6P_2ase · AMP complex (Pontremoli et al 1968b). AMP did not bind cooperatively to the enzyme in the absence of F1,6P_2 but the binding became increasingly cooperative at high concentrations of F1,6P_2.

Previous studies had indicated that the pH optimum of F1,6P_2ase in tissue extracts was in the neutral range but became distinctly alkaline upon purification. This change in pH optimum had first been noticed by Pogell and McGilvery (1952) who were able to activate the enzyme when assayed at alkaline pH, by incubating a partially purified preparation of F1,6P_2ase with a particulate fraction containing lysosomes. This effect could be mimicked by treating the enzyme with papain. Thus, the possibility was investigated that the enzyme was proteolytically modified during purification

(Nakashima and Horecker, 1971). The ratio of the activities of F1, $6P_2$ase at neutral pH compared with alkaline pH was measured throughout the purification procedure and expressed as the ratio of the activity at neutral pH/activity at alkaline pH. A distinct fall in this "pH ratio" from 3.16 to 0.38 was observed over the course of the preparation. A very similar result was obtained upon incubation of the partially purified enzyme with particulate cellular material. Moreover, treatment of the enzyme with papain, nagarse or pronase produced a small decrease in activity at pH 6.5 concomitant with a large increase in activity at pH 9.0 and shifted the pH optimum from neutral to alkaline (Geller et al, 1971). Thus, it appeared that "alkaline" F1,$6P_2$ase was an artefact produced by limited proteolytic modification of the "neutral" or "native" enzyme (Traniello et al, 1971). Confirmation of this conclusion came from experiments in which both the subunit size and the kinetic properties of pure "neutral" F1,$6P_2$ase were monitored during treatment with lysosomal proteases. The change in pH optimum, the decrease in sensitivity to AMP inhibition and a conversion of 36000 molecular weight subunits to subunits of 26000 and 29000 all occurred concomitantly (Pontremoli et al, 1973a; Botelho et al, 1977; Lazo et al, 1978). Thus, these findings have provided two useful criteria for detecting proteolysis of F1,$6P_2$ase: these are the pH ratio and the sensitivity to inhibition by AMP, both of which are considerably reduced by proteolysis.

1.2 The Properties of Neutral Fructose 1,6-Bisphosphatase

The first demonstration that F1,$6P_2$ase with a pH optimum in the range 7.0 - 7.5 could be isolated in homogeneous form was by Traniello et al (1971). Unlike the alkaline enzyme, neutral F1,$6P_2$ase

from rabbit liver had a molecular weight of 140000-144000 and was composed of 4 identical subunits of molecular weight 36000-39000 (Traniello et al, 1971; Traniello, 1974; Nimmo and Tipton, 1975a; Tejwani et al, 1976). Neutral F1,6P_2ase responded hyperbolically to F1,6P_2 and, similar to the alkaline enzyme, the K_m appeared to be in the 10^{-6} M range (Traniello, 1974). The enzyme was activated by Mg^{2+} ions and Mn^{2+} ions, but was inhibited by Ca^{2+} ions in a manner which was competitive with respect to the activating cations (Tejwani et al, 1976). The K_i for this inhibition was 14 μM with respect to Mg^{2+} ions (Rosenberg et al, 1973). NH_4^+ ions increased the maximum velocity by a factor of 2 but also reduced the affinity for substrate by an order of magnitude (Tejwani et al, 1976).

Inhibition of neutral ox liver F1,6P_2ase by AMP was reported to be non-competitive with respect to F1,6P_2 (Nimmo and Tipton, 1 75b). However, the finding that AMP was unable to bind to the rat liver enzyme except in the presence of F1,6P_2 (Tejwani et al, 1976) implied that AMP could be an uncompetitive inhibitor. Unlike alkaline F1,6P_2 ase, the neutral enzyme from several sources bound a maximum of 2 moles of AMP per molecule (as the tetramer). Neutral F1,6P_2ase from various sources responded sigmoidally to AMP and to activating cations. AMP increased the value of the Hill coefficient and decreased the affinity of the enzyme for Mg^{2+} ions (or Mn^{2+} ions): this effect was reciprocated upon AMP inhibition by activating cations. To explain these effects, Nimmo and Tipton (1975c) proposed that the allosteric properties of F1,6P_2ase from ox liver could be interpreted in terms of a two state concerted transition model (Monod et al, 1965) in which the activating cation played a dual role as both an allosteric activator of the enzyme and an essential cofactor for the reaction.

Neutral $F1,6P_2$ase was inhibited by high concentrations of $F1,6P_2$ at neutral pH but not at alkaline pH (Traniello et al, 1971; Traniello, 1974) and this inhibition was partially non-competitive with respect to Mg^{2+} ions (Nimmo and Tipton, 1975b). Traniello et al (1971) reported that 24 sulphydryl groups in the enzyme were available for reaction with thiol group reagents but in the presence of 1 mM $F1,6P_2$ only 20 of these groups were reactive. Similarly, Nimmo and Tipton (1975b) reported that 4 reactive thiol groups in the enzyme could be protected against modification by thiol group reagents if inhibitory, but not non-inhibitory, concentrations of $F1,6P_2$ were present. These results and the finding that modification of one thiol group per subunit almost completely abolished high substrate inhibition without altering any other properties of the enzyme, suggested that high substrate inhibition was mediated through an allosteric site at which the reactive thiol group was located.

$F1,6P_2$ase activity is greatly stimulated in the presence of chelating agents, especially EDTA, below pH 8.4. Rosenberg et al (1973) claimed that this activation was brought about by the interaction of the enzyme with a complex of the activating cation and EDTA. However, direct binding studies demonstrated that no direct interaction occurred between [$2-^{14}C$] EDTA and $F1,6P_2$ase (Nimmo and Tipton 1975a; Tejwani et al, 1976). Moreover, Nimmo and Tipton (1975a) showed that treatment of all components of the assay with Chelex 100 abolished the required for EDTA. These results clearly indicated that the role of EDTA is to remove inhibitory metal ions. Metabolites such as ATP, citrate and histidine might fulfil this role <u>in vivo</u>. Nimmo and Tipton (1975a) also found that the loss of inhibition by Zn^{2+} ions at alkaline pH in the chelexed system was dependent on the ionisation

state of a group or groups on the enzyme with an apparent pK_a value of 8.4. They suggested that this would explain why the enzyme did not appear to require EDTA at high pH and that this might be the basis of the change in kinetic properties of the enzyme between neutral and alkaline pH.

It has been suggested that the modification of neutral F1,6P_2ase by lysosomal proteases, over and above protein turnover, may be important in the control of F1,6P_2ase activity in vivo (Pontremoli et al, 1973a; 1973b; 1974). F1,6P_2ase activity appeared to be 50% lower in rabbit livers which were isolated during winter months or from "summer" animals which were fasted or exposed to cold temperature. These effects were attributed to a 6-fold increase in the intracellular soluble protease activity which arises under these conditions. More recent studies have shown that the concentration of F1,6P_2ase in the livers of animals on a balanced diet was in the range 3-4 μM, as measured by radioimmunoassay (Mazzota and Veneziale, 1980). Fasting produced a 40% increase in the amount of enzyme whereas a high protein diet or severe diabetes, more than doubled the intracellular level of F1,6P_2ase. Similar but less marked changes were observed in the kidney. In contrast to these findings, Zalitis and Pitot (1979) reported that a 50% increase in the activity of F1,6P_2ase in the livers of rats fed on a high protein diet was not the result of an increase in the concentration of F1,6P_2ase. Moreover, the rates of synthesis and degradation of F1,6P_2ase in rat liver and kidney remained constant under a variety of dietary and hormonal conditions.

1.3 The Regulatory Role of Fructose 1,6-Bisphosphatase in Gluconeogenesis

Gluconeogenesis is the metabolic pathway in which glucose is resynthesised from non-carbohydrate precursors, for the most part

amino acids, lactate from muscle metabolism and glycerol from lipolysis (figure 1.1). This pathway is unique to liver and kidney cortex in mammals (Newsholme and Gevers, 1967). The physiological importance of gluconeogenesis lies in its ability to satisfy the demands of the central nervous system and the red blood cells for glucose when the dietary supply of glucose is low and liver glycogen has been used up. The pathway may also play a role in the removal of excess lactate from the blood following strenuous exercise. Gluconeogenesis is not strictly a reversal of glycolysis since there are three sites at which different enzymes catalyse opposing reactions: these are the interconversions of (i) glucose and glucose 6-phosphate, (ii) fructose 6-phosphate and fructose 1,6-bisphosphate and (iii) phosphoenolpyruvate and pyruvate (figure 1.1). However, since most reactions are common to both processes, gluconeogenesis and glycolysis are very much interrelated and interdependent. For this reason, a complete understanding of the regulation of glucose metabolism can only be obtained if both gluconeogenesis and glycolysis are considered together.

$F1,6P_2$ase is situated in the gluconeogenic pathway at the interconversion of F6P and $F1,6P_2$ as illustrated in figure 1.1. The allosteric properties of the enzyme and the fact that its level can be modulated in the long term (see above) clearly indicate the possibility that the enzyme may be involved in the regulation of gluconeogenesis. Moreover, $F1,6P_2$ase satisfies several other criteria which indicate that an enzyme may be regulatory (Newsholme and Gevers 1967). Theenzyme catalyses a non-equilibrium, essentially irreversible reaction and has a low maximal activity which is of the same order as the overall flux rate of gluconeogenesis. $F1,6P_2$ase also catalyses a reaction for which the opposing reaction is catalysed by a different

Figure 1.1

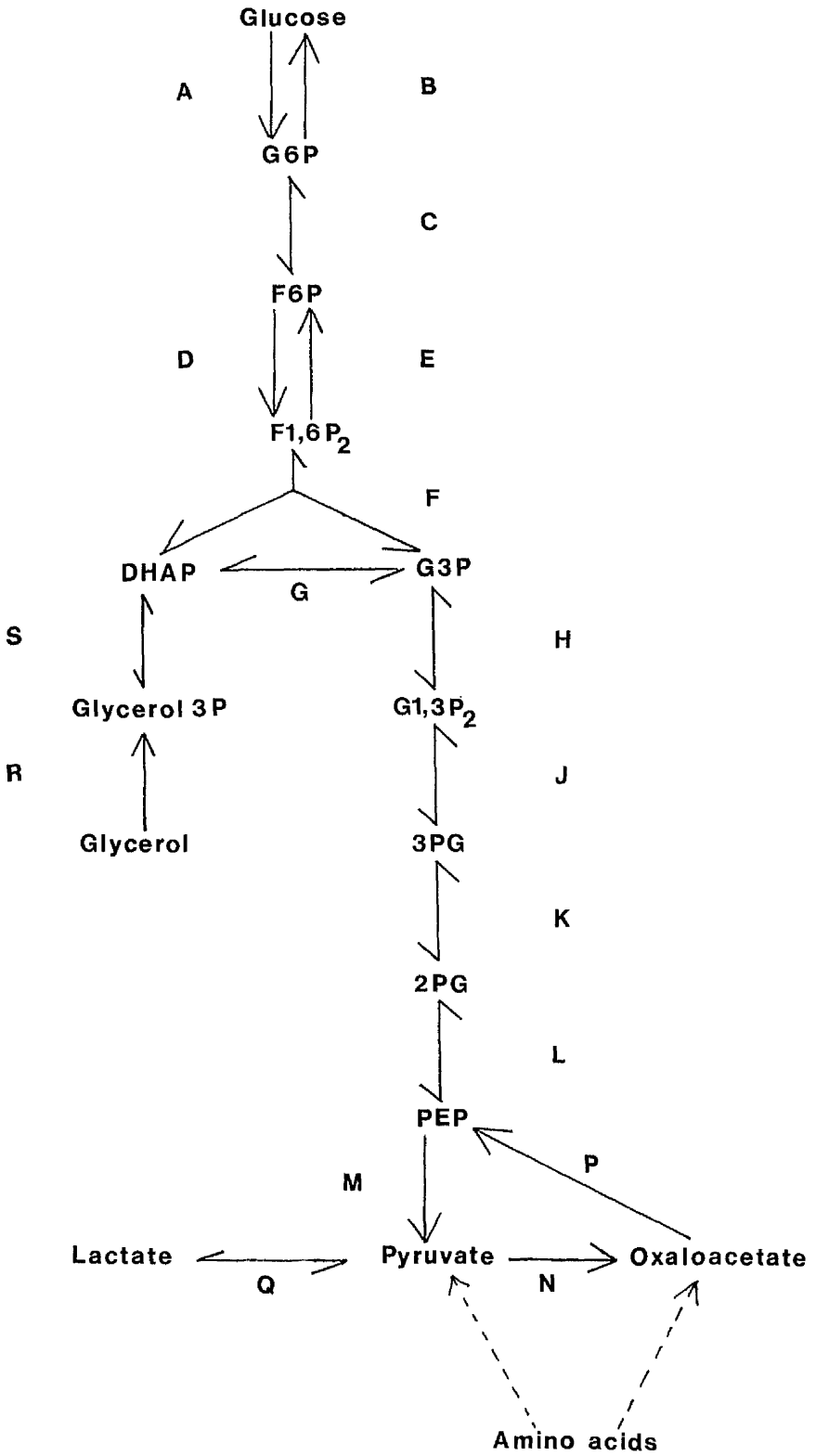
The pathways of gluconeogensis and glycolysis

The metabolites are:

G6P	glucose 6-phosphate
F6P	fructose 6-phosphate
$F1,6P_2$	fructose 1,6-bisphosphate
DHAP	dihydroxyacetone phosphate
G3P	glyceraldehyde 3-phosphate
$G1,3P_2$	1,3-diphosphoglycerate
3PG	3-phosphoglycerate
2PG	2-phosphoglycerate
PEP	phosphoenolpyruvate
Glycerol 3P	glycerol 3-phosphate

The enzymes are:

A	hexokinase/glucokinase
B	glucose 6-phosphatase
C	phosphoglucose isomerase
D	6-phosphofructo-1-Kinase (PFK-1)
E	fructose 1,6-bisphosphatase
F	fructose 1,6-bisphosphate aldolase
G	triose phosphate isomerase
H	glyceraldehyde 3-phosphate dehydrogenase
J	phosphoglycerate kinase
K	phosphoglycerate mutase
L	enolase
M	pyruvate kinase
N	pyruvate carboxylase
P	phosphoenolpyruvate carboxykinase
Q	lactate dehydrogenase
R	glycerol kinase
S	glycerol phosphate dehydrogenase


Glucose
A
B
G6P
C
F6P
D
E
F1,6P2
F
DHAP
G
G3P
S
H
Glycerol 3P
G1,3P2
R
J
Glycerol
3PG
K
2PG
L
PEP
P
M
Lactate
Q
Pyruvate
N
Oxaloacetate
Amino acids

enzyme. Furthermore, F1,6P_2ase is located at a branch point in metabolism at which, from the gluconeogenic point of view, the pathways of glucose synthesis from glycerol and from pyruvate converge at the triose phosphate level.

The findings that AMP and F1,6P_2 inhibit F1,6P_2ase but activate phosphofructokinase-1 (PFK-1) which catalyses the opposing reaction and is also inhibited by ATP and citrate led to a theory for the regulation of gluconeogenesis and glycolysis in which a major factor in determining the direction and rate of flux through these pathways is the "energy status" of the cell, expressed as the steady state concentrations of ATP, ADP and AMP relative to each other (Newsholme and Gevers, 1967). Thus a rise in the AMP concentration would simultaneously inhibit F1,6P_2ase and activate PFK-1, thereby favouring glycolysis and opposing gluconeogenesis; conversely, a fall in the AMP concentration would have the opposite effects. Newsholme and Gevers (1967) proposed a model in which the interconversion of F6P and F1,6P_2 operates as a "substrate cycle", as opposed to a situation in which the opposing enzymes are never simultaneously active. Although such a cycle would appear to be "futile" since it would operate at the expense of considerable hydrolysis of ATP, it may offer a significant regulatory advantage: if the rate of cycling were much greater than the overall net flux rate in either direction, small reciprocal changes in the activities of PFK-1 and F1,6P_2ase would produce a relatively large change in the magnitude and/or direction of the overall net flux. By this mechanism, the sensitivity of the system to small changes in the concentrations of effector molecules such as AMP would be greatly amplified. Evidence for the occurrence of substrate cycling at the F1,6P_2ase/PFK-1 level has come from studies

of gluconeogenesis from dihydroxyacetone in the presence of [1 -^{14}C] galactose in isolated rat hepatocytes (Rognstad and Katz, 1976). In theory, the ^{14}C in the C_1 position of galactose would be randomised between the upper and lower parts of the C_6 molecule by the actions of aldolase and triose phosphate isomerase. Thus if cycling were in operation, glucose would be formed with ^{14}C in the C_6 position, and this is, in fact, the result which was observed by experimentation. Moreover, Hers et al (1981) have shown that factors which affect the flux through gluconeogenesis and glycolysis also affect the rate of cycling.

The enzymes hexokinase and glucose 6-phosphatase which catalyse the interconversion of glucose and glucose 6-phosphate, and the enzymes pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase which catalyse the interconversion of pyruvate and phosphoenolpyruvate have also been identified as regulatory for gluconeogenesis and glycolysis. The regulatory role of these enzymes and the effects on the control of gluconeogenesis and glycolysis of intermediates which belong to other metabolic pathways, have been fully discussed by Newsholme and Gevers (1967).

1.4 The Mechanisms of Hormone Action

One of the major functions of the liver is to regulate the level of glucose in the blood and this is governed by the antagonistic actions of the hormones insulin and glucagon. A rise in the blood glucose level, in response to sugar intake from the gut, stimulates the secretion of insulin from the pancreas. Insulin, in turn, counteracts the rise in the glucose level by increasing the rate of utilisation of glucose by the liver through stimulation of hepatic glycogen synthesis, glycolysis, fatty acid and triglyceride synthesis and

simultaneous inhibition of glycogenolysis and gluconeogenesis. Conversely, when the blood glucose level is low, the liver, under the influence of glucagon, replenishes blood glucose by the opposite effects.

Although the physiological responses to these hormones are known, the molecular basis of hormone action on liver metabolism is still under active investigation. It is recognised that in the short term the sites of action of glucagon and insulin are the key enzymes in the metabolic pathways and the mechanism of action of the hormones appears to involve phosphorylation and dephosphorylation of these enzymes. By this mechanism, a regulatory enzyme can exist in either of two forms which are interconvertible through phosphorylation by kinases and dephosphorylation by phosphatases. Phosphorylation may alter the maximum catalytic activity and/or kinetic and regulatory properties of the enzyme and consequently may enhance or reduce the rate of flux through the respective metabolic pathway <u>in vivo</u>. It is known that the binding of glucagon to its receptor in the cell membrane stimulates the membrane bound enzyme adenylate cyclase which, utilising ATP, catalyses the formation of cyclic adenosine 3', 5', monophosphate (cyclic AMP), the hormonal "second messenger" (e.g. see Rodbell, 1980). Thus glucagon elevates the intracellular concentration of cyclic AMP (Johnson et al, 1972). Cyclic AMP, in turn, binds to the regulatory subunits of inactive cyclic AMP- dependent protein kinase (cAMP-PrK) holoenzyme which then dissociates to give regulatory subunit (R) dimers and active catalytic subunit (C) monomers according to the equation

$$R_2C_2 + 4cAMP \rightleftharpoons R_2cAMP_4 + 2C$$

(Builder et al, 1980). The catalytic subunits of cAMP-PrK catalyse

the phosphorylation by ATP, and corresponding activation or inactivation, of the key enzymes of the metabolic pathway. Although the mechanism of action of glucagon is reasonaby well defined, the molecular events which follow the binding of insulin to its receptor in the cell membrane and which mediate the physiological effects of insulin are still uncertain. The search for a "second messenger" of insulin action, which functions in a manner analogous to the role of cyclic AMP in glucagon action, has recently been focussed on an acid and heat stable "mediator substance" which is generated early in insulin action on skeletal muscle (Larner et al, 1979): this substance, which was believed to be a peptide with a molecular weight in the range 1000-1500, inhibited cAMP-PrK and activated glycogen synthase phosphatase. A similar peptide factor, which was identified by its ability to cause activation of pyruvate dehydrogenase (PDH) in mitochondria, could be generated by treatment of rat adipocyte plasma membranes with insulin, insulin agonists or trypsin (Seals and Czech, 1980). Treatment of the membranes with protease inhibitors prior to insulin treatment blocked the stimulation of PDH which suggested that insulin activated an endogenous membrane protease. The partially purified factor gave a variable 2-10 fold stimulation of PDH and this was attributed to the activation PDH phosphatase (Kiechle et al, 1981). However, the mechanism of this stimulation is unknown. Marchmont and Houslay (1980a; 1980b) have recently demonstrated that insulin triggers the cyclic AMP-dependent phosphorylation and activation of a membrane bound, low K_m phosphodiesterase, (the enzyme which catalyses the hydrolysis of cyclic AMP to give 5' AMP). They have also shown that two other peripheral membrane proteins are phosphorylated in response to insulin and cyclic AMP, but the functions

of these proteins are not yet known. Insulin dependent phosphorylation of cytosolic proteins has also been observed; for example, acetyl CoA carboxylase is phosphorylated and activated, at a site distinct from the cAMP-PrK site, in response to insulin (Brownsey and Denton, 1982). Insulin has recently been reported to produce a small but significant decrease in the activity of cAMP-PrK in hepatocytes when the enzyme was assayed at subsaturating concentrations of cyclic AMP (Mor et al, 1981). Thus it appears that insulin can either reduce the effectiveness of cyclic AMP or elevated cyclic AMP levels by inhibiting cAMP-PrK and activating phosphodiesterase respectively. The insulin-generated "mediator substance" may be involved in these effects and in the activation of phosphoprotein phosphatase but the mechanisms are as yet unclear.

1.5 Identification of the Hormone-Sensitive Sites of Gluconeogenesis

The identification of the hormone sensitive sites of gluconeogenesis came initially from studies of the changes in both the levels of intermediates and the flux through the pathway in response to hormone treatment. The presence of a hormone-sensitive site between triose phosphate and glucose in the gluconeogenic pathway was first observed by Veneziale (1971) who studied the effect of glucagon on gluconeogenesis from fructose in isolated, perfused rat liver. Fructose enters gluconeogenesis at the triose phosphate level and since phosphoenolpyruvate carboxykinase can be inhibited by perfusion with quinolate, gluconeogenesis from endogenous precursors entering at pyruvate or oxalocetate can be minimised, and the rate of gluconeogenesis from fructose can be monitored in the presence and absence of glucagon. In this way it was demonstrated that the rate

of gluconeogenesis from [U - ^{14}C]fructose was stimulated at least 2-fold in response to glucagon. Although this evidence suggested that there is a control site (or sites) between triose phosphate and glucose, an analysis of the radioactive metabolites did not reveal its location.

Rognstad and Katz (1976) have examined the effect of glucagon on gluconeogenesis from dihydroxyacetone in rat hepatcytes isolated by perfusion with collagenase, and have identified the site of hormonal control as the interconversion of F6P and F1,6P_2. Estimation of the flux through PFK-1 by isotopic techniques suggested that the glucagon-stimulated increase in gluconeogenesis was primarily the result of a suppression of PFK-1 activity. However, this study did not take into account possible changes in the activity of F1,6P_2ase. In a recent study, they have presented data which indicate that glucagon might also cause a small but significant stimulation of F1,6P_2ase (Rognstad and Katz, 1980). A number of groups (e.g. Feliu et al, 1976; Pilkis et al, 1976) have also investigated the effects of hormones on gluconeogenesis in isolated hepatocytes. Utilising [U - ^{14}C] dihydroxyacetone and [U - ^{14}C] glycerol, and estimating the metabolite levels and flux rates, Pilkis et al (1976) demonstrated that glucagon stimulates the conversion of F1,6P_2 to F6P and suppresses the conversion of PEP to pyruvate. Stimulation of gluconeogenesis by catecholamines in the 10^{-6}M range was also observed. This was mediated by α receptors and was cyclic AMP - independent. Insulin partially inhibited the stimulatory effect of glucagon. Direct measurements of pyruvate kinase activity in extracts from cells that had been incubated with or without glucagon, indicated that the suppression of the formation of pyruvate from PEP resulted from the inactivation of pyruvate kinase. The authors proposed that hormones could also influence the activities

of F1,6P_2ase and/or PFK-1.

The effects of hormones on the gluconeogenic and glycolytic regulatory enzymes in rat liver <u>in vivo</u> were investigated by Taunton et al (1972; 1974) and by Stifel et al (1974). Intravenous injections of hormones were given to anaesthetised rats and liver biopsies were taken in order to examine the specific activities of key enzymes and determine the level of cyclic AMP. The results demonstrated that glucagon produced a significant activation of F1,6P_2ase and a decrease in the activities of PFK-1 and pyruvate kinase, but gave no change in the activity of F1,6P_2 aldolase which is not a regulatory enzyme. These effects were concomitant with an increase in the intra ellular level of cyclic AMP and could be mimicked by administering exogenous cyclic AMP in place of glucagon. Adrenaline produced similar effects on these enzymes and, in contrast to the findings of others, (Pilkis et al, 1976), increased the cyclic AMP level in the cells. Insulin, however, produced the opposite response in each of these enzyme activities but had no effect on the cyclic AMP level. The effects of the hormones were rapid, producing maximal effects within 10 minutes and appeared to be dose dependent. Furthermore, insulin and glucagon could each reverse the effects of the other. Pretreatment of the rats with actinomycin D or puromycin did not alter any of the responses to hormones, which suggested that the activity changes were not the result of increased protein synthesis. Two other key enzymes in the gluconeogenic pathway, pyruvate ~~kinase~~ carboxylase and phosphoenolpyruvate carboxykinase, were not influenced by hormone treatment. The results of these experiments are surprising because the assays were all performed under V_{max} conditions and so one would not expect to observe changes in the activities of pyruvate kinase and PFK-1.

Chatterjee and Datta (1978) have also examined liver $F1,6P_2$ase activity following glucagon administration to mice and have shown, in agreement with the results of Taunton et al (1974), that there is a dose-dependent and cyclic AMP - dependent activation of $F1,6P_2$ase in response to glucagon. They have also suggested that the action by glucagon is facilitated by glucocorticoids since adrenalectomy abolishes the effect of glucagon, but not of dibutyryl cyclic AMP. In a similar type of study, using isolated hepatocytes, Castano et al (1979) demonstrated a glucagon and cyclic AMP-dependent inactivation of PFK-1 which was attributed to a decrease in the affinity for F6P concomitant with an increase in the Hill coefficient for F6P from 2.3 to 4.0.

These results clearly indicate that the sites of hormone action in the gluconeogenic and glycolytic pathways are the interconversion of F6P and $F1,6P_2$ and the interconversion of PEP and pyruvate. Moreover, the evidence suggests that the targets of hormone action in these pathways are the regulatory enzymes $F1,6P_2$ase, PFK-1 and pyruvate kinase but not pyruvate carboxylase or phospho-enolpyruvate carboxykinase. Cyclic AMP, which is known to be the intracellular "second messenger" of many hormones, has been shown to mimic the effects of glucagon on gluconeogenesis and this is a good indication that the key enzymes are controlled by phosphorylation and dephosphorylation mechanisms. Two other possible sites of regulation of gluconeogenesis exist. There is evidence to suggest that the plasma membrane transport of some amino acids may be regulated by hormones. There is also evidence to suggest that the availability of oxaloacetate from the mitochondrion is under hormonal control: glucagon and cyclic AMP-dependent stimulation of cytochrome C_1 increases proton efflux and this in turn leads to an increase in the efflux of dicarboxylate anions from

the mitochondrion. The possible contribution by these and other factors to the control of gluconeogenesis has been discussed in detail by Pilkis et al (1978).

1.6 The Phosphorylation and Dephosphorylation of Fructose 1,6-Bisphosphatase

The first report of the modification of F1,6P_2ase activity by phosphorylation and dephosphorylation *in vitro* was by Mendicino et al. (1966) who found that F1,6P_2ase in crude preparations from kidney could be inactivated by incubation with Mg^{2+} ions, ATP and cyclic AMP. When [γ - ^{32}P] ATP was used, the incorporation of ^{32}P into F1,6P_2ase paralleled the inactivation. F1,6P_2ase could be reactivated with the concomitant release of $^{32}P_i$ when the purified labelled enzyme was reincubated with a kidney extract. More recently, Mendicino et al (1978) have re-examined the phosphorylation of swine kidney F1,6P_2ase by purified cAMP-PrK *in vitro*. Although a maximum of 4 moles of phosphate were incorporated into serine residues in the enzyme (as the tetramer), no significant changes in the catalytic activity or the regulatory properties of the enzyme resulted from phosphorylation.

The phosphorylation of rat liver F1,6P_2ase by the catalytic subunit of cAMP-PrK *in vitro*, at physiological concentrations of these enzymes, has been reported by Riou et al (1976; 1977) who demonstrated that the incorporation of approximately 4 moles of phosphate per mole of tetrameric F1,6P_2ase resulted in a 40% increase in enzyme activity as measured under conditions of saturating substrate. However, no change in the K_m for F1,6P_2 or the K_i for AMP were observed, nor did saturating concentrations of these ligands alter the rate of phosphorylation of the enzyme. Riou et al (1977) were also able to isolate radioactively labelled F1,6P_2ase from the livers of rats which

had been injected with $^{32}P_i$, indicating that the enzyme was phosphorylated *in vivo*. However, treatment of the rats with glucagon did not appear to alter the phosphorylation state of the enzyme. Following acid hydrolysis of F1,6P_2ase which was phosphorylated *in vitro* residues of serine phosphate were isolated. Recently, Pilkis et al (1980) have demonstrated that the phosphorylation of F1,6P_2ase is reversible since the phosphorylated enzyme can be dephosphorylated by a partially purified phosphoprotein phosphatase from rat liver.

Pilkis et al (1980) have isolated a ^{32}P- containing fragment of molecular weight 6000 following treatment of phosphorylated rat liver F1,6P_2ase with cyanogen bromide. Moreover, brief exposure of the phosphorylated enzyme to trypsin resulted in the loss of a ^{32}P-phosphopeptide comprising 17 amino acids from the enzyme. The sequence of the first 8 amino acids of this peptide were found to be

Ser-Arg-Pro-Ser (^{32}P)-Leu-Pro-Leu-Pro

In a similar study, Humble et al (1980) found the sequence at the phosphorylation site to be

Ser-Arg-Tyr-Ser (^{32}P)-Leu-Pro-Leu-Pro

which differs from the sequence reported by Pilkis et al (1980) in that tyrosine was found in place of proline adjacent (on the N-terminal side) to phosphoserine. In common with other substrates for cAMP-PrK, rat liver F1,6P_2ase contains an arginine residue between 2 and 5 residues on the N-terminal side of the target serine (Nimmo and Cohen, 1977); however, the best substrates for cAMP-PrK contain two adjacent basic residues, at least one of which is arginine, in this position. In view of this hypothesis Pilkis et al (1980) have demonstrated that rat liver pyruvate kinase, which has two adjacent arginine residues at the phosphorylation site is in terms of the V_{max}/K_m ratio, a 10-fold

better substrate for cAMP-PrK than is rat liver F1,6P_2ase. However, since the physiological concentration of F1,6P_2ase is considerably greater than that of pyruvate kinase, the rate of phosphorylation of F1,6P_2ase *in vivo* may be 3-fold greater than for pyruvate kinase.

Hosey and Marcus (1981) found that rat liver F1,6P_2ase, but not F1,6P_2ases from mouse liver, rabbit liver or pig kidney, could be phosphorylated by cAMP-PrK *in vitro*. It appeared that the rat liver enzyme (subunit molecular weight 40000-42000) was larger than each of these other F1,6P_2ases (subunit molecular weight 36000), and that the phosphorylation site of rat liver F1,6P_2ase could be removed by partial proteolysis with trypsin to give an active species of molcular weight 36000. The authors suggested that a phosphorylation site might be removed from the other F1,6P_2ases by partial proteolysis during the purification procedure. Treatment with subtilisin has been shown to remove a peptide of molecular weight 6000 from the N-terminus of neutral F1,6P_2ase to give an "alkaline" form of the enzyme (Botelho et al, 1977; Lazo et al, 1978). Following treatment of phosphorylated F1,6P_2ase with subtilisin, ^{32}P remained associated with the enzyme, and this indicated that the phosphorylation site, which could be released by trypsin, was located close to the C-terminus. Information about the structure of phosphorylated F1,6P_2ase is summarised in figure 1.2.

It is interesting to note that control of F1,6P_2ase activity by phosphorylation has also been observed in micro-organisms. Yeast cells contain fully active F1,6P_2ase when grown in glucose-free medium containing acetate. However, on transfer to a medium containing glucose, F1,6P_2ase was rapidly phosphorylated at a serine residue and inactivated (Muller and Holzer, 1981). The evidence suggests that the phosphorylated enzyme is rapidly degraded by proteases. The finding

Figure 1.2

Summary of the information from proteolytic studies of phosphorylated rat liver fructose 1,6-bisphosphatase given in section 1.6

(a) The molecular weight of rat liver F1,6P_2ase is in the range 40000-42000 (Hosey and Marcus, 1981).

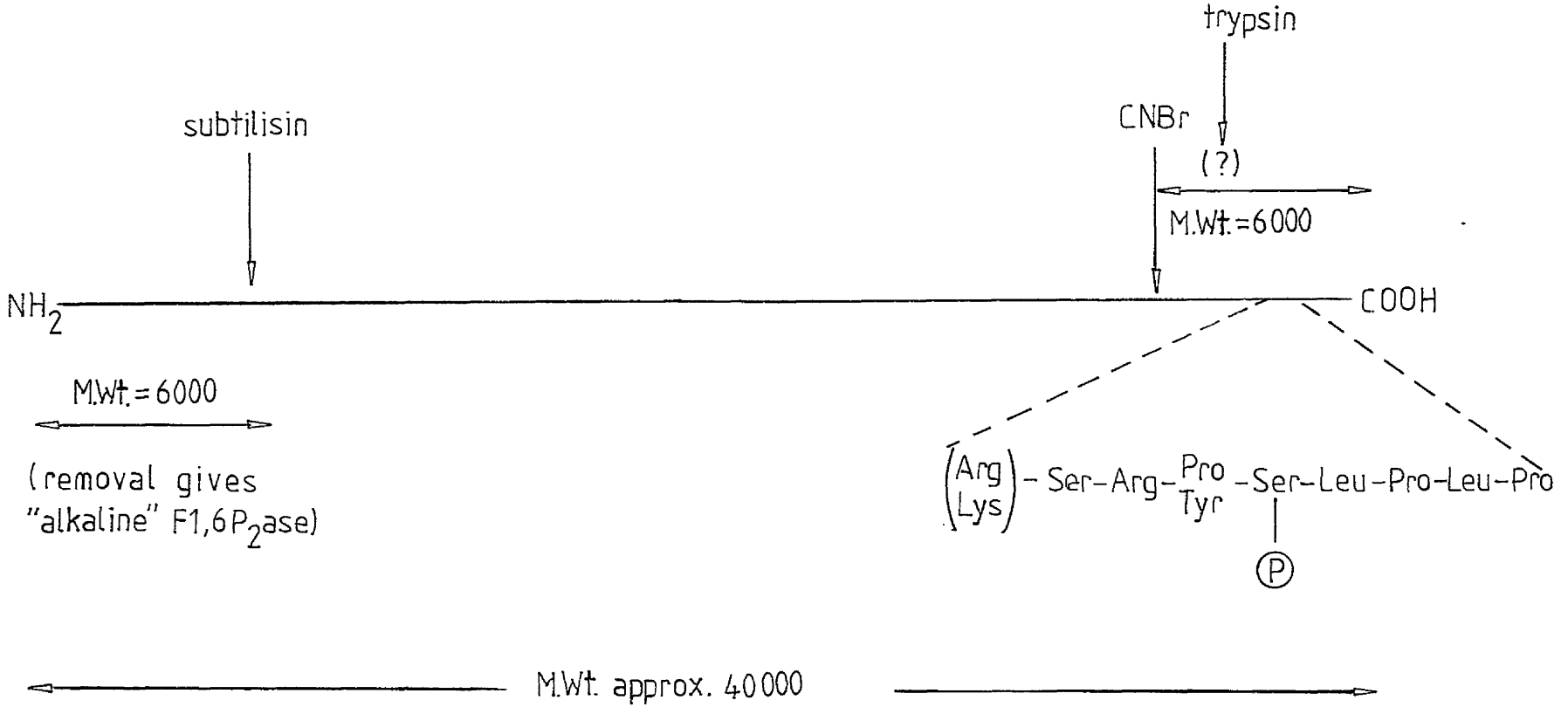
(b) Subtilisin removes a peptide of molecular weight 6000 from the N-terminus of F1,6P_2ase to yield alkaline F1,6P_2ase (e.g. see Botelho et al., 1977). This peptide does not contain the phosphorylation site (Pilkis et al., 1980; Humble et al., 1980; Hosey and Marcus, 1981).

(c) Cyanogen bromide removes a ^{32}P-containing peptide of molecular weight 6000 from the phosphorylated enzyme (Pilkis et al., 1980).

(d) Trypsin removes a ^{32}P-containing peptide comprising 17 amino acids (presumed molecular weight 2000) from the C-terminus of the phosphorylated enzyme (Pilkis et al., 1980; Hosey and Marcus, 1981).

(e) The phosphorylated residue is serine (Riou et al., 1977).

(f) The sequence at the phosphorylated site has been determined (Pilkis et al., 1980; Humble et al., 1980). Since trypsin releases the peptide from the C-terminus of the enzyme, the residue N-terminus adjacent to the peptide is presumed to be basic.


trypsin
subtilisin
CNBr
(?)
M.Wt.=6000
NH2
COOH
M.Wt.=6000
(removal gives
"alkaline" F1,6P2ase)
(Arg Lys) - Ser-Arg-Pro Tyr -Ser-Leu-Pro-Leu-Pro
P
M.Wt. approx. 40000

that the key enzymes in micro-organisms can be regulated by phosphorylation mechanisms may be of considerable evolutionary importance.

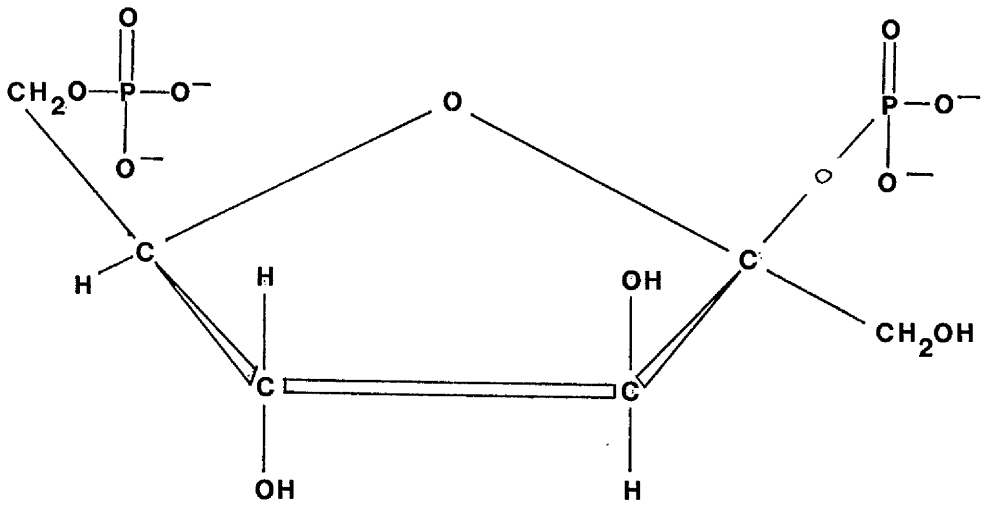
The phosphorylation of PFK-1 and of pyruvate kinase have also been studied. PFK-1 from swine kidney (Mendicino et al, 1978) and from rabbit muscle (Riquelme et al, 1978) have been phosphorylated in vitro using cAMP-PrK with stoichiometries approaching 1 mole of phosphate incorporated per mole of subunit. However, no change in the catalytic activity or regulatory properties of either enzyme could be discerned. In contrast, Foe and Kemp (1982) recently demonstrated that phosphorylation of muscle PFK-1 increased the sensitivity to inhibition by ATP and citrate and decreased the sensitivity to activation by AMP and $F2,6P_2$. Moreover, a 3-fold increase in the incorporation of ^{32}P into rat hepatic PFK-1 in response to glucagon administration in vivo led to a considerable increase in the inhibition of the enzyme given by ATP (Kagimoto and Uyeda, 1979). The phosphorylation of pyruvate kinase has received much attention and has been reviewed in detail by Pilkis et al (1978). Phosphorylation of hepatic pyruvate kinase leads to an increase in the K_m and Hill coefficient for PEP, enhances the sensitivity to inhibition by ATP and alanine and reduces the affinity for the activator $F1,6P_2$. Hepatic pyruvate kinase which has been phosphorylated in response to glucagon in vivo shows very similar properties to the enzyme phosphorylated in vitro.

1.7 The Discovery of Fructose 2,6-Bisphosphate

During attempts to purify rat liver PFK-1, Furuya and Uyeda (1980a) noticed that the sensitivity of the enzyme to inhibition by ATP increased at each fractionation step in the preparation. Moreover the purified enzyme, or PFK-1 in gel filtered liver extracts (Van

Figure 1.3

The β-anomer of fructose 2,6-bisphosphate



Schaftingen et al, 1980b) responded to inhibition by ATP with a sensitivity similar to that of PFK-1 in liver extracts from glucagon-treated rats (Kagimoto and Uyeda, 1979). A further examination showed that a low molecular weight component,which reversed the increase in the sensitivity of PFK-1 to inhibition by ATP, was present in ultra filtrates or heated samples of liver extracts and could be recovered following gel filtration of liver extracts (Claus et al, 1981a; Van Schaftingen et al, 1980b). The level of this activator in hepatocytes was increased by incubation with glucose and decreased upon administration of glucagon (Claus et al, 1981a; Van Schaftingen et al, 1980a).

The molecular weight of the stimulator was found to be similar to that of $F1,6P_2$ as estimated by gel filtration using Bio-Gel P-2 (Van Schaftingen et al, 1980b). The finding that the compound did not absorb light at 260nm and was not absorbed onto charcoal suggested that it was not a nucleotide or nucleoside (Claus et al, 1981a; Van Schaftingen et al 1980c). When the activator was partially purified, its elution profile from ion-exchange resins was similar to that of $F1,6P_2$. However, unlike $F1,6P_2$, ~~this~~ the activator was very acid labile which is characteristic of a phosphate group on a hemiacetal hydroxyl group. This result, and the finding that hydrolysis of the activator at pH2 and at 0^oC for 15 minutes produced equimolar amounts of F6P and P_i, suggested that the activator could be fructose 2,6-bisphosphate ($F2,6P_2$) (Claus et al, 1981a; Van Shaftingen et al, 1980b and 1980c; Pilkis et al, 1981c). Chemical synthesis of $F2,6P_2$ was successfully achieved using two different approaches and the synthetic molecule, which had identical properties to the natural activator was identified, using ^{13}C NMR spectroscopy, as the β anomer of $F2,6P_2$ (figure 1.3). The level of $F2,6P_2$ in hepatocytes and the concentration necessary to

stimulate PFK-1 have been estimated to be in the 10^{-7}M range (Van Schaftingen and Hers, 1980; Pilkis et al, 1981c; Van Schaftingen and Hers, 1981a; Van Schaftingen et al, 1980c).

The identification and properties of 6-phosphofructo 2-kinase (PFK-2) and fructose 2,6-bisphosphatase ($F2,6P_2$ase) which are responsible respectively for the synthesis of $F2,6P_2$ from F6P and ATP and its hydrolysis to give F6P and P_i, have been discussed by Hers and Van Schaftingen (1982). It appears that these two enzyme activities are on the same dimeric protein which can be rapidly phosphorylated in vitro using cAMP-PrK, with a stoichiometry of 1 mole of phosphate incorporated per subunit. Phosphorylation decreased the activity of PFK-2 and increased the activity of $F2,6P_2$ase. $F2,6P_2$ reduced the rate of phosphorylation of the enzyme (El-Maghrabi et al, 1982). These findings provide an explanation for the mechanism of the hormonal dependence of the intracellular $F2,6P_2$ concentration (Claus et al, 1981a; Van Schaftingen et al, 1980a); glucagon could cause a decrease in the level of $F2,6P_2$ by increasing the level of cyclic AMP. Thus $F2,6P_2$ provides a very sensitive system for the regulation of PFK-1 in response to hormones, and its profound effects on the regulatory and kinetic properties of PFK-1 have been discussed in detail by Hers and Van Schaftingen (1982). $F2,6P_2$ may also operate in concert with a phosphorylation mechanism. In this respect, Furuya and Uyeda (1980b) have isolated "phospho - " and "dephospho" forms of PFK-1 from rat liver. It appears that the dephospho-form of the enzyme has a higher affinity for $F2,6P_2$ than the phospho-form.

Factors which influence the activity of PFK-1 generally have reciprocal effects on the activity of $F1,6P_2$ ase. In view of this, it is reasonable to suggest that $F2,6P_2$ could play a role in the regulation

of F1,6P_2ase. Initial investigations of the effect of F2,6P_2 on F1,6P_2ase have produced contrasting results. Pilkis et al (1981a) claimed that F2,6P_2 behaved as a simple competitive inhibitor with respect to F1,6P_2. However, Van Schaftingen and Hers (1981b) reported that F2,6P_2 changed the substrate saturation curve from hyperbolic to sigmoidal. Both groups demonstrated that, similar to their stimulatory effects on PFK-1, F2,6P_2 and AMP interact synergistically to inhibit F1,6P_2ase. Clearly, the kinetics of inhibition by F2,6P_2 and possible differences in the reponse of non-phosphorylated and phosphorylated F1,6P_2ases to this regulator remain to be fully investigated.

1.8 The Aims of the Research

The evidence presented and discussed in this introduction clearly demonstrates that F1,6P_2ase plays a major role in the regulation of gluconeogenesis. However, the relative importance of the various factors that can regulate the activity of F1,6P_2ase is not fully understood. The enzyme activity appears to be modulated not only by allosteric factors, but also in response to hormonal signals, perhaps through phosphorylation/dephosphorylation mechanisms, or by changes in the level of F2,6P_2, or by both of these factors in concert. Therefore, in order to obtain a clearer understanding of the role of F1,6P_2ase in gluconeogenesis, a study of the regulation of rat liver F1,6P_2ase was undertaken and is presented in this thesis. The areas under investigation were as described below.

In order to study the regulation of rat liver F1,6P_2ase it was necessary to isolate the enzyme in a homogeneous form. In this respect, a procedure for the purification of neutral F1,6P_2ase has been developed and is described in Chapter 3.

The catalytic subunit of cAMP-PrK has been purified from ox liver and has been employed to study the phosphorylation of homogeneous rat liver F1,6P_2ase *in vitro*. Dephosphorylation has also been examined; the physiological roles of 4 phosphoprotein phosphatases have been defined (Cohen, 1982) and an initial study of the effects of 3 of these enzymes on phosphorylated F1,6P_2ase *in vitro* has been carried out. A brief investigation of the effects of the hormones insulin and glucagon on the phosphorylation state of F1,6P_2ase in isolated rat hepatocytes has also been carried out.

As reported in the literature, the phosphorylation of rat liver F1,6P_2ase by cAMP-PrK *in vitro* produced only a small increase in enzyme activity, and in preliminary studies, no change in the enzyme's affinity for substrate or sensitivity to effector molecules could be detected. In steady state pathways such as gluconeogenesis and glycolysis, the influence of allosteric ligands is of considerable importance with regard to regulation and the sensitivity to these effectors may depend on the phosphorylation state of the enzyme. For this reason , an extensive comparative study of the regulatory properties of non-phosphorylated and phosphorylated forms of rat liver F1,6P_2ase was carried out.

F2,6P_2 appears to be the most potent known regulator of PFK-1 activity and initial reports have suggested that it is also a potent effector of F1,6P_2ase. In view of these findings, further study of the inhibition of non-phosphorylated and phosphorylated forms of F1,6P_2ase by F2,6P_2 was carried out using F2,6P_2 which was synthesised by the method of Van Schaftingen and Hers (1981a). During these studies it became clear that F2,6P_2 had profound effects on the cooperative interactions of the substrate and effectors of F1,6P_2ase.

These results, and a study of the type of inhibition given by $F2,6P_2$, made it necessary to construct a new model for the allosteric transitions of $F1,6P_2$ase. Such a model is essential in order to provide a clear understanding of regulation in a situation in which phosphorylation modulates enzyme activity rather than operates as a direct on/off mechanism of control.

Several important communications which were published while this work was in progress have been discussed in relation to the findings reported in this thesis. In the final Chapter, the contribution of the regulation of $F1,6P_2$ase to the coordinated control of gluconeogenesis and glycolysis is considered.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1 (a) Biochemicals

Adenosine 5' diphosphate, adenosine 5' monophosphate, adenosine 5' triphosphate, fructose 6-phosphate, fructose 1,6-bis-phosphate, glucose 6-phosphate, nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide (NADH), oxaloacetate, phosphoenolypyruvate, pyruvate and triethanolamine hydrochloride were all purchased from the Boehringer Corporation (London) Ltd, Lewes, Sussex U.K. 2 - Mercaptoethanol, glucose, glycine, imidazole, o-nitrophenyl β-D-galactopyranoside, sucrose and urea were all obtained from BDH Chemicals, Poole, Dorset, U.K. Dithiothreitol was purchased from Koch Light Laboratories, Colnbrook, Berks, U.K. and benzamidine hydrochloride, bovine serum albumin, histone type IIA (from calf thymus), morpholino propane sulphonic acid (MOPS), phenyl-methane sulphonyl fluoride (PMSF) and tris (hydroxymethyl) amino methane (Tris) were bought from the Sigma (London) Chemical Company, Fancy Road, Poole, Dorset BH17 7NH, U.K.

2.1 (b) Enzymes

Commercial enzyme preparations, which were obtained from the Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K. were as listed below :

aldolase (EC 4.1.2.13) from rabbit muscle

carbonic anhydrase (EC4.2.1.1) from bovine erythrocytes

fructose 1,6-bisphosphatase (EC 3.1.3.11) from rabbit muscle

β - galactosidase (EC 3.2.1.23) from E. coli

glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from yeast

glyceraldehyde 3 - phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle.

glycerol 3 - phosphate dehydrogenase (EC 1.1.1.8) from rabbit muscle.

hexokinase (EC 2.7.1.1.) from yeast

lactate dehydrogenase (EC 1.1.1.27) from pig heart

malate dehydrogenase (EC 1.1.1.37) from pig heart mitochrondria

myokinase (EC 2.7.4.3) from rabbit skeletal muscle

phosphofructokinase - 1 (EC 2.7.1.11) from rabbit muscle

phosphoglucose isomerase (EC 5.3.1.9) from yeast

pyruvate kinase (EC 2.7.1.40) from rabbit muscle

triose phosphate isomerase (EC 5.3.1.1) from rabbit muscle

*TPCK - treated trypsin (EC 3.4.21.4) from bovine pancreas and α-chymotrypsin (EC 3.4.4.5) from bovine pancreas were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A. Bovine liver fructose 1,6-bisphosphatase, which was prepared as described by Nimmo and Tipton (1975a), and rabbit muscle glycogen phosphorylase b (EC 2.4.1.1), which was prepared by the method of Fischer and Krebs (1958), were both kind gifts from Dr. H.G. Nimmo. Rabbit muscle glycogen phosphorylase kinase (EC 2.7.1.38), which was prepared according to the procedure of Cohen (1973), and protein phosphatases 1,2A and 2C (Cohen 1982) were kind gifts from Prof. P. Cohen of the Department of Biochemistry in the University of Dundee.

2.1 (c) Polyacrylamide gel electrophoresis components

Acrylamide monomer and N,N' methylenebis - acrylamide (specially purified for gel electrophoresis), ammonium persulphate, sodium dodecyl sulphate and N,N,N',N' tetra-methyl ethylenediamine were "analaR" grade materials which were obtained from BDH Chemicals, Poole, Dorset, U.K.

* TPCK is tosyl phenylalanine chloromethyl ketone

2.1 (d) Radiochemicals

All radiochemicals were purchased from Amersham International Bucks, U.K. and were as listed below :

[2 - ^{3}H] adenosine 5' monophosphate (20C_i/mmol)

adenosine 5' -[γ- ^{32}P] triphosphate (5000C_i/mmol)

D - [U - ^{14}C] fructose 1,6-bisphosphate (239mC_i/mmol)

phenylmethane [^{35}S] sulphonyl fluoride (25 mC_i/mmol)

2.1 (e) Chromatographic Media

Dowex 1 - X8 and Dowex 50W - X8 were procured from BDH Chemicals,Poole, Dorset, U.K. Hydroxylapatite HTP was from Bio-Rad Laboratories, 32nd and Griffin, Richmond, California, U.S.A. Silica gel thin layer chromatography plates were obtained from Macherey - Nagel and Company, 5160 Duren, Werkestrasse 6-8, Postfach 307, West Germany and Sephadex G25 (coarse grade), Sephadex G200 (superfine grade) and Sepharose 6B were purchased from Pharmacia (GB) Ltd., London W5, U.K. Chromatography paper (types 1 and 3mm), and diethyl-aminoethyl cellulose (microgranular form, DE52) were obtained from Whatman Biochemicals, Madistone, Kent, U.K.

2.1. (f) Miscellaneous Materials

Ethylenediamine tetracetic acid, 8-hydroxy-quinoline, iod-ocecetic acid and N-ethyl maleimide (all analaR grade) were purchased from BDH Chemicals, Poole, Dorset, U.K. Coomassie Brilliant Blue G250 was obtained from Serva Feinbiochemica, Heidelberg, West Germany. Procion red - HE3B was a kind gift from Dr. L. Jervis of Paisley College of Technology. All other reagents were of the highest grade commercially available.

2.2 General Methods

2.2 (a) Glassware

All items of glassware were washed in solutions of the detergent "Haemo-sol" (Alfred Cox (Surgical) Ltd., Edward Road, Coulsdon, Surrey, U.K.), rinsed thoroughly with tap water, then distilled water, and dried in an oven at 100°C. For amino acid analysis and peptide mapping, glassware was additionally treated by immersion in concentrated nitric acid for 1 hour, followed by rinsing with distilled water and drying. Pipettes were washed in solutions of the detergent Decon 75 (Decon Laboratories Ltd, Conway Street, Hove BN3 3 LY) and thoroughly rinsed using an automatic pipette washer. The pipettes were then rinsed with distilled water and dried in an automatic pipette dryer.

2.2 (b) Micropipetting

Solutions in the range 10 μl to 1 ml were transferred reproducibly using either adjustable "Finnpipettes" (Finnpipette Ky, Pultittie 9, SF-00810, Helsinki 81, Finland) or fixed volume Oxford pipettes (BCL, Bell Lane, Lewes, Sussex). Volumes in the range 0.5 μl to 50 μl were transferred accurately using microsyringes (Scientific Glass Engineering Pty Ltd., 111 Arden Street, North Melbourne, Australia 3051).

2.2 (c) pH and Conductivity Measurements

Measurements of pH were carried out using a Radiometer Copenhagen Type PHM 26 pH meter equipped with a glass calomel electrode type GK 2301. This apparatus was standardised regularly using solutions of pH 7 and pH 4 supplied by the manufacturer.

Conductivity measurements were made using a Radiometer Copenhagen Conductivity Meter Type CDM 2e.

2.2 (d) Centrifugation

Preparative centrifugation was routinely performed at +4°C using an MSE High Speed 18 refrigerated centrifuge, giving forces of up to 43000g. Microcentrifugation was carried out using an Eppendorf Microfuge.

2.2 (e) Preparation of Chromatographic Media

Sephadex gels were swollen and poured according to the manufacturer's instructions and were stored at 0-4°C in 0.02% (W/V) sodium azide.

Hydroxylapatite HTP was defined and equilibrated in starting buffer as described by the manufacturer. Prior to recycling, used material was washed thoroughly in 2M NaCl to remove any bound protein.

Dowex 1-X8 (Cl^- form) and Dowex 50W-X8 (H^+ form) were prepared according to the manufacturer's directions.

As instructed by Whatman, DEAE-cellulose (DE52) was defined and suspended in a large excess of the acid component of the starting buffer, followed by filtration to the appropriate pH with 2N KOH. Before use, the material was washed with starting buffer until the desired conductivity had been achieved. Recycling involved washing thoroughly in 2M NaCl and treatment with 0.5N HCl and 0.5N NaOH as described by the manufacturer.

Procion red - sepharose (cross-linked) was prepared as follows. To cross-link the sepharose, 10ml of epichlorohydrin and 0.5g of $NaBH_4$ were added to 100ml of washed, settled sepharose (6B or 4B). This was stirred at 60°C for 2 hours and washed thoroughly. The 100ml of cross-linked sepharose was then suspended in 100ml of 12.5% (w/v) NaCl containing 3.3% (w/v) procion red HE3B, and was heated at 60°C for 30 minutes. 2g of Na_2CO_3 were added and heating was continued

at 80°C for 2 hours. The material was washed thoroughly and stored at 0-4°C in 0.02% sodium azide. Procion red sepharose was equilibrated in starting buffer immediately before use.

2.2 (f) Dialysis and Vacuum Dialysis

Dialyses were carried out using Visking Tubing (Scientific Instrument Centre Ltd., 1 Leeke Street, London WC1), which had been immersed in boiling 1mM EDTA, pH 7.0, for 15 minutes, and washed thoroughly in distilled water.

Protein solutions were concentrated by dialysis under vacuum.

2.2 (g) Desalting Coupling Enzymes

Coupling enzymes to be used in F1,6P_2ase assays and PFK-1 assays were desalted by overnight dialysis against 50mM Treacl - KOH pH 7.5 plus 50 mM 2 - mercaptoethanol.

2.2 (h) Measurement of Protein Concentrations

All protein concentrations were estimated by the method of Bradford (1976). Protein reagent was prepared, comprising 0.01% (w/v) Coomassie Brilliant Blue G250, 4.7% (v/v) ethanol and 8.5% (v/v) phosphoric acid in H_2O. 5ml of protein reagent were added to test-tubes, each containing 0.1ml of a sample of unknown protein concentration and also to tubes containing known amounts of bovine serum albumin, in the range 0-100 μg in 0.1 ml, as a means of standardisation. All samples were vortexed and allowed to stand for 2 minutes. The optical densities were measured at 595 nm and a standard curve of E_{595} against protein concentration was constructed from which the protein concentrations of the unknown samples were estimated.

2.2 (i) Distilled Water

Unless otherwise stated, glass distilled water, stored in polythene containers, was used in all experiments.

2.3 Enzyme Assays and Standardisation of Solutions

2.3 (a) Enzyme Assays

All spectrophotometric assays were performed at 37°C using a Gilford Unicam SP500 spectrophotometer, equipped with a Gilford photoelectric detector and recorder, and with quartz cells of pathlength 1cm. Although Beer's law was obeyed up to approximately 2.0 absorbance units, absorbance measurements rarely exceeded 1.2 units.

Samples from assays in which radioactive phosphorus was used were counted on an Intertechnique SL 4000 liquid scintillation counter.

Unless otherwise stated, one unit of enzyme activity is defined as the amount of enzyme required to catalyse the disappearance of one micromole of substrate, or the appearance of one micromole of product, per minute.

Fructose 1,6-bisphosphatase

F1,6P_2ase was assayed as described by Nimmo and Tipton (1975a) in a coupled system in which fructose 6-phosphate, isomerised to glucose 6-phosphate in the presence of excess phosphglucose isomerase, was used to reduce **$NADP^+$** in the presence of excess glucose 6-phosphate dehyrogenase. Each cuvette contained, in a total volume of 1 ml, 50mM Treacl-KOH buffer, pH 7.4 (or 50 mM glycine -KOH,pH 9·6), plus 100mM KCl, 2.1mM $MgCl_2$, 0.1mM EDTA, 100μM F1,6P_2, 0.15mM $NADP^+$, 5 units of phosphoglucose isomerase and 3 units of glucose 6-phosphate dehydrogenase. The reaction was initiated by the addition of enzyme and the reduction of $NADP^+$ was monitored at 340nm.

Cyclic AMP-dependent Protein Kinase (EC 2.7.1.37)

The protein kinase assay was based on the phosphorylation of histone which was then adsorbed onto phsphocellulose paper (Witt and Roskowski, 1975). The reaction mixture contained in a total volume

of 80μl, 2 mg/ml histone (Sigma, Type IIA), 20mM potassium phosphate, pH 6.8, 2mM $MgCl_2$, 10 μM cyclic AMP, 0.2mM [γ - ^{32}P] ATP (circa 10^7 cpm/μmol) and protein kinase, suitably diluted . (For control samples, protein kinase was omitted). The reaction was initiated by the addition of ATP and incubated at 30^{o}C for 15 minutes after which a 50 μl sample was removed, placed on a 2x2cm square of phosphocellulose paper and dropped into a beaker of cold tap water. When all the reactions had been completed, the papers were rinsed 5 times with cold tap water (50ml per paper) washed twice with acetone and dried. Each paper was placed in a scintillation vial containing 10 ml of scintillant. (80g naphthalene, 4g 2.5 diphenyloxazole, 300ml ethoxyethanol, 300 ml dioxan and made up to 1000ml with toluene) and counted.

Protein kinase activity is expressed in units where one unit is defined as the amount of enzyme needed to catalyse the incorporation of one picomole of ^{32}P into histone per minute.

β - Galactosidase was assayed by the method of Craven et al (1965). Each cuvette contained, in a total volume of 1 ml, 10mM Tris acetate buffer, pH 7.5, 10mM $MgCl_2$, 100mM 2-mercaptoethanol, 100mM NaCl, 1.75mM o-nitrophenyl -β - D - galactopyranoside and enzyme. The production of o-nitrophenol was monitored at 412 nm.

Glucose 6-phosphate dehydrogenase was assayed by the method of Noltmann et al (1961). The reaction mixture contained 50mM Treacl-KOH buffer, pH 7.2, 100mM KCl, 0.15mM $NADP^+$, 1mM glucose 6-phosphate and enzyme. The appearance of NADPH was monitored at 340nm.

Lactate dehydrogenase was assayed as described by Kornberg (1955). The assay medium contained, in a total volume of 1ml, 100mM potassium phosphate buffer, pH 7.0, 0.2mM NADH, 1mM sodium pyruvate and enzyme. The oxidation of NADH was monitored at 340nm.

Malate dehydrogenase was assayed as described by Mehler et al (1948). Each cuvette contained, in a total volume of 1ml, 100mM potassium phosphate buffer, pH 7.0, 0.2mM NADH, 1mM oxaloacetate and enzyme. The oxidation of NADH was monitored at 340nm.

Phosphofructokinase 1 was assayed essentially by the method of Castano et al (1979). Each cuvette contained in a total volume of 1ml, 50mM MOPS-KOH buffer, pH 7.0, 100mM KCl, 5mM $MgCl_2$, 1.5mM MgATP, 0.15mM NADH, 5mM potassium phosphate, 0.1mM AMP, 0.5 units of both aldolase and glycerol 3 - phosphate dehydrogenase, 5 units of triose phosphate isomerase, fructose 6-phosphate and enzyme. The activity was measured at 0.12mM fructose 6-phosphate (subsaturating) and at 5mM fructose 6-phosphate (saturating), and was expressed as the activity ratio, ($v_{0.12}/V_{max}$). The oxidation of NADH was monitored at 340nm.

Phosphoglucose isomerase was assayed as described by Noltmann (1966). Each cuvette contained, in a final volume of 1ml, 100mM Treacl-KOH buffer, pH 8.0, 0.15mM $NADP^+$, 1mM fructose 6-phosphate, 3 units of glucose 6-phosphate dehydrogenase and enzyme. The reduction of $NADP^+$ was monitored at 340nm.

Pyruvate kinase was assayed as described by Valentine and Tamara (1966). Each cuvette contained, in a total volume of 1ml, 50mM Treacl-KOH buffer, pH 7.2, 100mM KCl, 20mM $MgSO_4$, 1 mM phosphenolpyruvate, 2mM ADP, 0.2mM NADH, 2 units of lactate dehydrogenase and enzyme. The oxidation of NADH was monitored at 340nm.

2.3 (b) Standardisation of Solutions

Fructose 1, 6-bisphosphate concentrations were measured essentially as described by Bucher and Hohorst, (1963). Each cuvette contained in a final volume of 1ml, 50 mM MOPS-KOH, pH 7.0, 100mM KCl, 5mM $MgCl_2$, 0.2mM NADH, 0.5 units of each of aldolase and glycerol 3-phosphate

dehydrogenase, 5 units of triose phosphate isomerase and fructose 1, 6-bisphosphate. On addition of the sample, the change in absorbance at 340nm was measured.

Adenosine 5' monophosphate concentrations were measured by the method of Adam (1963). Each cuvette contained, in a final volume of 1ml, 50mM Treacl-KOH, pH 7.5, 100mM KCl, 0.15 mM NADH, 0.04 mM ATP, 1mM phosphoenolpyruvate, 2.1mM Mg, 0.1mM EDTA, 2 μg of lactate dehydrogenase, 2μg of pyruvate kinase, 5 μg of myokinase and AMP. On addition of myokinase the change in absorbance at 340nm was measured.

Fructose 2,6-bisphosphate ($F2,6P_2$)was measured as acid-revealed fructose 6-phosphate essentially as described by Van Schaftingen et al (1980c). Samples containing $F2,6P_2$ were brought to pH 2.0 by the addition of 0.1N HCl and were incubated at 20^{o}C for 10 minutes to allow complete hydrolysis of the 2-phosphate. Samples were then neutralised with 0.1N NaOH and fructose 6-phosphate was measured using the $F1,6P_2$ase assay system described in the previous section (minus $F1,6P_2$ase).

2.4 Polyacrylamide Gel Electrophoretic Techniques

2.4 (a) Native Polyacrylamide Gel Electrophoresis

Electrophoresis under non-denaturing conditions was performed in 7% (w/v) polyacrylamide gels at pH 8.9 and at 4^{o}C essentially as described by Davis (1964).

The following stock solutions were made up and stored at 4^{o}C.

A. 36.3g tris (hydroxymethy) aminomethane (Tris)

48 ml 1N HCl

H_2O to 100 ml

B. 28g acrylamide

0.74g N,N' - methylenebis acrylamide (bis)

H_2O to 100ml

C. 6g Tris

28.8g glycine

H_2O to 1000ml

The gel solution was prepared by mixing 4ml of solution A with 8 ml of solution B and 20ml of H_2O and adding to this 15 mg of solid ammonium persulphate and 10 μl N,N,N',N' tetramethyl ethylene diamine (TEMED). The solution was poured into gel tubes of dimensions 0.5 x 10 cm which had been coated with Repelcote 2% (v/v) dimethyl-chlorosilane in CCl_4 (Hopkin and Williams Ltd., Chadwell Heath, Essex), carefully overlaid with H_2O and allowed to set.

Electrophoresis was carried out in buffer C which had been diluted 5-fold with H_2O and brought to 0.1% (v/v) with 2-mercaptoethanol. The gels were pre-electrophoresed for 30 minutes at 3mA per tube, constant current, after which 10 μl of tracking dye (a 0.01% bromo-phenol blue solution in 20% (v/v) glycerol) was layered onto the gel surface and electrophoresis was carried out until the dye had penetrated about 1 mm into the gel. The samples were carefully loaded onto the gel surface in 20% (v/v) glycerol and electrophoresis was performed at 3mA per tube until the tracking dye had run the length of the gel. Gels were stained for protein by immersion for 1 hour at 37°C in 0.1% Coomassie Brilliant Blue G250 in methanol/acetic acid/water, 5/1/4 (v/v/v), and destained at 37°C by bathing in methanol/acetic acid/water, 1/1/8 (v/v/v), with several changes of this solution over two days.

2.4 (b) <u>F1,6P_2ase activity stain</u>

Alternatively, native gels were stained for F1,6P_2ase activity by the method of Nimmo and Tipton (1975a). Firstly, gels

were washed once in 50mM glycine-KOH buffer, pH 10.0, containing 100mM KCl and this was followed by incubation at 37°C in the F1,6P_2ase activity stain cocktail which comprised 50 mM glycine-KOH buffer, pH 10.0 containing 100mM KCl, 1mM F1,6P_2, 8mM $MgCl_2$ and 8mM $CaCl_2$. The stain appeared as a white band of precipitated calcium phosphate in the gel. This method could be used to detect F1,6P_2ase in a sample of rat liver extract electrophoresed under native conditions as described above.

2.4 (c) Urea Polyacrylamide Gel Electrophoresis

Electrophoresis of protein samples, carboxymethylated as described in section 2.5(b), in 7% polyacrylamide gels in the presence of 8M urea was carried out essentially as described above except that carboxymethylated 9.6g of urea (recrystallised from ethanol to remove ammonium cyanate) were dissolved in 12ml of gel mixture prior to the addition of TEMED. Furthermore, the electrophoresis buffer was a 10-fold dilution of buffer C and did not contain 2-mercaptoethanol. Gels were stained for protein as described in 2.4 (a).

2.4 (d) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (Phosphate System)

Electrophoresis in 7.5% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulphate (SDS) was performed essentially by the method of Weber and Osborn (1975). The following stock solutions were made up :

A. 0.6M sodium phosphate buffer, pH 6.5

B. 10% (w/v) SDS

C. 30% acrylamide/0.8% bisacrylamide (stored at 4°C)

D 10mM sodium phosphate, pH 6.5, 20% (v/v) glycerol, 1% (w/v) SDS and 0.04% (w/v) bromophenol blue

Gel mixture was prepared by mixing 4 ml of solution A, 0.4 ml of solution B, 10ml of solution C and 25.6 ml H_2O and to this, 40mg of solid ammonium persulphate and 20 μl of TEMED were added. The gel mixture was poured into tubes, overlaid with H_2O and allowed to set, as described in section 2.5(a). Electrophoresis buffer consisted of 200ml of solution A, 20ml of solution B and 1780ml H_2O. Protein samples to be denatured were mixed with an equal volume of "sample buffer" (solution D) and 5 μl of 2-mercaptoethanol then heated to 100^{o} C for 3 minutes in a boiling water bath. When cool, the samples were carefully layered onto the top of each gel and electrophoresis was carried out at 8 mA per tube constant current, until the tracking dye had run the length of the gel. The dye front of each gel was tagged with a piece of steel wire, and gels were stained for protein as described in section 2.4(a).

The destained gels were scanned at 600nm using a Gilford Unicam SP 500 spectrophotometer equipped with a Gilford gel scanner and recorder, and the electrophoretic mobility, defined as :

$$\frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}}$$

was calculated for each protein. Standard proteins were as follows :

Protein	(Subunit) Molecular Weight	Reference
bovine serum albumin	67000	Phelps & Putnam,1960
pyruvate kinase	57000	Steinmetz & Deal,1966
aldolase	40000	Kawahara & Tanford,1966
glyceraldehyde 3-phosphate dehydrogenase	36000	Harrington & Karr,1965
Carbonic anhydrase	29000	Weber & Osborn ,1975
myoglobin(from sperm whale)	17200	Weber & Osborn ,1975

A standard curve of electrophoretic mobility against log molecular weight was constructed.

2.4 (e) SDS Polyacrylamide Gel Electrophoresis (Discontinuous System)

Electrophoresis in 10% or 15% polyacrylamide slab gels in the presence of 0.1% SDS was carried out essentially by the discontinuous system described by Laemmli, (1970). The following stock solutions were made up :

A 30% acrylamide/0.8% bisacrylamide (stored at $4^{o}C$)

B. 1.5M Tris - HCl, pH 8.8 (stored at $4^{o}C$)

C. 0.5M Tris - HCl, pH 6.8 (stored at $4^{o}C$)

D. 10% (w/v) SDS

E. 0.05m Tris - HCl, pH 6.8, 1% (w/v) SDS and 10% (v/v) glycerol

A separating gel was prepared by mixing components according to the following table :

Component	10% gel	15% gel
Solution A	10 ml	15 ml
Solution B	7.5 ml	7.5 ml
H_2O	12.2 ml	7.2 ml
Solution D	0.3 ml	0.3 ml
Solid ammonium persulphate	15 mg	15 mg
TEMED	10 μl	10 μl

The gel solution was poured between glass plates, separated by 1.5 mm spacers and sealed with 1% (w/v) agarose, and was overlaid with water-saturated - butanol. When the separating gel had set, the water-saturated butanol was removed, a comb for forming lanes was inserted between the plates and a stacking gel, comprising 1.5 ml solution A, 3.75 ml solution C, 9.6 ml H_2O, 0.15 ml solution D, 10 mg solid ammonium persulphate and 10 μl TEMED was prepared, poured onto

the separating gel and allowed to set. Electrophoresis buffer consisted of 3g Tris base, 14.4g glycine and 10ml of solution D made up to 1000ml with H_2O. Protein samples to be denatured were mixed with an equal volume of "sample buffer" (solution E) and 5 μl of 2-mercaptoethanol, then heated to 100^{o}C in a boiling water bath for 3 minutes. When cooled, samples were carefully layered into each lane of the gel and electrophoresis was carried out at 50mA, constant current, until the tracking dye had travelled the length of the gel. The gel was stained for protein as described in section 2.4 (a). Standard proteins were as described in section 2.4 (d).

2.4 (f) Cleveland Mapping

One dimensional peptide mapping of proteins isolated from SDS gels was performed by the method of Cleveland et al (1977), utilising the gel system of Laemmli (1970). A 15% SDS slab gel, with stacking gel was prepared as described in the previous section (2.4(e)), and bands from SDS gels stained with Coomassie Blue were sliced out with a scalpel and cut into square "chips". Each chip was loaded into a lane on the slab gel, was overlaid with 20 μl of electrophoresis buffer containing 10% (v/v) glycerol, and was further overlaid with 20 μl of "sample buffer" containing 0.2 mg/ml α- chymotrypsin from bovine pancreas. Electrophoresis was carried out at 50mA, constant current, until the tracking dye had reached the end of the stacking gel, when the current was switched off for 30 minutes to allow digestion of proteins by the α- chymotrypsin to take place. The current was then restored and electrophoresis was continued until the tracking dye had travelled the length of the separating gel. The gel was stained for protein by the method of Wray et al (1981).

2.5 Characterisation of Rat Liver Fructose 1,6-Bisphosphatase

2.5 (a) Amino Acid Analysis

All glassware was acid washed as described in section 2.2(a).

Dialysis. 0.75 mg of homogeneous rat liver fructose 1,6-bisphosphatase were dialysed firstly against 250ml of 20mM Treacl-KOH buffer, pH 7.5 at 0.4°C overnight, and then against 3 changes, each of 5000ml for 24 hours of 0.5% (w/v) ammonium bicarbonate.

Internal Standardisation. The dialysed F1,6P_2ase was placed in a 100ml round bottom flask and to this 200 nmoles of DL-norleucine was added as internal standard. The material was subsequently lyophilised, then redissolved and lyophilised at least twice to ensure the removal of as much ammonia as possible.

Performic Acid Oxidation, was carried out by the method of Hirs (1967). This method converts cysteine to cysteic acid and methionine to methionine sulphone. The reagent was prepared by mixing 95 volumes of formic acid with 5 volumes of 30% (v/v) hydrogen peroxide and incubating the mixture for 120 minutes at -5°C, using a water /methanol/dry ice mixture to maintain the temperature. To the lyophilised protein, 0.5 ml of formic acid and 0.1ml of methanol were added and this was kept at -5°C for 30 minutes, after which 1 ml of the performic acid reagent was added to the protein and the mixture was incubated for 150 minutes at -5°C. The reaction was terminated by the addition of 15ml of H_2O and the material was lyophilised.

Acid Hydrolysis. 1.75 ml H_2O, 1.75 ml "Aristar" concentrated HCl, 3.5 μl 2-mercaptoethanol and one drop of 5% (w/v) phenol were added to the oxidised, lyophilised protein and 0.4 ml of this mixture were placed in each of 8 test tubes. The tubes were evacuated and sealed, and were heated on a block at 110° C in duplicate for periods of 24,48,

72 and 96 hours. After opening the tubes the samples were immediately lyophilised with sodium hydroxide pellets in the dessicator.

Analysis. Samples were analysed on a JLC-5AH amino acid analyser.

2.5 (b) Carboxymethylation and Peptide Fingerprinting

All glassware was acid washed as described in section 2.2 (a) Carboxymethylation was carried out essentially as described by Anfinsen and Haber (1961). A 1mg sample of rat liver fructose 1,6-bisphosphatase was dialysed exhaustively against H_2O and then against 0.1M Tris-HCl, pH 8.2. Solid, recrystallised urea was added to the sample in a small test-tube to a final concentration of 8M, and freshly prepared 0.1M dithiothreitol (DTT) was added to give a final concentration of 2mM. After thorough mixing, the tube was flushed with N_2, sealed and incubated in the dark at room temperature for at least 60 minutes to ensure complete reduction of the sample. A solution of recrystallised iodoacetic acid, 0.5M in 0.1M Tris-HCl, pH 8.2, was used to carboxymethylate the sample, and was added to a final concentration of 10mM. Again the tube was flushed with N_2 and incubated in the dark at room temperature for 60 minutes. The reaction was stopped by addition of 0.1M DTT to a final concentration of 10mM and the sample was dialysed against 2 changes of 0.1% (w/v) ammonium bicarbonate and then lyophilised.

Tryptic digest. The lyophilised, carboxymethylated protein was dissolved in 1ml of 0.5% (w/v) ammonium bicarbonate followed by addition of 1mg/ml TPCK- treated trypsin to give a protein: trypsin ratio of 100:1 by weight.

The mixture was incubated at 37°C for 16 hours with vigorous shaking and was subsequently lyophilised, dissolved in 30 μl of 30mM ammonia and stored at -20°C.

Peptide Mapping. A 5 μl sample of the tryptic digest was carefully loaded onto a 10 x 10 cm silica gel thin layer plate and electrophoresis was performed at 400V for 20 minutes in 10% (v/v) pyridine/0.5% (v/v) acetic acid, pH 6.5. When the plate was dry, chromotography of the electrophoretically separated peptides was carried out in butan-1-ol /acetic acid/water/pyridine, 15/3/10/12 by volume. Peptides were stained by spraying the plate with "ninhydrin reagent" (20 volumes 0.25% (w/v) ninhydrin in acetone plus 3 volumes 0.67% (w/v) cadmium acetate/0.33% acetic acid in H_2O) and heating it gently.

2.5 (c) Molecular Weight Estimation by Gel Filtration Chromatography

The method of Andrews (1965) was employed to estimate the molecular weight of native rat liver fructose 1,6-bisphosphatase. All steps in the procedure were carried out at 4^{o}C. A 1.1 x 32 cm column (bed volume 30.4 ml) of Sephadex G200 (superfine grade) was packed and equilibrated in 20mM Treacl-KOH buffer, pH 7.5, containing 100mM KCl, 2mM $MgCl_2$, 1mM EDTA and 0.1mM DTT. Before use, 10 mg of bovine serum albumin were applied and run through the column to saturate any possible protein binding sites. F1,6P_2ase and marker proteins of known molecular weight were loaded onto the column and run through at a flow rate of of 2.5ml/hour. Fractions of 1.25ml were collected and a plot of elution volume (Ve)/void volume (Vo) versus log molecular weight was constructed and used to estimate the molecular weight of F1,6P_2ase. The marker proteins used were as follows :

Protein	Molecular Weight	Reference
β-galactosidase	520000	Ullman et al, 1968
pyruvate kinase	237000	Steinmetz & Deal,1966
lactate dehydrogenase	144000	Castellino & Barker, 1968
malate dehydrogenase	65000	Andrews, 1965

2.5 (d) Sedimentation Equilibrium Analysis

The molecular weight of rat liver fructose 1,6-bisphosphatase was estimated by sedimentation equilibrium analysis performed in a Beckman Model E Analytical Ultracentrifuge equipped with an AN-G-T-I rotor and 12 mm double sector charcoal filled Epon cells with quartz windows. F1,6P_2ase and a sample of glyceraldehyde 3-phosphate dehydrogenase as a control, were dialysed into 20mM Treacl-KOH, pH 7.5, containing 100mM KCl, 2mM $MgCl_2$, 1mM EDTA and 0.1mM DTT. To eliminate optical artefacts, absorbances were read against this buffer and the proteins were spun down at 30000 rpm at the end of the run to allow measurement of any background absorbance. Centrifugation was carried out at 9000 rpm for 48 hours at 20^{o}C, and the molecular weights were calculated using the equation

$$M_w = \frac{2RT}{\omega^2 (1-v\rho)} = \frac{d\ln c}{dr^2}$$

where M_w is the weight average molecular weight

R is the gas constant which is 8.314×10^7 erg/degree/mol.

T is the absolute temperature in degrees K

ω is the angular velocity in radians/second and is $2\pi/60$ revs per minute

v is the partial specific volume in ml/g

ρ is the density of the solvent in g/ml

and c is the absolute concentration of the protein in mg/ml at a distance, r , in cm from the axis of rotation.

A value for the partial specific volume of F1,6P_2 ase was calculated from the amino acid composition by the method of Schachman (1957) and was found to be 0.734 ml/g. A value of 0.729 for the partial specific volume of glyceraldehyde 3-phosphate dehydrogenase was assumed (Harrington and Karr, 1965).

TABLE 2.1

Purification of the Catalytic Subunit of Cyclic AMP Dependent-Protein Kinase from Bovine Liver

Step	Volume (ml)	[Protein] (mg/ml)	Activity (unit/ml)	Specific Activity (units/mg)	Yield (%)
10 000g extract	2300	60	6700	114	100
DEAE Cellulose	1310	0.030	8233	274430	70
HAP I	61	0.083	131120	1579800	52
HAP II	10	0.289	506146	1751400	33

2.6 Purification of Cyclic AMP-Dependent Protein Kinase

The catalytic subunit of bovine liver adenosine 3' : 5' cyclic monophosphate - dependent protein kinase (EC 2.7.1.37), hereafter cAMP-PrK, was purified by the method of Sugden et al (1976), scaled down by a factor of 4 and omitting the final gel filtration step.

The purification of the catalytic subunit of cAMP-PrK is summarised in table 2.1. Following the second hydroxylapatite step the catalytic subunit was found to be 91% pure with one contaminating minor band of molecular weight 67000, as judged by SDS gel electrophoresis. The enzyme was stored in 350mM potassium phosphate, pH 6.8/0.1mM DTT at 4^{o} C with negligible loss of activity over 4 weeks.

2.7 Phosphorylation/Dephosphorylation Studies

2.7 (a) In vitro Phosphorylation of Rat Liver Fructose 1,6 Bisphosphatase

Phosphorylation of homogeneous rat liver $F1,6P_2$ase was performed at 30^{o}C in a reaction mixture which contained, in a total volume of 200 μl, 5 μM $F1,6P_2$ase (as the tetramer) 10000 units (final concentration 0.72 μM) catalytic subunit of cAMP-PrK, 2.1mM $MgCl_2$, 0.1mM EDTA, 0.4mM DTT, 35mM potassium phosphate, pH 6.8 and 0.2mM [γ-^{32}P] ATP (circa 10^7cpm/ μmole). In control experiments, either protein kinase or $F1,6P_2$ase was omitted. The reactions were initiated by the addition of the ATP and after appropriate time intervals, 20 μl samples were withdrawn and placed in microfuge tubes containing 10 μl of 50 mg/ml BSA, immediately after which 1 ml aliquots of ice cold 5% trichloroacetic acid (TCA) were added to each tube. The samples were mixed, placed on ice for at least 10 minutes to allow the protein to precipitate and spun in an Eppendorf Microfuge for 2 minutes. The supernatant fractions were discarded and the pellets were redissolved in 0.5 ml of 0.1N NaOH after which 0.5 ml aliquots of 10% TCA were added. The samples were mixed and placed on ice for 10 minutes.

Samples were again centrifuged for 2 minutes and having discarded the supernatant fractions, the pellets were washed once with 1 ml of 5% TCA and redissolved in 0.1 ml aliquots of 90% formic acid. The samples were placed in scintillation vials each containing 2 ml of scintillant (4g 2,5 diphenyloxazole in 1000 ml triton : toluene, 1:2 by volume) and counted in an Intertechnique SL 4000 liquid scintillation counter.

2.7 (b) Slicing and Counting Gels

In order to check that the ^{32}P was covalently associated with F1,6P_2ase, samples of the phosphorylated enzyme were run on native 7% polyacrylamide gels as described in section 2.4 (a), and were stained for protein or for F1, 6P_2ase activity. Gels were scanned then frozen using powdered dry ice and were sliced into 1 mm segments with a gel slicer. The segments were placed sequentially, in pairs, into scintillation vials and were covered with 0.3 ml portions of hydrogen peroxide and incubated at 37^{o}C for 24 hours or until the segments had dissolved. 3 ml portions of scintillant, (4g diphenyloxazole in 1000ml triton: toluene, 1:2 by volume), were added to each vial and the samples were then counted for ^{32}P.

2.7 (c) Preparation of Phosphorylated Rat Liver Fructose 1,6-Bisphosphatase for Kinetic and Dephosphorylation Studies

F1,6P_2ase was phosphorylated using the procedure described in the previous section (2.7(a)), scaled up by a factor of 10. Samples were taken at various times and when the incorporation of ^{32}P, was determined to be complete, the reaction mixture was brought to 2mM EDTA/100mM NaF to "freeze" the phosphorylation state and was placed on ice. In order to remove ATP and catalytic subunit, the material was filtered at 4^{o}C, through a 1.1 x 32 cm column of Sephadex G200

(superfine), equilibrated in 50mM Treacl-KOH, pH 7.5, containing 100mM KCl, 2mM EDTA, 10mM NaF and 0.1mM DTT, by the procedure described in section 2.5 (c). Fractions containing F1,6P_2ase activity were pooled, concentrated by vacuum dialysis, dialysed against the above buffer containing 40% (v/v) glycerol and stored at -20^{o} C.

2.7 (d) In Vitro Dephosphorylation of Rat Liver Fructose 1, 6 Bisphosphatase

Dephosphorylation experiments, using protein phosphatases 1, 2A and 2C from rabbit liver (Cohen 1982) were carried out at the Dept. of Biochemistry, University of Dundee in collaboration with Dr. T. Ingebritsen. Assay mixtures for each of the three protein phosphatases contained in a total volume of 60 μl in plastic Eppendorf centrifuge tubes, 50mM Tris-HCl, pH 7.0, 0.2mg/ml BSA, 10mM 2-mercaptoethanol, 1mM EGTA, 0.01% Brij 35, 1 μM fructose 1,6-bisphosphatase (containing 4 moles ^{32}P/tetramer and prepared as described in section 2.7 (b) above) and the appropriate protein phosphatase. Additionally, dephosphorylation assay mixtures for protein phosphatase contained 2mM $MnCl_2$, those for protein phosphatase 2A contained 2mM $MnCl_2$ plus 100 units of protein phosphatase 1 inhibitor 2, and those for protein phosphatase 2C contained 11mM Mg acetate plus 100 units of protein phosphatase inhibitor 2. Protein phosphatases were omitted in control samples. Following a 5 minute incubation period at 30^{o} C of all components except fructose 1,6 bisphosphatase, the reaction was initiated by the addition of substrate and was incubated at 30^{o} C for 5 minutes or for 30 minutes. To stop the reaction 100 μl of ice cold 20% trichloracetic acid were added, followed by 100 μl of 10mg/ml ovalbumin. Samples were immediately vortexed and allowed to stand on ice for 10 minutes to allow the protein to precipitate. Samples were subsequently centrifuged for 2 minutes and 200 μl of the

supernatant fractions were transferred to vials containing 1 ml scintillant ("Unisolve 100", Koch Light Laboratories Ltd, Colnbrook, Berks, U.K.). Vials were then sealed and counted. Rates of dephosphorylation were calculated and expressed as percentage release of phosphate per minute. As a comparison, phosphorylase kinase labelled with ^{32}P in the α and β sites, replaced F1,6P_2ase at the same protein concentration as a substrate for the phosphatases.

2.7 (e) In vivo Phosphorylation and Dephosphorylation of Rat Liver Fructose 1,6-Bisphosphatase

Isolated rat hepatocytes were used to determine whether the hormones insulin and glucagon had any effect on the phosphorylation or dephosphorylation of rat liver F1,6P_2ase. These experiments were performed at the Dept. of Biochemistry, University of Dundee. Hepatocytes were prepared according to the method of Seglen (1976), by Mr. Ross Holland, using one male rat fed on a high carbohydrate diet.

Hepatocytes were suspended in 40ml of Eagle's minimal essential medium containing 1.2mM phosphate and were incubated at 37^{o}C for 30 minutes. The cells were then washed with this buffer, resuspended in 40ml of this buffer containing 0.2mM phosphate plus 5mC_i of $^{32}P_i$ and incubated for 1 hour at 37^{o}C to allow the adenine nucleotide pool to become equilibrated with the ^{32}P. The suspension was divided into two vessels, hormone was added (one vessel receiving either 10^{-7}M glucagon or 10^{-9}M insulin, the other, as control having no additions), and the vessels were incubated at 37^{o}C for 15 minutes. The cells were then centrifuged at 100g for 1 minute, the supernatant fractions were removed, and the cells were resuspended in 4 ml of ice-cold homogenisation buffer, which consisted of 0.1M Tris HCl, pH 7.5, containing 2mM EDTA, 10mM 2-mercaptoethanol, 100mM NaF, 0.25M sucrose, 40 μg/ml leupeptin, 35 μg/ml TPCK, 37 μg/ml TLCK, 17 μg/ml PMSF, 156 μg/ml

benzamidine, 4 μg/ml soyabean trypsin inhibitor, 50 μg/ml antipain and 50 μg/ml pepstatin. The hepatocytes were homogenised by 40 strokes with a Dounce homogeniser and the extracts were centrifuged at 100000g for 1 hour. The supernatant fractions were collected and the pellets were discarded.

1mg of total soluble protein was run on each of twelve 7% polyacryamide gels, (as described in section 2.4 (a), 4 gels for samples from untreated cells, 4 gels for insulin treated cells and 4 gels for glucagon treated cells. As standards, another 4 gels were run, each containing 5 μg of homogeneous rat liver F1,6P_2ase. Electrophoresis was performed as described in section 2.4 (a) with 10mM NaF in the upper tank buffer, and the gels were stained overnight for F1,6P_2ase activity (as described in 2.4 (b). Bands containing F1,6P_2ase, which coincided with the bands on the standard gels, were excised, along with the standards. Each slice was placed in a plastic test tube containing 50 μl "Laemmli sample buffer" (described in 2.4 (e) and heated to 100^{o} C in a boiling water bath, during which time the gel slices absorbed the sample buffer. A 10% SDS polyacrylamide slab gel, with stacking gel, was prepared as previously described (2.4 (e), and a square "chip" dimensions 5 x 5 x 2mm was cut from each of the boiled gel samples and placed in a lane in the slab gel. Each chip was overlaid with 20 μl of sample buffer and electrophoresis was carried out as described above (2.4 (e)). The slab gel was stained for protein as previously described (2.4 (a)).

When completely destained, the gel was dried onto a piece of chromatography paper (Whatman type 3MM), placed between two glass plates with a piece of X-ray film (Kodak X-Omat) then placed in a sealed black envelope and autoradiographed. After a suitable time the film was developed and fixed. Protein bands on the gel, which were

identified as F1,6P_2ase, along with the standards, were excised and "Cleveland mapped" as described in section 2.4 (f).

2.7 (f) Estimation of Alkali-Labile Phosphate

The alkali-labile phosphate content of a freshly prepared sample of homogeneous rat liver fructose 1,6 bisphosphatase was measured by the method of Nimmo et al (1976) using glycogen phosphorylases b and a as control and standard respectively.

A sample of glycogen phosphorylase a was prepared in a reaction mixture which contained, in a total volume of 2.5 ml, 40mM Tris - 40mM α- glycerol phosphate, pH 6.8, 0.13 mg/ml glycogen phosphorylase kinase, 3.5 mg/ml glycogen phosphorylase b (freed of AMP by treatment with charcoal), 20 μM $CaCl_2$, 8.8 mM $MgCl_2$ and 0.4 mM [$\gamma - ^{32}P$] ATP (circa 10^7 cpm/μmol). The mixture was incubated at 30^0C for 1 hour after which a sample was taken and acid precipitated (as described for the in vitro phosphorylation of F1,6P_2ase in section 2.7(a)) to verify that the incorporation of ^{32}P was complete. Phosphorylase a was precipitated by adding an equal volume of ice cold 90% (w/v) ammonium sulphate, mixing and leaving on ice for 10 minutes. Following centrifugation for 2 minutes in the microfuge, the supernatant fraction was discarded, the pellet was redissolved in 50mM Tris HCl, pH 7.0 containing 50mM 2-mercaptoethanol and 1mM EDTA, and the precipitation procedure was repeated. The pellet was then dissolved in 1-2 ml of the above buffer containing 250mM NaCl and was dialysed against this buffer.

Precipitation and Hydrolysis 10 n moles (in terms of subunits) of each of F1, 6P_2ase, phosphorylase a and phosphorylase b (free of AMP) were precipitated by adding an equal volume of 10% (w/v) TCA, placing on ice for 10 minutes and centrifuging for 2 minutes in a microfuge. On removal of the supernatant fractions, the pellets were redissolved in

0.5 ml of 0.1N NaOH, reprecipitated by adding 0.5 ml of 10% TCA and placed on ice for 10 minutes. Following centrifugation for 2 minutes, the pellets were washed twice with 1ml of 5% TCA, dissolved in 0.3 ml of 0.1N NaOH and incubated at 37°C for 20 hours to hydrolyse alkali-labile bonds.

Assay for Inorganic Phosphate. After the hydrolysis, 0.1 ml portions of 100% TCA were added to each of the samples which were then placed on ice for 10 minutes and centrifuged for 2 minutes to remove any unhydrolysed polypeptide. 0.3 ml of the supernatant fractions were removed for the assay of phosphate, and 0.2 ml of 25% TCA were added to the remainder of the samples in order to wash the pellets. After centrifugation for 2 minutes, the supernatant fractions were removed for the assay of phosphate. 0.7 ml of freshly prepared molybolate/ascorbate reagent, consisting of 1 volume of 10% (w/v) ascorbic acid plus 6 volumes of 0.42% (w/v) ammonium molybdate in 1N H_2SO_4, were added to each of the samples and also to 0.3ml samples containing 0-15 n moles of KH_2PO4 in 25% TCA as a means of standardisation. Samples and standards were incubated at 45°C for 20 minutes and the absorbance of each was measured at 820 nm. A standard curve of E_{820} against n moles of phosphate was constructed and used to estimate the amount of phosphate present in each of the samples.

2.8 Synthesis and Purification of Fructose 2,6-Bisphosphate

A slight modification of the method of Van Schaftingen and Hers (1981a) was employed for the synthesis and purification of fructose 2,6-bisphosphate ($F2,6P_2$). This method involves the formation of fructose 1:2 cyclic, 6-bisphosphate by treatment of fructose 1,6-bisphosphate with dicyclohexylcarbodimide in aqueous pyridine, and the subsequent formation of a fructose 1,6-bisphosphate/fructose 2,6-

bisphosphate mixture by alkaline hydrolysis of the cyclic diester. $F2,6P_2$ was detected by its ability to stimulate phosphfructokinase - 1 activity, as measured by an increase in the activity ratio of the enzyme, (Van Schaftingen et al, 1980a). The concentration of $F2,6P_2$ was measured as acid revealed F6P as described in section 2.3 (b).

Synthesis. The synthesis of $F2,6P_2$ in the presence of dicyclohexylcarbodiimide and alkaline hydrolysis of the cyclic intermediate were carried out as previously described (Van Schaftingen and Hers, 1981a), starting with 0.72 m moles of [U-^{14}C]$F1,6P_2$, specific activity 1 μC_i/mmole. The time course for the synthesis of the cyclic diester is presented in figure 2.1. After the alkaline hydrolysis, the mixture was cooled on ice and glycine and $MgCl_2$ were added to give final concentrations of 20mM and 10mM respectively in a final volume of 90ml. The pH was then adjusted to 9.4 by the dropwise addition of 2N HCl. $F1,6P_2$ was hydrolysed to F6P and P_i by incubation of the mixture at 30^{o}C for 3 hours in the presence of 0.2 units/ml rabbit muscle $F1,6P_2$ase. The decrease in $F1,6P_2$ concentration was monitored throughout the incubation as described in section 2.3 (b).

Purification. When $F1,6P_2$ was completely hydrolysed, the solution was diluted 5-fold with H_2O and loaded onto a 1.1 x 12 cm column of Dowex 1-X8 (Cl^- form) at a flow rate of 114 ml/hour. The column was washed with 100ml of 0.1M NaCl after which $F2,6P_2$ was eluted with a linear gradient of 0.1 - 0.4M NaCl, flow rate 22ml/hour and 2.3 ml fractions were collected. Fractions containing $F2,6P_2$ were pooled and stored at -20^{o}C. (A profile of the elution of $F2,6P_2$ from Dowex 1-X8 is given in figure 2.2). The overall yield of $F2,6P_2$, as measured enzymatically and by radioactivity, was 4% which is comparable with the value of 7% obtained by Van Schaftingen and Hers (1981a).

Figure 2.1

Time Course of the Synthesis of Fructose 1:2 cyclic, 6-Bisphosphate

150 μl samples were taken, added to 2 ml H_2O and extracted 3 times with 5 ml of diethyl ether. The samples were assayed for $F1,6P_2$ as described in 2.3(b) and 20 μl aliquots were counted for ^{14}C. Prior to measurement of acid revealed-F6P as described in 2.3(b), aliquots were brought to 0.5 N with NaOH and incubated at $37^{o}C$ for 30 minutes to open up the cyclic diester.

The symbols are as follows:

$F1,6P_2$	○
acid revealed F6P	□
Radio^activity	◊

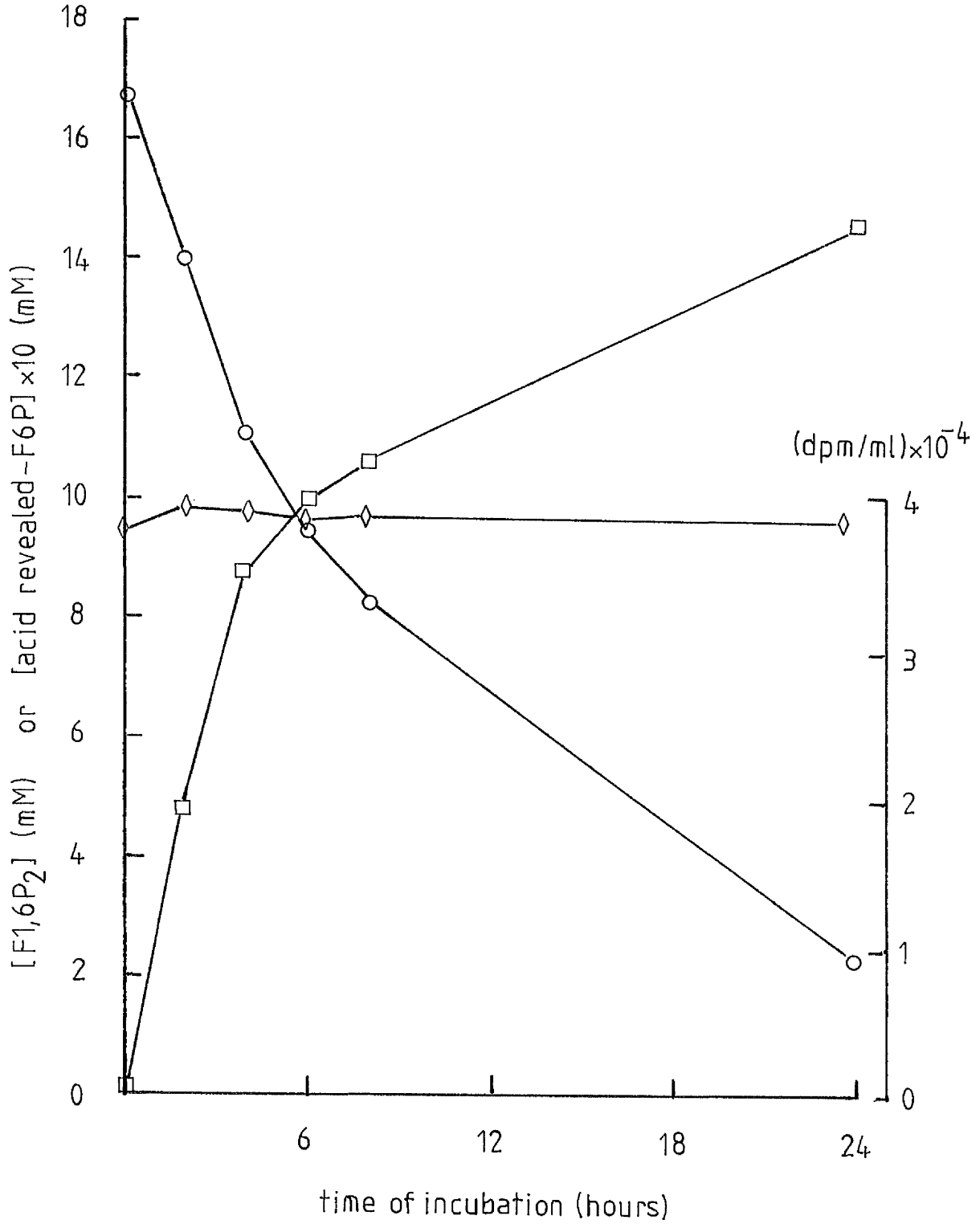

18
16
14
12
10
8
6
4
2
0
[F1,6P2] (mM) or [acid revealed-F6P] x10 (mM)
(dpm/ml)x10⁻⁴
4
3
2
1
0
6
12
18
24
time of incubation (hours)

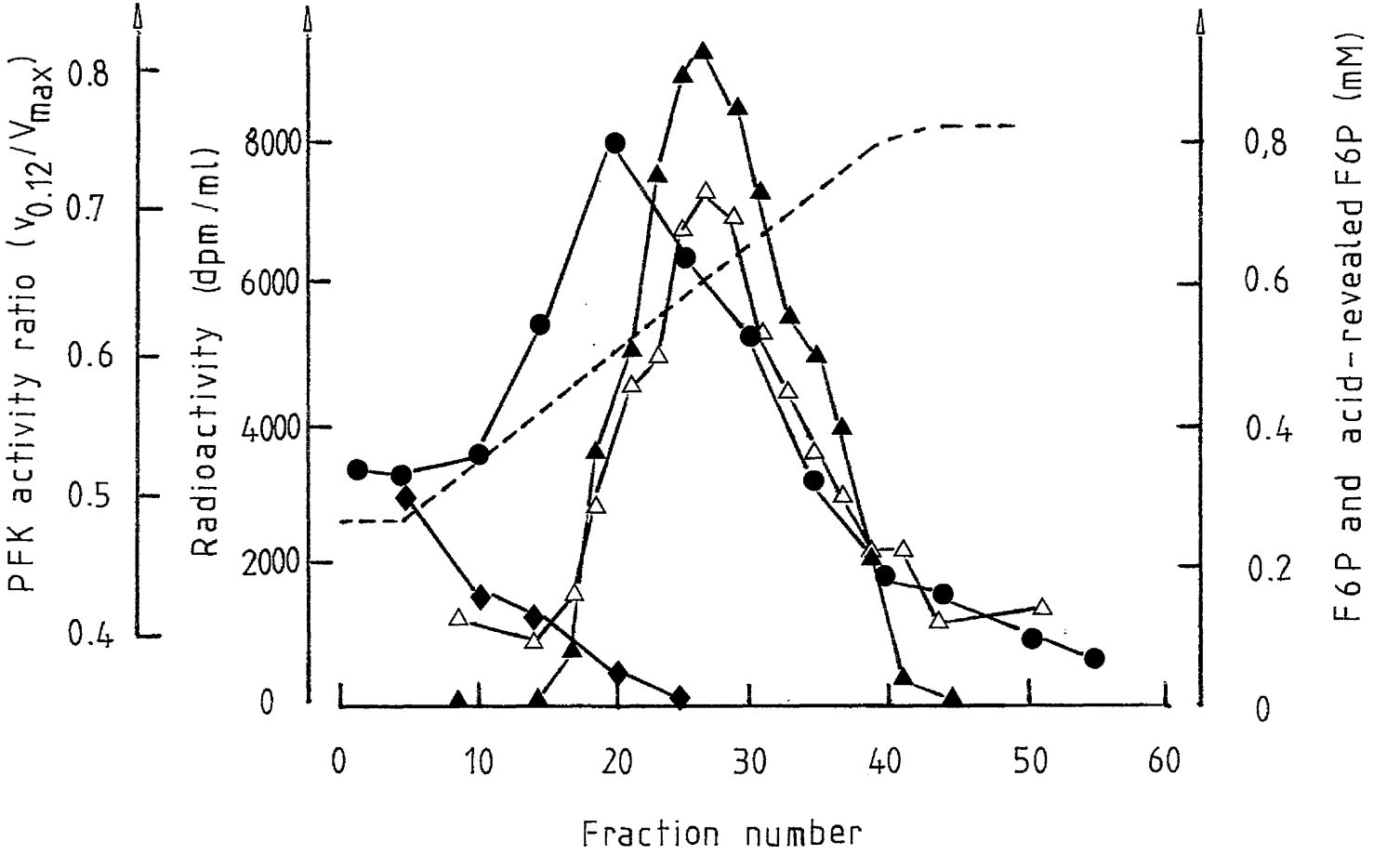
Figure 2.2

Elution of Fructose 2,6-Bisphosphate from Dowex 1-X8

Fructose 2,6-bisphosphate was eluted from a column of Dowex 1-X8 using a linear gradient of 0.1 - 0.4 M NaCl as described in the text.

The symbols are as follows:

●	Radioactivity
◆	Fructose-6-phosphate
▲	Acid revealed Fructose-6-phosphate
△	PFK-1 Activity Ratio
--------	NaCl gradient


PFK activity ratio ($v_{0.12}/V_{max}$)
0.8
0.7
0.6
0.5
0.4
Radioactivity (dpm/ml)
8000
6000
4000
2000
0
F6P and acid-revealed F6P (mM)
0,8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
Fraction number

Purity. Samples of purified $F2,6P_2$ (1-5 μg) were spotted onto Whatman chromatography paper (type 1) along with samples of $F1,6P_2$, F6P and P_i as standards, and were chromatographed in isobutanol: ethanol: water: ammonia solution (specific gravity 0.88), (26:34:40:0.35 by volume) as described by Pollard and Nickless (1965) for phosphorus containing anions. Chromatograms were stained by spraying with a reagent, consisting of 1 volume 3.3% (w/v) $AgNO_3$ plus 1 volume of 0.3% (w/v) sodium fluorescein, which gave a strong blue colour with phosphate containing compounds. Alternatively, using the method of Hough (1954), chromatograms were stained for reducing sugars by spraying with a reagent which consisted of 1 volume of 0.1N $AgNO_3$ plus 1 volume of 5N ammonia, followed by heating at 100-110^{o}C for 5 minutes. By this method, reducing sugars appeared as dark brown spots on a light brown background.

Using either method of staining, a single spot was obtained with $F2,6P_2$. The Rf values for $F2,6P_2$ and the standards are given below :

Compound	Rf value
$F2,6P_2$	0.33
$F1,6P_2$	0.32
F6P	0.39
P_i	0.26

2.9 Kinetic and Binding Studies

2.9 (a) Kinetic Studies

Kinetic analyses of rat liver fructose 1,6-bisphosphatase were carried out using the coupled system described in section 2.3 (a) with slight modifications. Each cuvette contained, in a total volume of 2 ml, 50mM Treacl-KOH buffer, pH 7.2, 100mM KCl, 0.01mM EDTA, 5 units of phosphoglucose isomerase and 3 units of glucose 6 - phosphate

dehydrogenase. The concentrations of free Mg^{2+} ions, AMP and $Fl,6P_2$ were calculated using values of $250M^{-1}$ and $35M^{-1}$ for the association constants of $MgFl,6P_2$ and MgAMP respectively, (Nimmo and Tipton, 1975b). A value of $350M^{-1}$ for the association constant of $MgF2,6P_2$ was obtained using the method of Burton (1959). The reaction was initiated by the addition of enzyme, and the reduction of $NADP^+$ was monitored at 25°C on a Hitachi Perkin-Elmer MPF 2A fluorescence spectrophotometer with the excitation wavelength set at 340nm and the emission wavelength set at 460nm. The chart recorder was calibrated to give a full scale deflection of 0.5 μM NADPH at 25°C.

Statistical treatment of the kinetic data presented as double reciprocal plots was carried out using a program which performed a weighted least squares linear regression analysis modified to determine the kinetic parameters K_m and V_{max} and their respective errors (Roberts, 1977). The data presented as Hill plots were processed using a program which carried out a non-weighted least squares fit to a polynomial (Bevington, 1969).

2.9 (b) Binding Studies

Measurement of the binding of AMP to rat liver fructose 1,6-bisphosphatase was performed by equilibrium dialysis, using a small volume multichamber equilibrium dialysis apparatus with rotator described by Furlong et al (1972) and constructed by Mr N. Harvey in the Biochemistry Department of Glasgow University. Single thickness membranes, which were cut from dialysis tubing and pretreated as described in section 2.2 (f), were blotted and inserted between the chambers as previously described (Furlong et al, 1972). Chambers were filled using glass microsyringes. Fructose 1,6-bisphosphatase was dialysed into 50mM Treacl-KOH, pH 7.5, containing 100mM KCl, 10 μM EDTA and 0.1mM DTT and 100 μl of 4 μM enzyme (in terms of subunits)

was placed in the left hand side of each chamber. The 100 μl of solution placed in the right hand side of each chamber contained [2- ^{3}H]AMP, specific activity 680 μC_i/ μmole with or without fructose 1,6-bisphosphate or fructose 2,6-bisphosphate made up in the buffer described above. When all the chambers had been filled the ports were sealed with a single strip of "lasso" tape and the chambers were rotated at 2.5 rpm at $4^{o}C$ overnight to allow the AMP to equilibrate across the membrane. Control experiments with buffer on the left hand side and buffer containing AMP on the right hand side of the chamber, or vice versa showed that after incubation overnight the total numbers of counts on each side of the membrane were equal and that equilibrium had therefore been reached. To measure the binding of AMP to the enzyme 80 μl samples were withdrawn at equilibrium from both sides of each chamber and placed in vials containing 2 ml scintillant (4g diphenyloxazole in 1000 ml triton: toluene, 1:2 by volume). The samples were counted and the concentration of free AMP was calculated from the number of counts on the side of the membrane which did not contain any enzyme. Since the free AMP concentration is equal on both sides of the membrane at equilibrium, the difference in counts between the two sides represents the bound AMP.

CHAPTER 3

THE PURIFICATION AND CHARACTERISATION OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE

3.1 Introduction

A procedure for the purification of fructose 1,6-bisphosphatase from rat liver was developed with the objective of reproducibly providing good yields of homogeneous, unproteolysed, fully active enzyme with intact regulatory properties.

In all recent reports of the study of rat liver F1,6P_2ase, the enzyme has been purified using a procedure described in either one of the following communications :

Traniello, 1974

Tejwani et al, 1976

Riou et al, 1977

Zalitis and Pitot, 1979

All of these methods employ adsorption on negatively charged cellulose resins followed by substrate elution, a procedure which is of considerable advantage because F1,6P_2ase is eluted specifically with a corresponding increase in specific activity of up to 250-fold (Traniello, 1974). For this reason, a step involving the specific elution of F1,6P_2ase from immobilised procion Red HE-3B (Watson et al, 1978) has been included in the procedure described below. Most methods also utilise heat treatment at 65^{o}C as a means of fractionation. This step, however, provides at best a 4-fold increase in the specific activity of the enzyme, (Tejwani et al, 1976) and it might be argued that heating at 65^{o}C could significantly impair or alter the regulatory properties of the enzyme. A heat step has therefore been omitted from the procedure described below.

Two methods of preventing proteolysis which have been included in the purification procedure are the homogenisation of livers in isotonic sucrose solution to prevent disruption of lysosomes,

followed by centrifugation to remove particulate material (Traniello et al, 1972), and the inclusion of protease inhibitors in all the buffers.

3.2 Purification of Rat Liver Fructose 1,6-Bisphosphatase

All stages in the preparation were carried out as quickly as possible and at 0-4^{o}C

(a) Preparation of a crude extract

Six male or female Wistar rats, fed on a normal diet, were killed by cervical dislocation and the livers were immediately removed and immersed in ice-cold 0.25M sucrose solution. After several washings in this solution to remove any blood, the livers were placed in 4 volumes of 0.25M sucrose containing 2mM $MgCl_2$, 1mM EDTA, 0.4mM DTT and 1mM benzamidine, then homogenised for 3 bursts of 10 seconds in a Waring Blendor. A solution of phenylmethane sulphonyl fluoride (PMSF) in 95% (v/v) ethanol was prepared, (200 mg PMSF/35 ml ethanol/ litre of homogenate), and was stirred into the homogenate. This was immediately centrifuged at 23000 g for 30 minutes after which the supernatant fraction was filtered through muslin and the pellet was discarded.

(b) Ammonium Sulphate precipitation

The supernatant fraction from the previous step was brought to pH 7.4 by the dropwise addition of ice-cold 1N KOH and was then brought to 30% saturation by the slow addition of crushed ammonium sulphate, with stirring,keeping the pH between 7.0 and 7.4. This was kept on ice, stirring, for 20 minutes and was subsequently centrifuged at 23000g for 30 minutes. The supernatant fraction was removed, discarding the pellet, and brought to 50% saturation with ammonium sulphate.

After 20 minutes stirring, on ice, centrifugation was performed at 23000g for 30 minutes. The supernatant fraction was discarded and the pellet was redissolved in a minimal volume of 20mM triethanolamine hydrochloride (Treacl) KOH buffer, pH 7.5, containing 1mM $MgCl_2$, 0.4mM DTT and 1mM benzamidine, (hereafter buffer A). This material was then desalted by passage through a 4.4 x 14 cm column of Sephadex G25 (coarse grade), equilibrated in buffer A.

(c) Ion exchange chromatography

The desalted material was loaded onto a 4.4 x 8 cm column of diethylaminoethyl cellulose (DEAE - cellulose) equilibrated in buffer A, and was washed with this buffer until the effluent was protein free as measured by the Bradford method, (Bradford, 1976). The column was developed with a 1200ml linear gradient of 0-0.2M KCl in bufferA. The flow rate was 240ml/hour and 12 ml fractions were collected. Fl,6P_2ase was eluted as a single peak in the range 0.07 to 0.13 M KCl, (for a profile, see figure 3.1). Fractions containing Fl,6P_2ase activity greater than 0.5 units/ml were pooled and desalted by overnight dialysis against 10 volumes of buffer A.

(d) Dye-ligand chromatography

The desalted material was loaded onto a 4.4 x 4 cm column of Procion Red HE-3B-Sepharose 6B (cross-linked) equilibrated in buffer A and was washed with this buffer until the effluent was protein free as judged by the Bradford method. Fl,6P_2ase was specifically eluted with a 500ml step of 100 μM Fl,6P_2/100 μM AMP in buffer A. The activity profile showed a single broad peak, (figure 3.2). The flow rate was 180 ml/hour and 16 ml fractions were collected. Fl,6P_2ase was concentrated by loading the pooled fractions from the peak onto a 1 ml column of DEAE-cellulose equilibrated in buffer A, and was

eluted with a 5ml step of 0.2M KCl in buffer A.

(e) Gel filtration chromatography

The concentrated material from the previous stage was carefully loaded onto a 2.2 x 83cm column of Sephadex G200 (superfine grade) which had been equilibrated in 20 mM Treacl-KOH, pH 7.5, containing 2mM $MgCl_2$, 1mM EDTA, 100mM KCl, 0.4mM DTT and 1mM benzamidine, (buffer B), and was developed with buffer B at a constant flow rate of 10 ml/hour, (for a profile see figure 3.3). Fractions of 5 ml were collected and those containing Fl,6P_2ase activity were pooled and concentrated by vacuum dialysis. The concentrated Fl,6P_2ase was dialysed against 40% (v/v) glycerol inbuffer B and was kept at -20^{o}C. When stored in this way, enzyme activity, "pH ratio" and sensitivity to AMP inhibition remained constant over a period of three months.

3.3 Purity and Molecular Weight

The purified Fl,6P_2ase appeared to be homogeneous by the criterion of polyacrylamide gel electrophoresis. Native 7% polyacrylamide gels stained for protein showed a single band which coincided with a band of precipitated calcium phosphate on gels stained for Fl,6P_2ase activity, (figure 3.4). The purified enzyme also gave a single band on 7.5% polyacrylamide gels run in the presence of 0.1% (w/v) SDS (figure 3.5).

The subunit molecular weight of rat liver Fl,6P_2ase was measured by SDS polyacrylamide gel electrophoresis using the system of Weber and Osborn (1975) and also the system of Laemmli (1970). The mobilities of marker proteins of known molecular weight were measured and were used to construct standard curves of electrophoretic mobility against log (subunit) molecular weight (figures 3.6 and 3.7). Comparison of the mobility of Fl,6P_2ase with these markers gave values

in the range 39000-40000 and 40000--42000 with the Weber and Osborn and the Laemmli systems respectively. To verify this value, Fl,6P_2ase was run along with internal marker proteins. Rat liver Fl,6P_2ase has a smaller electrophoretic mobility than glyceraldehyde 3-phosphate dehydrogenase (figure 3.5) or rabbit muscle Fl,6P_2ase (not illustrated) and therefore has a greater subunit molecular weight. Fl,6P_2ase and aldolase were coincident, however, which suggests that both proteins have very similar subunit molecular weights.

The molecular weight of undissociated Fl,6P_2ase was estimated by gel filtration chromatography. Standard proteins of known molecular weight were used to calibrate a column of Sephadex G200 and a standard curve of elution volume (V_e) /void volume (V_o) against log molecular weight was constructed (figure 3.8). The ratio Ve/Vo for rat liver Fl, 6P_2ase corresponds to a molecular weight of 158000 for the undissociated enzyme. Sedimentation equilibrium analysis was also employed to measure the molecular weight of the enzyme. Fl,6P_2ase gave a linear plot of ln c against r^2 which is typical of a non-associating species (figure 3.9). A value of 0.71 was obtained for the slope, from which a molecular weight of 148000 was calculated for the enzyme. As a control, the molecular weight of glyceraldehyde 3-phosphate dehydrogenase was also measured. Figure 3.10 gives a value of 0.75 for the slope from which a molecular weight of 151000 was obtained. This value is in close agreement with the known molecular weight of 144000 for glyceraldehyde 3-phosphate dehydrogenase, (Harrington and Karr, 1965).

Polyacrylamide gel electrophoresis of carboxymethylated Fl,6P_2ase was carried out in the presence of 8M urea and a single protein band was observed, indicating that the enzyme contains only

Table 3.1

The amino and composition of rat liver fructose 1,6-bisphosphatase

Performic acid-oxidised rat liver F1,6P_2ase was hydrolysed and analysed for amino acid content as described in the text. The presence of tryptophan in a sample which had not been hydrolysed was assayed by fluorimetry. Owing to the performic acid oxidation, cysteine and methionine were measured as cysteic acid and methionine sulphone respectively. All values are based on a subunit molecular weight of 40000.

Amino acid	Content		
	average residues/subunit	± S.D.	assumed residues/tetramer
lysine	24.3	1.8	96
histidine	4.7	0.7	20
arginine	14.2	1.5	56
cysteine	9.1	0.5	36
aspartic acid	44.8	3.2	180
threonine	19.6	0.7	80
serine	32.0	1.2	128
glutamic acid	36.6	1.5	148
proline	19.9	2.1	80
glycine	29.9	2.7	120
alanine	31.0	1.4	124
valine	22.7	1.5	92
methionine	8.7	0.6	36
isoleucine	27.0	1.9	108
leucine	32.5	1.0	132
tyrosine	15.5	0.6	64
phenylalanine	10.3	0.4	40
tryptophan	0		0

To account for the loss of serine and threonine throughout the hydrolysis, the values for these amino acids were obtained by extrapolation to zero time. The values for isoleucine and valine were estimated from the samples taken at 72 and 96 hours since the recovery of each of these two amino acids was not complete before 72 hours of acid hydrolysis.

Table 3.2

Comparison of the amino acid content of rat liver fructose 1,6-bisphosphatase from this study with analyses performed by Tejwani et al., 1976 (based on a subunit molecular weight of 38000) and Traniello, 1974 (based on a subunit molecular weight of 36000)

	Content/tetramer		
	This study	Tejwani et al., 1976	Traniello, 1974
lysine	96	86	172
histidine	20	15	48
arginine	56	48	155
cysteine	36	28	24
aspartic acid	180	148	125
threonine	80	62	49
serine	129	96	56
glutamic acid	148	114	136
proline	80	64	55
glycine	120	100	49
alanine	124	120	60
valine	92	79	89
methionine	36	24	39
isoleucine	108	92	77
leucine	132	110	104
tyrosine	64	52	70
phenylalanine	40	37	60
tryptophan	0	0	8
	1541	1275	1376

(gives M_r = 166000)

one type of subunit (figure 3.11). This conclusion is supported by the 2-dimensional "fingerprint" of peptides produced by an extensive tryptic digest of carboxymethylated Fl,6P_2ase, (figure 3.12) which shows that 27-30 peptides were separated. This value compares well with 24 lysines and 14 arginines per subunit of molecular weight 40000 as shown in table 3.1. It can therefore be concluded that the undissociated rat liver Fl,6P_2ase is a tetramer composed of very similar or identical subunits.

3.4 Amino Acid Composition

The amino acid composition of rat liver Fl,6P_2ase which is presented in table 3.1 and compared to other analyses communicated in the literature in table 3.2, shows close similarity to the composition reported by Tejwani et al (1976) but differs significantly from the analysis reported by Traniello (1974). To determine whether tryptophan was present, a sample of unhydrolysed rat liver Fl,6P_2ase was scanned at 25^{o} C in a Hitachi Perkin-Elmer MPF 2A fluorescence spectrophotometer. Although the fluorescence spectrum (A in figure 3.13) shows an emission peak at 318 nm, an emission peak at 340 nm characteristic of tryptophan containing proteins was not observed. When a sample of rat liver Fl,6P_2ase was unfolded in the presence of 4M urea (b in figure 3.13), the peak at 318 nm was absent from the fluorescence spectrum, but an emission peak at 305 nm was observed. The fluorescence spectrum of tyrosine (C in figure 3.13) also shows an emission peak at 305 nm. These results suggest that rat liver Fl,6P_2ase does not contain tryptophan, and that the emission peak observed at 318 nm with the undissociated enzyme is due to "red shifted" tyrosine residues.

PURIFICATION TABLE FOR RAT LIVER

FRUCTOSE 1,6 BISPHOSPHATASE

Step	Volume (ml)	Activity (units/ml)	Recovery (units)	Yield (%)	Protein (mg/ml)	Specific Activity (units/mg)	% inhibition by 50uM AMP	"pH ratio"	Purification factor
Crude extract	280	3.8	1064	100	18	0.21	53	4.1	1
30-50% $(NH_4)_2SO_4$	40	21.3	851	80	35	0.61	50	3.9	2.9
Ion exchange chromatography on DEAE-cellulose	260	2.5	649	61	1.2	2.08	50	4.0	9.9
Dye-ligand chromatography on procion red sepharose	500	0.53	265	25	0.02	26.5	52	3.9	126
Gel filtration chromatography on Sephadex G200	24	7.5	180	17	0.125	60.0	49	3.9	286

Table 3.4

Comparison of values reported for the specific activity of rat liver fructose 1,6-bisphosphatase

Source	Specific Activity
This study	60 units/mg at 37°C
Traniello, 1974	21 units/mg at 23°C
Tejwani et al., 1976	29 units/mg at 25°C
Riou et al., 1977	34-46 units/mg at 30°C
Zalitis and Pitot, 1979	34-36 units/mg at 30°C
Ekman and Dahlqvist-Edberg, 1981	10 units/mg at 30°C

3.5 Discussion

The procedure for the purification of rat liver Fl,6P_2ase described in this study routinely and reproducibly produced homogeneous enzyme, as judged by polyacrylamide gel electrophoresis, in an overall yield of 20-25%. A summary of the procedure is presented in table 3.3, which shows that a 286-fold purification was achieved with a corresponding final specific activity of 60 units/mg at 37^{o}C. Taking into account differences in temperature and assay conditions, this value compares well with the values reported by other workers (table 3.4).

Artifacts arising as a result of proteolytic degradation of Fl,6P_2ase during the purification procedure have been discussed in Chapter 1. In order to prevent proteolysis occurring, PMSF was added at the homogenisation stage and benzamidine was included in all the buffers. Since PMSF irreversibly modifies serine residues in the serine proteases, the possibility that PMSF might also modify serine residues which are phosphorylated by cyclic AMP-dependent protein kinase was considered. To test this hypothesis, ^{35}S-labelled PMSF was included in one preparation. No incorporation of ^{35}S into Fl,6P_2ase could be detected, and the purified enzyme could be phosphorylated to the extent of 4 moles of phosphate per mole of Fl,6P_2ase (as the tetramer) as has been previously reported, (Riou et al, 1977). Two criteria for proteolytic degradation of Fl,6P_2ase during the procedure are the "pH ratio" and the sensitivity to inhibition by AMP (Nimmo and Tipton, 1975a). As discussed in Chapter 1, proteolysed Fl,6P_2ase has a pH optimum in the range 9.0-9.6 and therefore the "pH ratio", that is the ratio of activities of Fl,6P_2ase at pH 7.5 and pH 9.6, was employed to determine whether proteolysis

occurred at any stage of the purification procedure. Proteolysis also results in a loss of sensitivity to inhibition by AMP and therefore proteolysis could be detected by a fall in the percentage inhibition given by 50 μM AMP. The data in table 3.3 clearly demonstrate that the pH ratio and the percentage inhibition given by 50 μM AMP remained constant throughout the preparation and therefore no proteolysis occurred as judged by these criteria.

During the development of the purification procedure detailed in this Chapter, several types of resin were examined with respect to substrate elution. Fl,6P_2ase did not bind significantly to either carboxymethyl-cellulose (CM52) or phosphocellulose (P11) above pH 5.5. Purification was also attempted using "blue-sepharose" and "affi-gel blue" columns, but the enzyme did not bind to either of these resins at neutral pH. However, Fl,6P_2ase was successfully retained by a column of cross-linked Procion Red HE-3B -Sepharose 6B, then eluted with a step of 100 μM Fl,6P_2/100 μM AMP. The enzyme could not be eluted using either 100 μM Fl,6P_2 or 100 μM AMP alone. The elution profile (figure 3.2) shows that Fl,6P_2ase was eluted as a broad peak of low activity in a volume of about 500 ml. This material could easily be concentrated since the Fl,6P_2ase could be loaded onto and retained by a 1 ml column of DEAE-cellulose equilibrated in buffer A, then eluted with a 5 ml step of 0.2M KCl in buffer A. By increasing the eluting power five-fold, the enzyme could be brought off the procion red-sepharose column in a much smaller volume, but this resulted in several other proteins also being eluted. Following concentration using the small DEAE-cellulose column, three minor bands of molecular weights 220000, 33000 and 57000 which were present, as judged by SDS gel electrophoresis, could be removed by passage through a column of Sephadex G200. The elution profile (figure 3.3) indicates that the pH

Table 3.5

The reported subunit molecular weights of rat liver fructose 1,6-bisphosphatase

Source	Subunit molecular weight	Gel system used
This study	39000-40000	Weber and Osborn , 1975
This study	40000-42000	Laemmli, 1970
Traniello, 1974	36000	Weber and Osborn , 1969
Tejwani et al., 1976	38000-39000	Weber and Osborn , 1969
Riou et al., 1977	36000	Fairbanks et al., 1971
Zalitis and Pitot, 1979	36000	Swictz and Ip, 1974
Hosey and Marcus, 1981	40000-42000	Laemmli, 1970
Ekman and Dalqvist-Edberg, 1981	37000	not specified
Claus et al., 1981b	36000	Fairbanks et al., 1971
Claus et al., 1981b	41000	Laemmli, 1970

ratio and the sensitivity to inhibition by AMP were constant in all fractions of the peak, and therefore the enzyme was unproteolysed at this, the final stage in the purification.

Although a subunit molecular weight of 36000 has consistently been reported for "native" mammalian Fl,6P_2ases (Horecker et al, 1975), there has been some disagreement in the literature as to the subunit molecular weight of the rat liver enzyme (table 3.5). In this study, a subunit molecular weight in the range 39000 - 42000 was obtained using two systems of SDS polyacrylamide gel electrophoresis, and the use of internal marker proteins has clearly supported this result. The inconsistency in the values reported for the subunit molecular weight might be due to limited proteolysis of Fl,6P_2ase during purification of the enzyme, bearing in mind that Hosey and Marcus (1981) have observed that preparations of rat liver Fl,6P_2ase consisted of several closely migrating bands of protein, each having a molecular weight greater than 36000 on SDS polyacrylamide gels. One possible approach to testing the proteolysis hypothesis would be to determine whether Fl,6P_2ase immunoprecipitated from a freshly prepared rat liver extract and purified Fl,6P_2ase mixed together could be separated by gel electrophoresis. Alternatively, iso-electric focussing would determine whether purified and immunoprecipitated Fl,6P_2ase had the same isoelectric point. Claus et al (1981b) have suggested that the molecular weight value obtained depends on the particular gel system used. The comparison given in table 3.5 suggests that slightly higher molecular weight values are consistently obtained using the Laemmli (1970) system, which would appear to support this hypothesis to some extent. The explanation for these differences, however, remains unclear.

Figure 3.1

Ion-exchange chromatography of rat liver fructose 1,6-bisphosphatase using DEAE-cellulose.

The column was equilibrated, loaded and developed as described in the text. The symbols are as follows:

○ protein concentration

□ $F1,6P_2$ase activity

X KC1 concentration

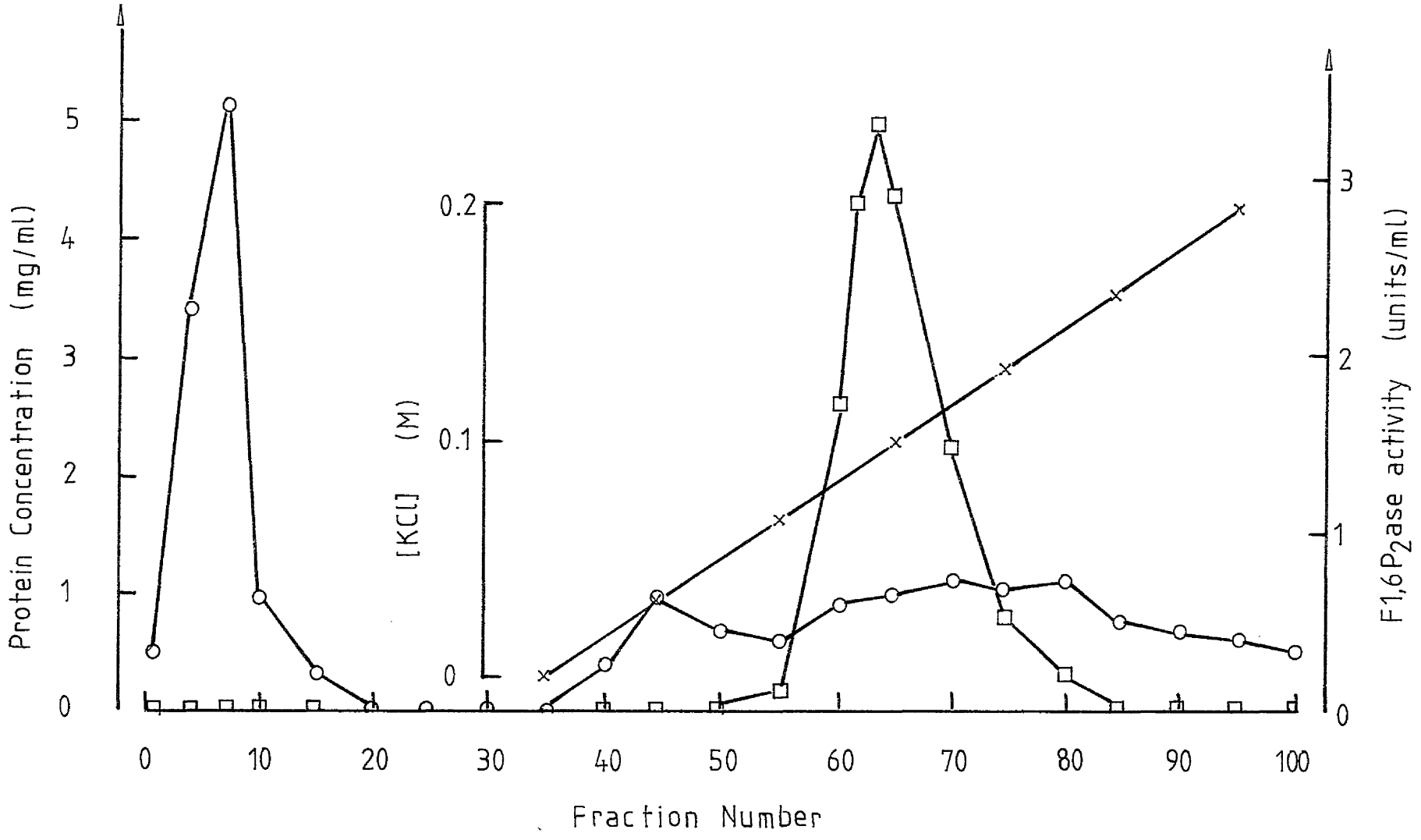

Protein Concentration (mg/ml)
[KCl] (M)
F1,6P2ase activity (units/ml)
Fraction Number
0
1
2
3
4
5
0
0.1
0.2
0
1
2
3
0
10
20
30
40
50
60
70
80
90
100

Figure 3.2

Dye-ligand chromatography of rat liver fructose 1,6-bisphosphatase using cross-linked Procion Red HE-3B-Sepharose 6B

Details of the procedure are given in the text.

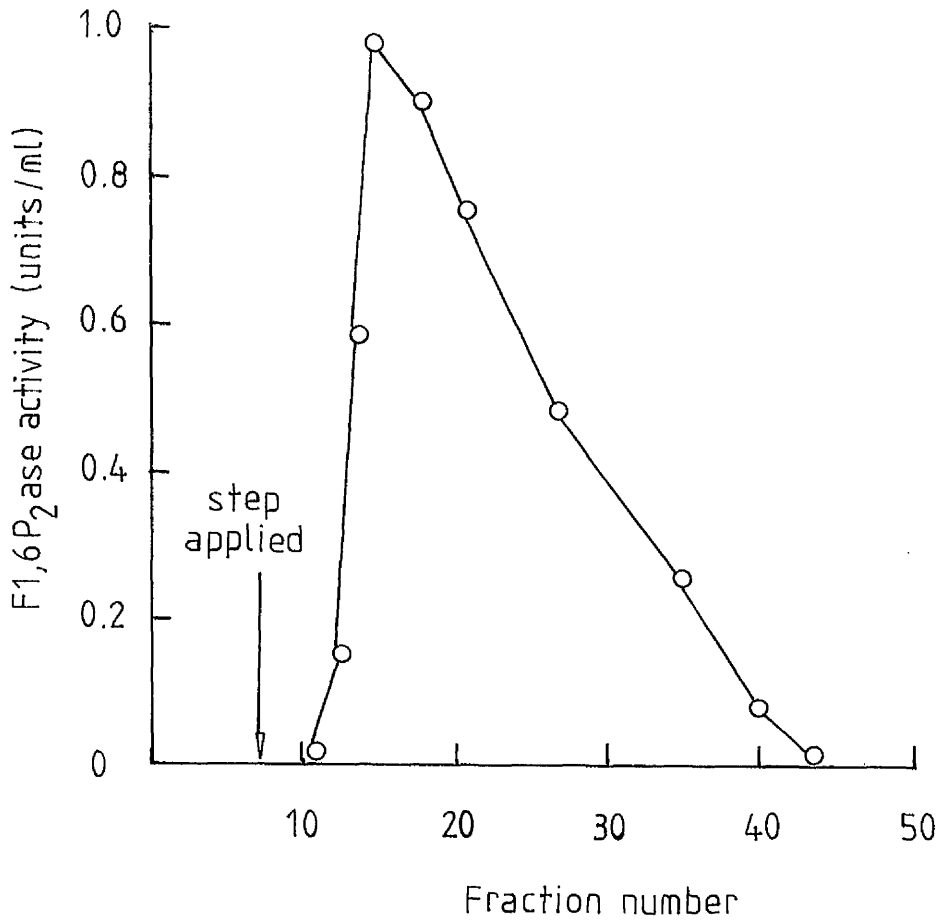

1.0
0.8
0.6
0.4
0.2
0
F1,6P2ase activity (units/ml)
step
applied
10
20
30
40
50
Fraction number

Figure 3.3

Gel filtration chromatography of rat liver fructose 1,6-bisphosphatase on Sephadex G200

Details of the procedure are given in the text. Each fraction was assayed at pH 7.5 in the presence and absence of 50 μM AMP and at pH 9.6 as described in section 2.3(a). The symbols are as follows:

○ $F1,6P_2$ase activity

■ pH ratio

▲ percentage inhibition given by 50 μM AMP, which is:

$$\frac{\text{activity at pH 7.5 in the presence of 50 } \mu\text{M AMP}}{\text{activity at pH 7.5 in the absence of 50 } \mu\text{M AMP}} \times 100\%$$

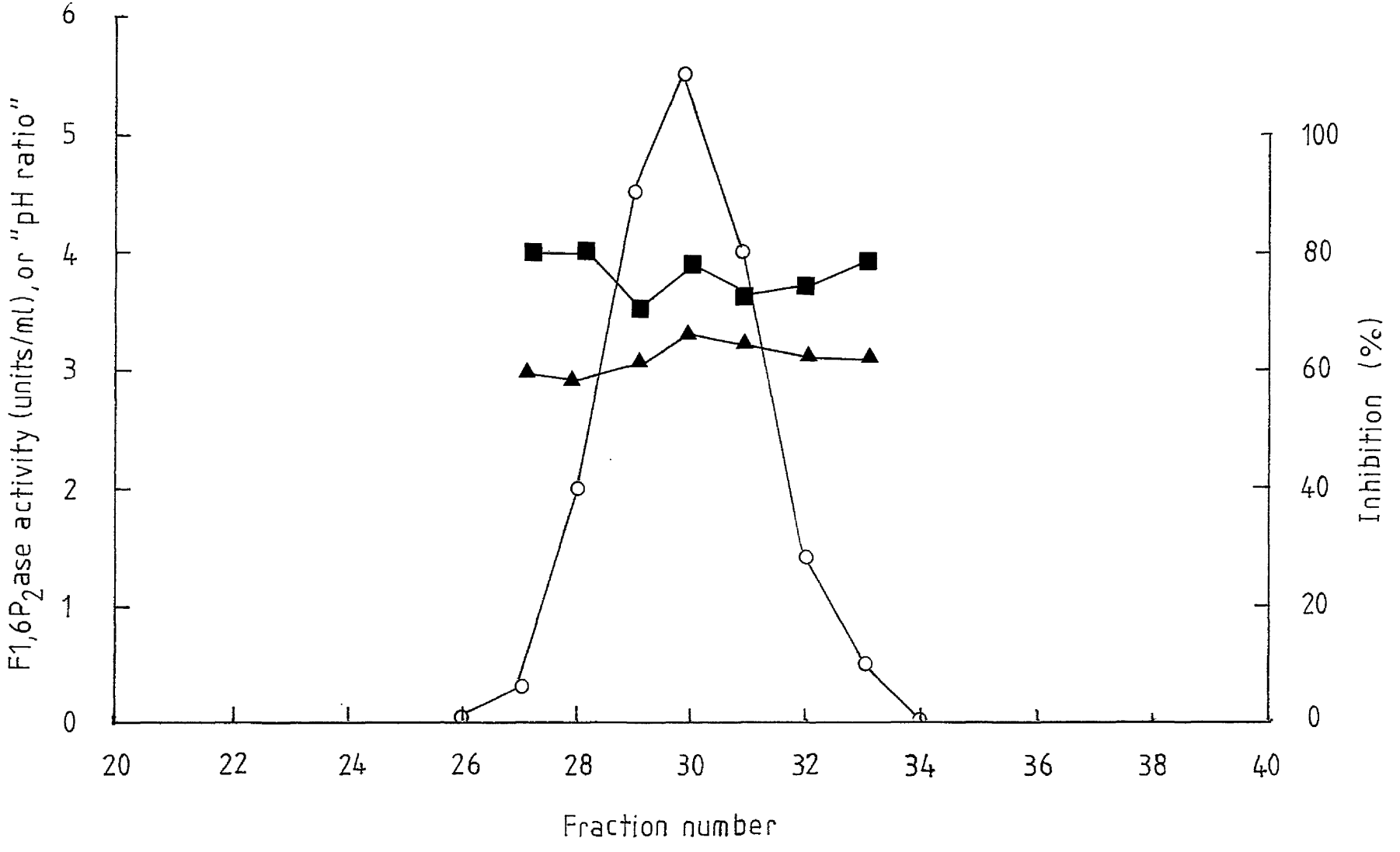

F1,6P2ase activity (units/ml), or "pH ratio"
Inhibition (%)
Fraction number
0
1
2
3
4
5
6
0
20
40
60
80
100
20
22
24
26
28
30
32
34
36
38
40

Figure 3.4

Polyacrylamide gel electrophoresis of purified rat liver fructose 1,6-bisphosphatase

Details of the electrophoretic and staining procedures are given in sections 2.4(a) and 2.4(b). 7% polyacrylamide gels were used and each gel was loaded with 10 μg of the purified enzyme. A gel on which no protein had been loaded was stained for F1,6P_2ase activity as a control. The gels were as follows:

A Control stained for F1,6P_2ase activity

B Purified F1,6P_2ase stained for F1,6P_2ase activity

C Purified F1,6P_2ase stained for protein

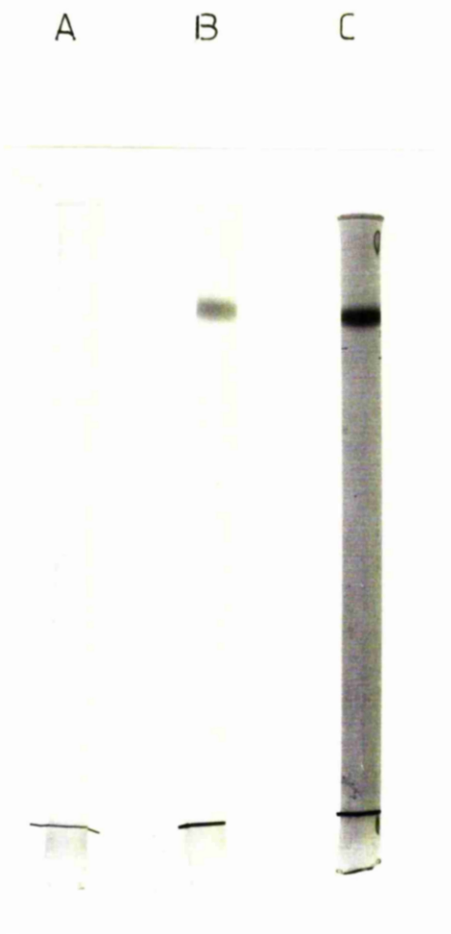

A
B
C

Figure 3.5

SDS polyacrylamide gel electrophoresis of purified rat liver fructose 1,6-bisphosphatase

7.5% polyacrylamide gels in the presence of 0.1% SDS were loaded with 10 μg of each protein and electrophoresis was performed using the system of Weber and Osborn as described in section 2.4(d). The gels were as follows:

A Standard proteins bovine serum albumin (B.S.A.), pyruvate kinase (P.K.), aldolase (Ald.), glyceraldehyde 3-phosphate dehydrogenase (G.A.P.D.H.), carbonic anhydrase (C.A.) and myoglobin (Mb)

B Purified rat liver F1,6P_2ase

C Purified rat liver F1,6P_2ase plus aldolase

D Purified rat liver F1,6P_2ase plus glyceraldehyde 3-phosphate dehydrogenase

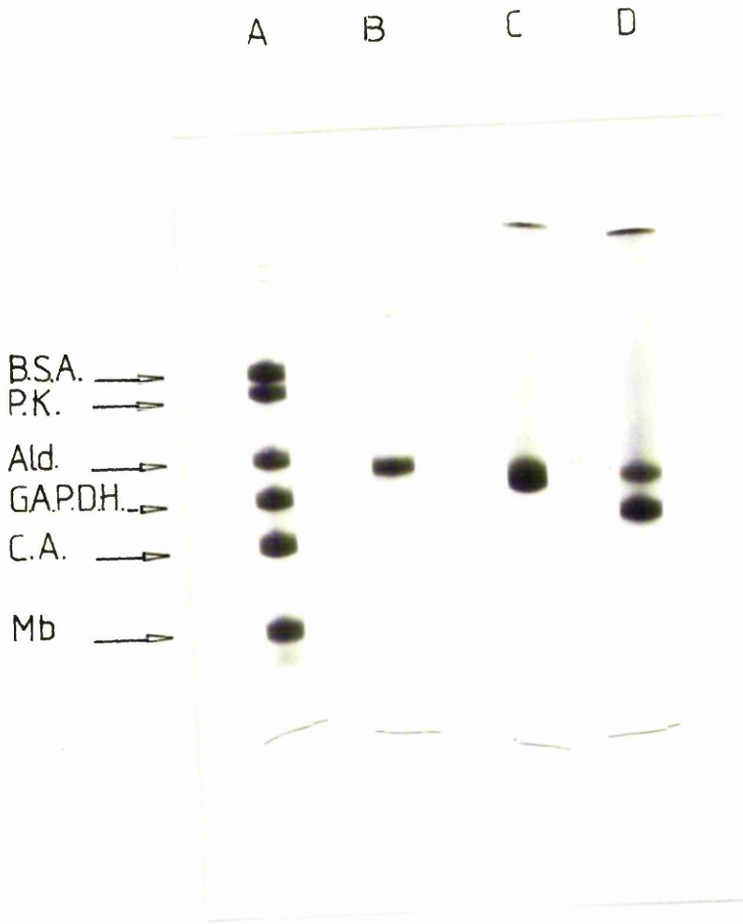

A
B
C
D
B.S.A.
P.K.
Ald.
G.A.P.D.H.
C.A.
Mb

Figure 3.6

Standard curve of electrophoretic mobility against log (subunit) molecular weight

Electrophoresis in 7.5% polyacrylamide gels in the presence of 0.1% SDS, performed using the system of Weber and Osborn (1975), and the gel scanning procedure are described in section 2.4(d). The standard proteins are also given in section 2.4(d) and in the legend to figure 3.5.

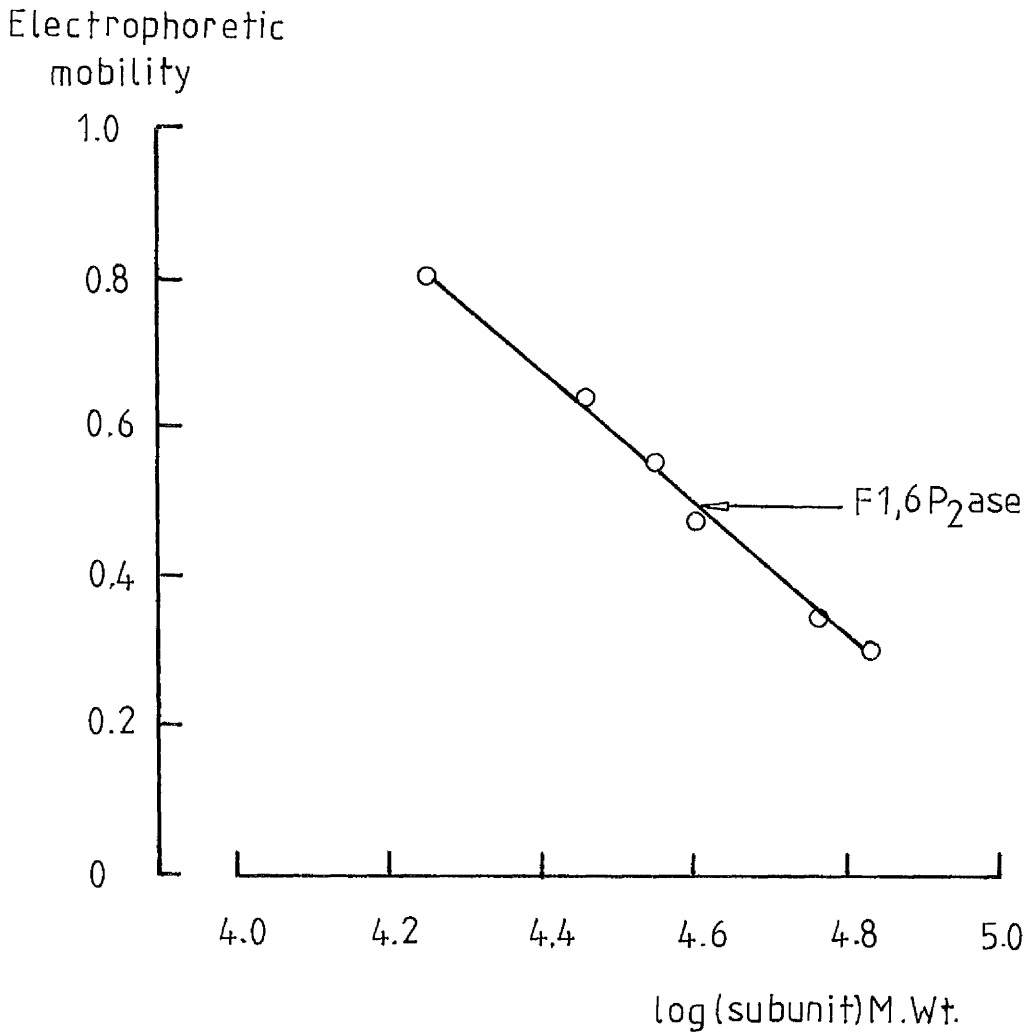

Electrophoretic mobility
1.0
0.8
0.6
0.4
0.2
0
F1,6P2ase
4.0
4.2
4.4
4.6
4.8
5.0
log (subunit) M.Wt.

Figure 3.7

Standard curve of electrophoretic mobility against log (subunit) molecular weight

Electrophoresis in 10% polyacrylamide tube gels in the presence of 0.1% SDS, performed using the system of Laemmli (1970), and the gel scanning procedure are described in section 2.4(e) and 2.4(d) respectively. The standard proteins are given in section 2.4(d).

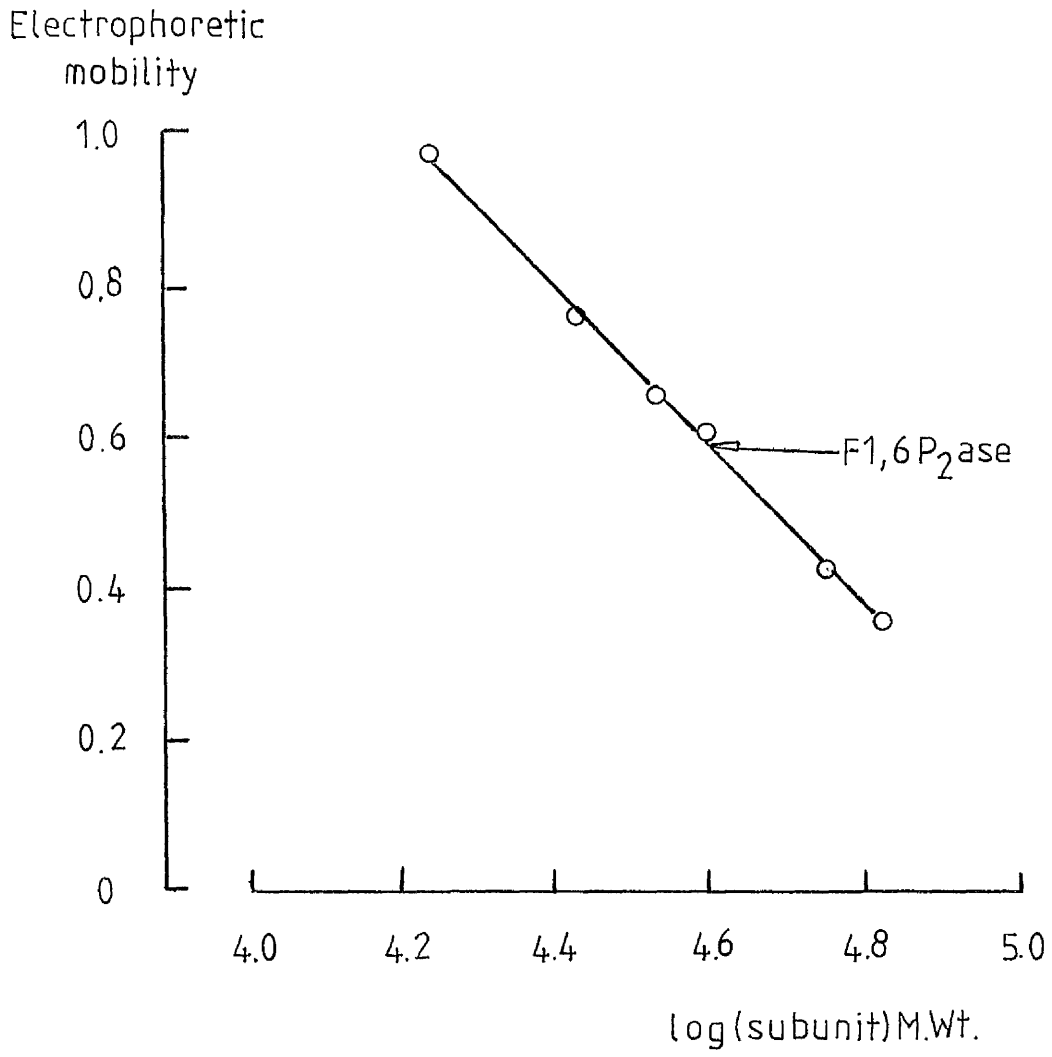

Electrophoretic mobility
1.0
0.8
0.6
0.4
0.2
0
F1,6P2ase
4.0
4.2
4.4
4.6
4.8
5.0
log (subunit) M.Wt.

Figure 3.8

Standard curve of V_e/V_o against log molecular weight

The procedure for the estimation of molecular weight by gel filtration chromatography using Sephadex G200 and the marker proteins used to calibrate the column are described in section 2.5(c).

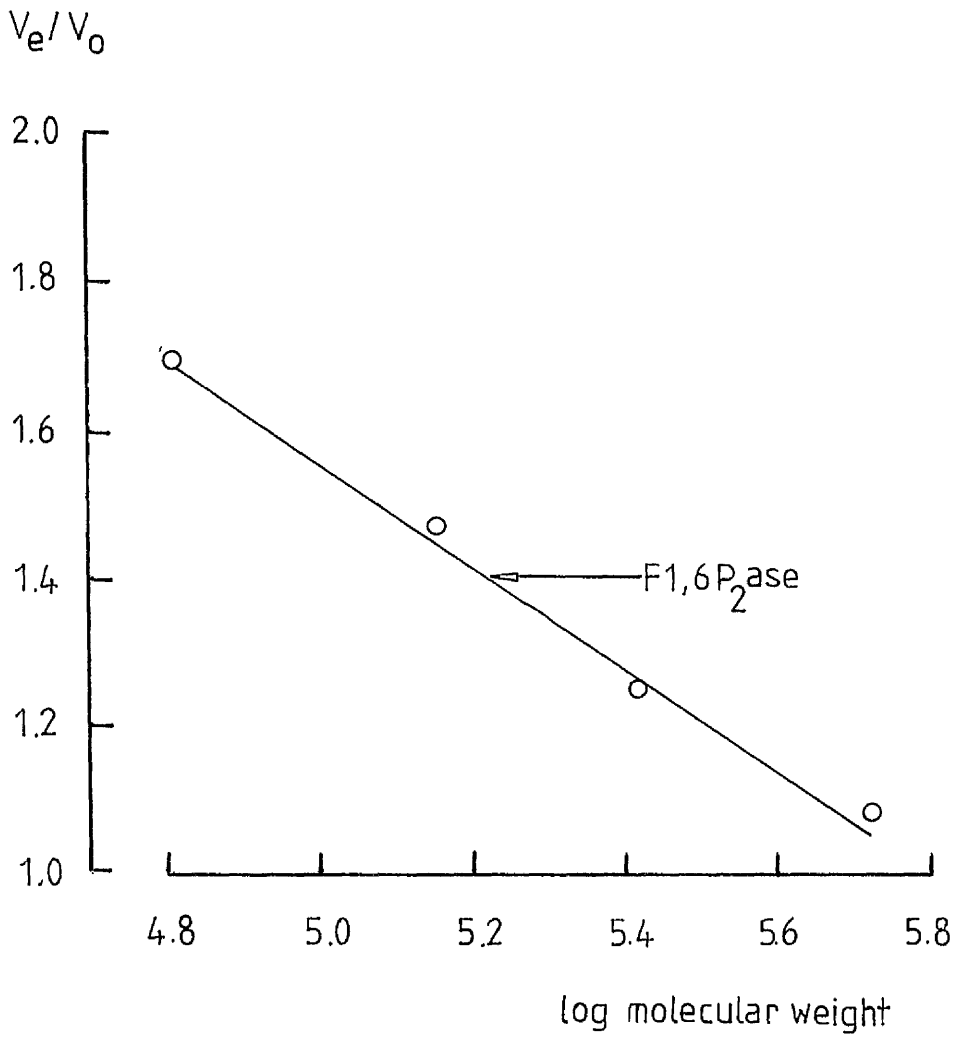

Ve/Vo
2.0
1.8
1.6
1.4
1.2
1.0
F1,6P2ase
4.8
5.0
5.2
5.4
5.6
5.8
log molecular weight

Figure 3.9

Sedimentation equilibrium analysis of rat liver fructose 1,6-bisphosphatase

Details of the centrifugation procedure and calculation of the molecular weight from the plot of ln c against r^2 are given in section 2.5(d).

The protein concentration at the start of the experiment was 0.5 mg/ml. A very similar result was obtained when the starting protein concentration was 0.35 mg/ml (not illustrated).

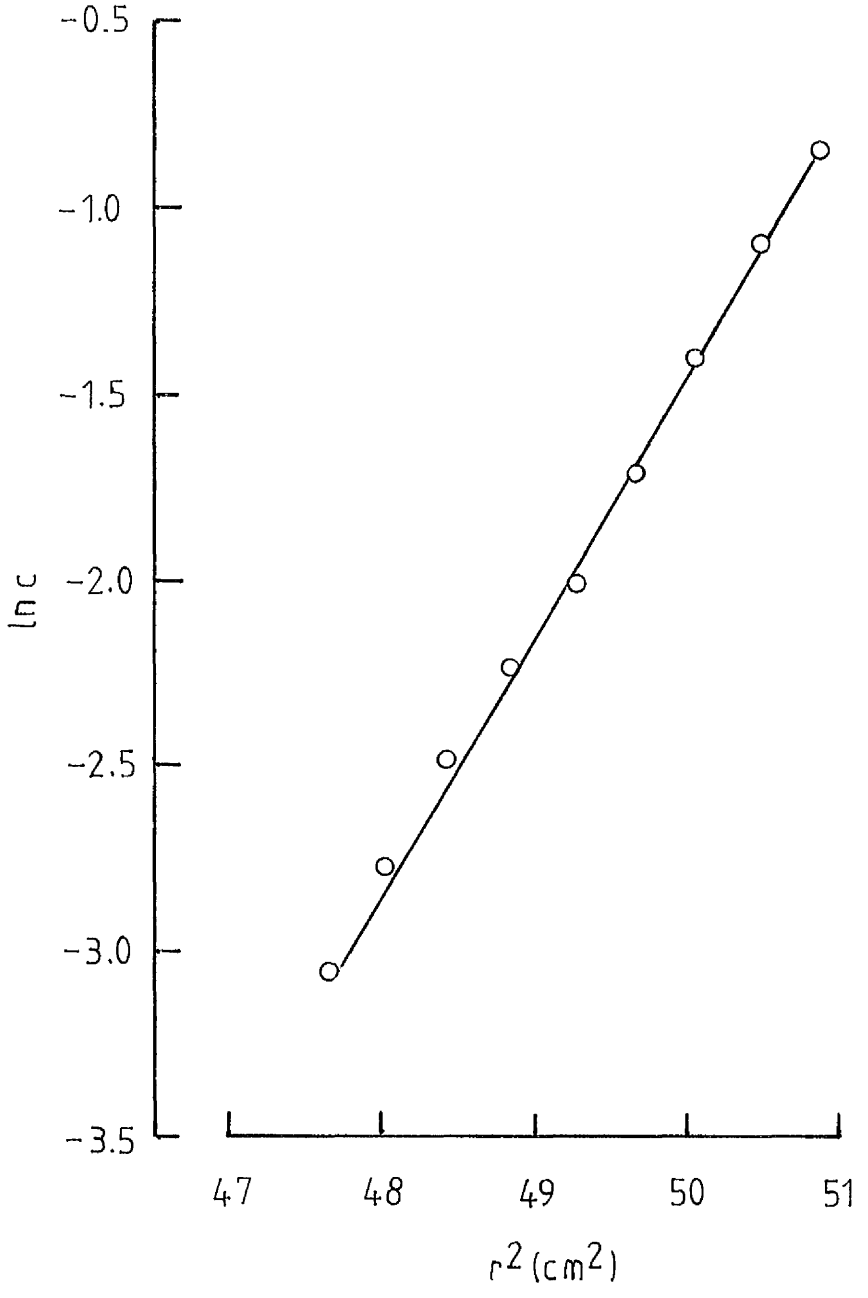

-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
ln c
47
48
49
50
51
r2 (cm2)

Figure 3.10

Sedimentation equilibrium analysis of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase

Details of the centrifugation procedure and calculation of molecular weight from the plot of ln c against r^2 are given in section 2.5(d).

The protein concentration at the start of the experiment was 0.2 mg/ml.

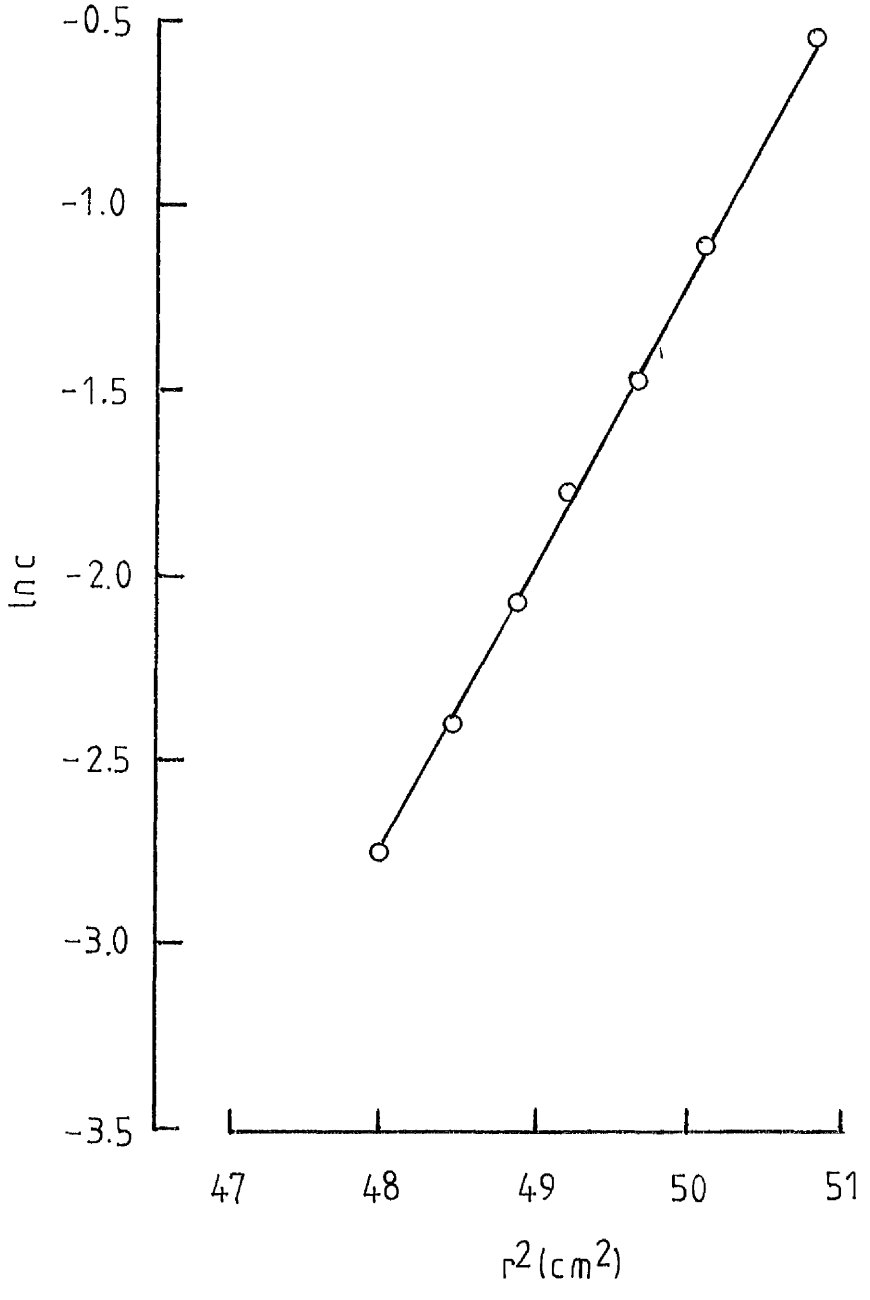

-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
ln c
47
48
49
50
51
r2 (cm2)

Figure 3.11

Polyacrylamide gel electrophoresis of carboxymethylated rat liver fructose 1,6-bisphosphatase in the presence of 8 M area

Details of the electrophoretic procedure are given in section 2.4(c). 7% polyacrylamide gels were used and each gel was loaded with 10 μg of protein.

Figure 3.12

Tryptic "fingerprint" of purified rat liver fructose 1,6-bisphosphatase

Carboxymethylated rat liver F1,6P_2ase was digested with trypsin and the resulting peptides were separated by electrophoresis and chromatography as described in section 2.5(b). The peptides indicated by broken lines stained only very faintly.

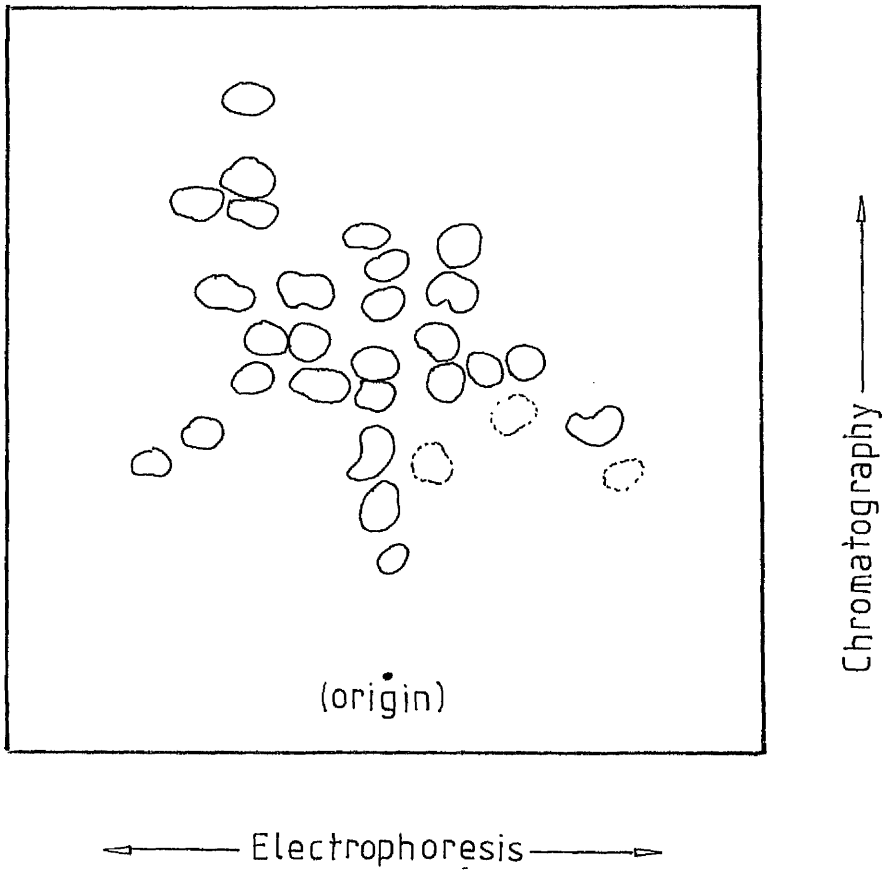

(origin)
Chromatography
Electrophoresis

Figure 3.13

The fluorescence spectrum of rat liver fructose 1,6-bisphosphatase

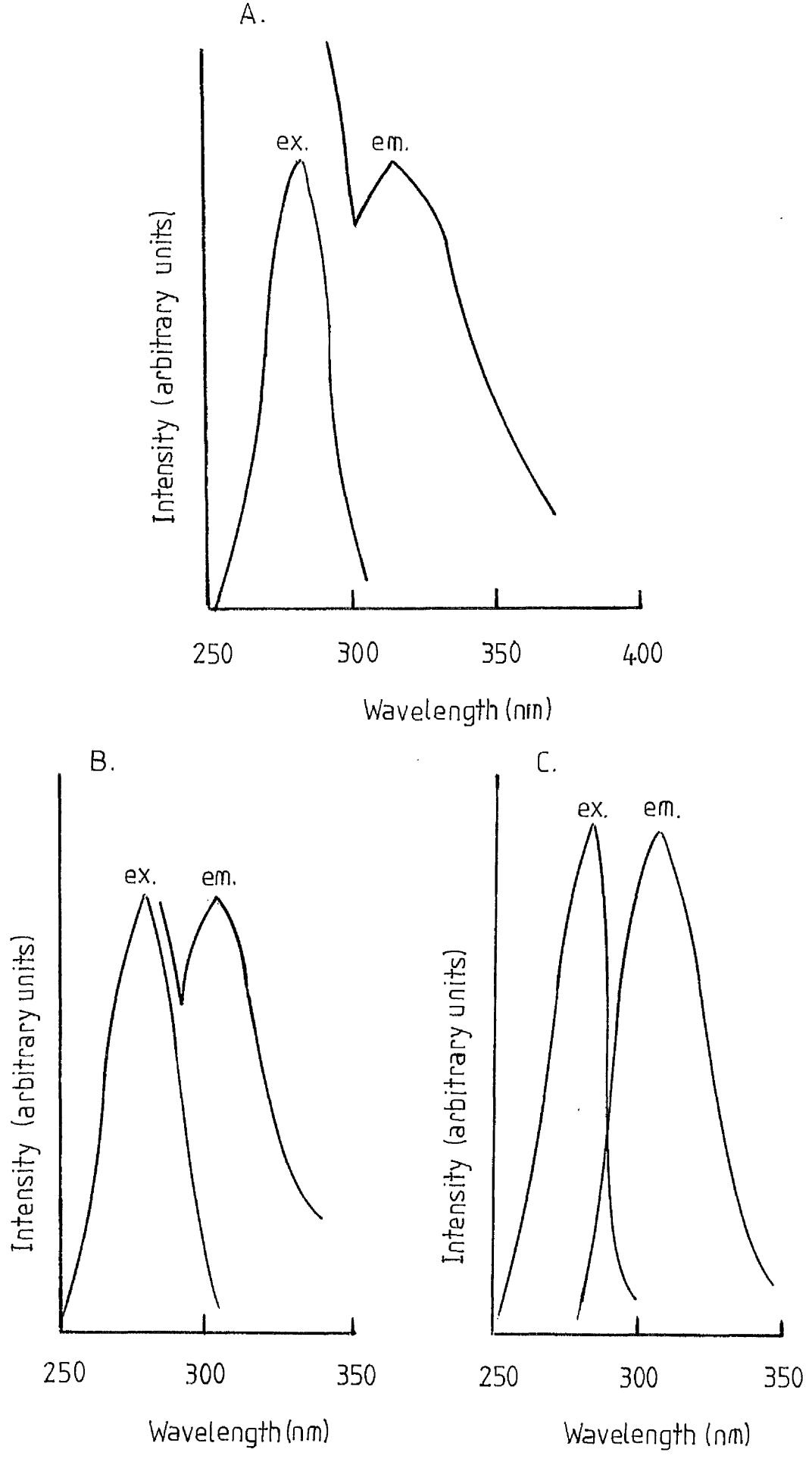
Samples of rat liver F1,6P_2ase in 0.1 M Treacl-KOH, pH 7.5, containing 2 mM $MgCl_2$ and 1 mM EDTA were scanned at 25°C in a Hitachi Perkin-Elmer MPF 2A fluorescence spectrophometer. The scans are as follows:

A 60 µg/ml rat liver F1,6P_2ase

B 60 µg/ml rat liver F1,6P_2ase in 4 M urea

C 0.1 mg/ml tyrosine in 0.1 M Treacl-KOH,pH 7.5, containing 2 mM $MgCl_2$ and 1 mM EDTA

ex. and em. represent the excitation and emission spectra respectively.


A.
ex.
em.
Intensity (arbitrary units)
250
300
350
400
Wavelength (nm)
B.
ex.
em.
Intensity (arbitrary units)
250
300
350
Wavelength (nm)
C.
ex.
em.
Intensity (arbitrary units)
250
300
350
Wavelength (nm)

CHAPTER 4

A STUDY OF THE PHOSPHORYLATION AND DEPHOSPHORYLATION OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE

4.1 Introduction

The work presented in this chapter covers three of the areas for further investigation which have emerged as a result of the studies of the phosphorylation and dephosphorylation *in vitro* and *in vivo*, of mammalian fructose, 1-6,bisphosphatases reviewed in Chapter 1.

Several workers have reported that cyclic AMP - dependent protein kinase (cAMP-PrK) catalyses the incorporation of phosphate into rat liver Fl,6P_2ase with a stoichiometry of 1 mole of phosphate per mole of subunits (Riou et al, 1977; Pilkis et al, 1980). Humble et al (1980) were only able to achieve the incorporation of 0.36 or 0.65 moles of phosphate per mole of subunit, in twoseparate experiments. In this respect, Ekman and Dalqvist-Edberg (1981) have shown that a very small peptide, which contains the serine residue recognised and phosphorylated by cAMP-PrK, can easily be removed proteolytically without any noticeable change in molecular weight, activity or "pH ratio" Bearing in mind that the loss of this peptide during purification of the enzyme would result in inability to obtain complete phosphorylation of Fl,6P_2ase the stoichiometry of phosphate incorporation catalysed by cAMP-PrK was examined. Two other aspects of cyclic AMP-dependent phosphorylation which have also been studied are the effect of fructose 2,6-bisphosphate on the phosphorylation of rat liver Fl,6P_2ase and the phosphorylation of two other mammalian Fl,6P_2ases.

Pilkis et al (1980) and Ekman and Dalqvist-Edberg (1981) have suggested that phosphorylated Fl,6P_2ase can be dephosphorylated *in vitro* by an unspecified, partially purified rat liver phosphoprotein phosphatase. Cohen (1982) has reported that three protein phosphatases, designated protein phosphatases 1, 2A and 2C, which are present in liver, have broad substrate specificities and appear to be the major

Table 4.1

The endogenous phosphate content of rat liver fructose 1,6-bisphosphatase

Samples of purified rat liver $F1,6P_2$ase, rabbit muscle glycogen phosphorylase *a* and glycogen phosphorylase *b* (free of AMP) were treated as described in section 2.7(f) to estimate the content of alkali-labile phosphate.

Sample	Amount of protein used in the assay	Amount of phosphate present in the sample	moles of phosphate per mole of subunit
	n. moles	n. moles	
rat liver $F1,6P_2$ase	10.0	5.4	0.54
glycogen phosphorylase *b*	15.4	0	0
glycogen phosphorylase *a*	13.5	17.1	1.27

phosphatases involved in gluconeogenesis, fatty acid synthesis and cholesterol synthesis. Since the literature does not contain any reports of the dephosphorylation of Fl,6P_2ase by well characterised enzymes, an examination of the effects of 3 highly purified rat liver protein phosphatases on phosphorylated rat liver Fl,6P_2ase was undertaken.

The procedure adopted by Riou et al (1977) to investigate the phosphorylation of rat liver Fl,6P_2ase *in vivo* involved the injection of rats with radioactive inorganic phosphate, then administration of glucagon or, as a control, saline. Following isolation of the Fl,6P_2ase by purification or by immunoprecipitation, they demonstrated that phosphate was incorporated into the enzyme *in vivo* but could not show any change in the extent of phosphorylation resulting from glucagon treatment. An alternative approach to examining the phosphorylation of enzymes *in vivo* involves the use of isolated hepatocytes. This method, in which the isolated cells are treated with radioactive inorganic phosphate followed by hormone administration, was used to study the effects of glucagon and insulin on the phosphorylation of rat liver, Fl,6P_2ase *in vivo*.

Recent reports concerning the phosphorylation and dephosphorylation of Fl,6P_2ase are discussed in relation to the studies presented in this chapter.

4.2 Measurement of Alkali-Labile Phosphate

As a prerequisite to studies of *in vitro* phosphorylation the endogenous phosphate content of rat liver Fl,6P_2ase was measured in a sample of the purified enzyme. Table 4.1 shows that rat liver Fl,6P_2ase contained 0.54 moles of phosphate per mole of subunit which is approximately 2 moles of phosphate per mole of tetramer. This value

should be compared with the value of 1.27 moles of phosphate per mole of subunit obtained with glycogen phosphorylase a. As a control glycogen phosphorylase b was found not to contain any endogenous phosphate. In respect of this result, Pilkis et al (1980) have recently commented that their purified Fl,6P_2ase also contained endogenous phosphate. The quantity however was considerably less than the value given in table 4.1, being in the range 0.2-0.3 moles of phosphate per mole of tetramer.

4.3 In vitro Phosphorylation of Fructose 1,6-Bisphosphatase

The time course for the phosphorylation of rat liver Fl,6P_2ase by the catalytic subunit of cyclic AMP-dependent protein kinase, *in vitro* is shown in figure 4.1. Under the conditions used, a maximum incorporation of 3.9 moles of phosphate per mole of tetrameric Fl,6P_2ase, which is apparently one mole of phosphate per mole of subunit, was reached in one hour. No incorporation of phosphate was detected when either protein kinase, ATP or Fl,6P_2ase was omitted. Figure 4.1 also suggests that there was no significant change in the activity of the enzyme as a result of phosphorylation when assayed under the conditions decribed in section 2.3(a).

In order to confirm that radioactive phosphate was incorporated into Fl,6P_2ase, samples of the phosphorylated enzyme were loaded onto native 7% polyacrylamide gels and electrophoresed as described in section 2.4 (a). The gels were stained for protein or for Fl,6P_2ase activity, then sliced and counted for ^{32}P. As demonstrated in figure 4.2, the major protein-staining band coincided with the band that stained for Fl,6P_2ase and the peak of radioactivity in either gel was associated with these bands.

Table 4.2

Dephosphorylation of phosphorylated rat liver fructose 1,6-bisphosphatase by phosphoprotein phosphatases 1, 2A and 2C from rat liver

Phosphorylated F1,6P_2ase was prepared as described in section 2.7(c) and the dephosphorylation experiments were performed according to the method described in section 2.7(d). The amount of phosphatase present in each assay was measured in terms of its ability to dephosphorylate glycogen phosphorylase kinase under the same conditions used for F1,6P_2ase and is expressed as percentage release of phosphate/minute from phosphorylase kinase. Thus, the dephosphorylation mixtures contained phosphoprotein phosphatases 1, 2A or 2C in amounts required to catalyse 3.4% release/min. from the β subunit, 2.7% release/min. from the α subunit and 4.8% release from the α subunit respectively.

Protein phosphatase	Period of incubation	Release of $^{32}P_i$	Rate of release of $^{32}P_i$	Percentage of rate with phosphorylase kinase
	min.	%	%/min	%
1	5	2.8	0.57	16.8
1	30	11.5	0.38	11.2
2A	5	17.3	3.45	127.8
2A	30	62.2	2.07	76.7
2C	5	1.27	0.25	5.2
2C	30	7.8	0.26	5.4

The effects of various ligands on the phosphorylation of Fl,6P_2ase were also tested. Figure 4.3 shows that the presence of saturating concentrations of fructose 1,6-bisphosphate (10mM) or fructose 2,6-bisphosphate (100 μM) in the presence or absence of a saturating level of AMP (200 μM) caused a noticeable decrease in the rate of phosphorylation under the conditions used. Samples taken after 3 and 4 hours confirmed that the phosphorylation of the enzyme was complete with a stoichiometry of 1 mole of phosphate incorporated per subunit. A saturating concentration of AMP (200 μM) alone did not alter the rate of phosphorylation.

Attempts were also made to phosphorylate bovine liver Fl,6P_2ase and rabbit muscle Fl,6P_2ase <u>in vitro</u> using the catalytic subunit of cAMP-PrK under the same conditions as were employed to phosphorylate the rat liver enzyme. The concentrations of bovine liver and rabbit muscle Fl,6P_2ases in the incubation mixture were 7.1μM and 3.5μM respectively. Under these conditions phosphate was not incorporated into either enzyme.

4.4 <u>In vitro Dephosphorylation of Rat Liver Fructose 1,6 - Bisphosphatase</u>

The dephosphorylation of rat liver Fl,6P_2ase by phosphoprotein phosphatases 1, 2A and 2C was investigated. The results, which are presented in table 4.2, indicate that Fl,6P_2ase was dephosphorylated by all three phosphoprotein phosphatases. When the rates of dephosphorylation of Fl,6P_2ase are expressed as percentages of the rates of dephosphorylation of phosphorylase kinase (table 4.2), a comparison shows that the rate of dephosphorylation of Fl,6P_2ase with phosphatase 2A was approximately 7 times greater than with phosphatase 1 and 25 times greater than with phosphatase 2C. This clearly demonstrates that phosphoprotein phosphatase 2A was most active

against Fl,6P_2ase. These results also show that Fl,6P_2ase compares well with glycogen phosphorylase kinase as a substrate for phosphoprotein phosphatase 2A.

4.5 In vivo Phosphorylation of Rat Liver Fructose 1,6-Bisphosphatase

In order to determine whether the hormones glucagon and insulin could influence the phosphorylation state of rat liver Fl,6P_2ase, isolated hepatcytes, which had been incubated for 1 hour in the presence of radioactive inorganic phosphate, were given saturating doses of glucagon (10^{-7}M), insulin (10^{-9}M), or as a control, saline. The enzyme was isolated by native polyacrylamide gel electrophoresis followed by SDS polyacrylamide gel electrophoresis as described in section 2.7 (e), using purified rat liver Fl,6P_2ase as an identification marker and running samples in quadrupicate to assess the reproducibility of the method. The photograph of the SDS gel presented in figure 4.4, shows that the reproducibility was fairly good, apart from the "saline-treated" samples. In each of the lanes containing cell extract samples, a protein band coincided with the purified Fl,6P_2ase marker and was therefore identified as Fl,6P_2ase. The corresponding autoradiograph, which is given in figure 4.5, clearly demonstrates that the ^{32}P was incorporated into Fl,6P_2ase <u>in vivo</u>. Although this particular approach does not yield quantitative results, there appears to be similar levels of ^{32}P in the enzyme from both saline treated and insulin treated cells. Slightly darker bands can be discerned on the autoradiograph of glucagon treated samples, indicating that glucagon increased the amount of ^{32}P incorporated into Fl,6P_2ase. (In comparison, a very noticeable glucagon effect can be seen on the autoradiograph with a protein of higher molecular weight than Fl,6P_2ase.) In order to check the authenticity of the Fl,6P_2ase in the extract

samples, the protein bands containing the putative Fl,6P_2ase, and the markers, were excised from the gel. A one-dimensional peptide "fingerprint", which compared the marker with the putative Fl,6P_2ase was produced by the method of Cleveland et al (1977), as described in section 2.4 (f). Figure 4.6 clearly demonstrates that both proteins generated identical banding patterns. This confirms that the protein coincident with the marker was Fl,6P_2ase and that no other protein(s) co-migrated with the enzyme.

4.6 Discussion

The results provide clear evidence that rat liver Fl,6P_2ase can be phosphorylated, *in vitro*, by the catalytic subunit of cyclic AMP-dependent protein kinase. Both Fl,6P_2ase and protein kinase were present in the *in vitro* incubation in physiological concentrations, (Mazzota and Veneziale, 1980; Sugden et al, 1976). The stoichiometry of 1 mole of phosphate incorporated per subunit indicates that the phosphorylation site had not been removed proteolytically during the purification, as has been found by other workers. (Ekman and Dalqvist-Edberg, 1981). The result from the native polyacrylamide gel electrophoresis demonstrates that all of the incorporated phosphate was associated covalently with the Fl,6P_2ase.

The finding that the rate of phosphorylation of Fl,6P_2ase was not altered by the presence of a saturating concentration of AMP (200 μM) is in agreement with previous reports, (Riou et al, 1977; Pilkis et al, 1980). This result would of course be expected from the observation that AMP is unable to bind to Fl,6P_2ase except in the presence of Fl,6P_2 or F2,6P_2 (see Chapters 5 and 6). However, saturating levels of F2,6P_2 or Fl,6P_2 caused a noticeable reduction in the phosphorylation rate. In a separate experiment, neither 10^{-2}M

$Fl,6P_2$ nor 10^{-4}M $F2,6P_2$ altered the activity of cAMP-PrK when assayed as described in section 2.3 (a). Since the concentration of $F2,6P_2$ in liver is increased upon glucose uptake and decreased 10-fold by glucagon (Van Schaftingen et al, 1980c) the inhibition of phosphorylation of $Fl,6P_2$ase might be physiologically significant. On the other hand, the inhibition effect is small and the concentration of $F2,6P_2$ used was 10^{-4}M; this is considerably greater than the physiological concentration of $F2,6P_2$ which has been estimated to be in the range 10^{-7} - 10^{-6}M (Van Schaftingen et al, 1980c). The effects of such concentrations of $F2,6P_2$ on the rate of phosphorylation of Fl, $6P_2$ase should be tested. Riou et al (1977) and Pilkis et al (1980) have ascertained that 10^{-4}M $Fl,6P_2$ does not influence the rate of phosphorylation of $Fl,6P_2$ase. The demonstration that 10^{-2}M $Fl,6P_2$ inhibits the phosphorylation reaction might be due to $Fl,6P_2$ binding as a low affinity ligand to a $F2,6P_2$ site on $Fl,6P_2$ase. There is some evidence that binding of this sort does occur (see Chapter 6). Since the concentration of $Fl,6P_2$ used in the experiment was approximately 3 orders of magnitude greater than the likely <u>in vivo</u> free concentration (Van Schaftingen et al, 1980a), it is unlikely to be of any physiological significance.

There has been some disagreement in the literature concerning activity changes resulting from phosphorylation. In this study, phosphorylation did not produce any change in the activity of rat liver $Fl,6P_2$ase when assayed under the conditions described in section 2.3 (a) (but see Chapter 5). Similarly, an activity change was not observed when swine kidney $Fl,6P_2$ase was phosphorylated <u>in vitro</u> (Mendicino et al, 1978). In contrast, Riou et al (1977) demonstrated

Table 4.3

Conditions reported in the literature for the assay of fructose 1,6-bisphosphatase

All the methods outlined below are variations on the coupled system described in section 2.3(c)

Source	buffer		2-Me	Mg^{2+}	EDTA	$(NH_4)_2SO_4$	KCl	$F1,6P_2$	Activity change
			mM	mM	mM	mM	mM	mM	
This study	50 mM Treacl-KOH	7.4	-	2.0	0.1	-	100	0.10	No
Taunton et al. (1974)	120 mM Tris-HCl	8.8	-	60.0	2.0	-	-	1.20	Yes
Riou et al. (1977)	100 mM Tris-HCl	7.5	2.5	2.0	-	2.0	-	0.07	Yes
Chatterjee and Datta (1978)	200 mM Treacl/DEA	7.2	-	2.0	0.1	40.0	-	0.10	Yes
Mendicino et al. (1978)	40 mM Tris-HCl	7.4	10.0	10.0	-	-	-	0.20	No
Ekman and Dalqvist-Edberg (1981)	10 mM K Phosphate	7.5	25.0	7.5	-	-	-	0.05	Yes
Claus et al. (1981b)	_____ assay conditions not described _____								No

a 40% increase in the activity of rat liver Fl,6P_2ase, as measured in the absence of EDTA, which paralleled *in vitro* phosphorylation. Support for this result comes from studies of the effects of hormones on enzyme activities carried out by Taunton et al (1974) and Chatterjee and Datta (1978) who have reported increases in activity of 100% in each case for Fl,6P_2ase from rat liver and mouse liver respectively. However, Claus et al (1981b) failed to detect any increase in enzyme activity resulting from *in vivo* phosphorylation. Ekman and Dalqvist-Edberg (1981) claim to have obtained a small increase in the activity of rat liver Fl,6P_2ase concomitant withphosphorylation *in vitro*, but they also comment that non-phosphorylated and phosphorylated forms of the enzyme had the same specific activity. Since, under the assay conditions adopted by these workers, the Fl,6P_2 concentrations were subsaturating, the observed increase in activity could be attributed to a change in the K_m value produced by phosphorylation. Ekman and Dalqvist - Edberg (1981) have also emphasised that the choice of assay buffer significantly affects the kinetic parameters of the enzyme. Moreover if one extends this argument to include other components in the assay mixture, then clearly the concentrations of Mg^{2+}, EDTA, ammonium sulphate, potassium chloride and Fl,6P_2, which influence the behaviour of the enzyme, might also highlight or conceal any differences between non-phosphorylated and phosphorylated forms of the enzyme. In this respect, a comparison of the assay conditions employed in each of the studies discussed above which is given in table 4.3, does not show any obvious similarities or differences in the compositions of the assay media which would account for the ability or inability to detect activity changes. The explanation for the inconsistency in discerning a phosphorylation-dependent increase in activity therefore remains unclear.

Although rat liver Fl,6P_2ase was a substrate for cAMP-PrK, rabbit muscle and ox liver Fl,6P_2ases could not be phosphorylated. Hosey and Marcus (1981) were unable to phosphorylate Fl,6P_2ases from mouse liver, rabbit liver and pig kidney. It has been postulated that the inability to phosphorylate these enzymes might be due to removal of the phosphorylation site by proteolysis during the purification procedure. (Hosey and Marcus, 1981; Ekman and Dalqvist - Edberg 1981). The finding that pig kidney Fl,6P_2ase can be phosphorylated with a stoichiometry of 1 mole of phosphate per subunit (Mendicino et al 1978) proves that this enzyme does contain a phosphorylation site and this would therefore appear to support the hypothesis of proteolysis. Two possible approaches might provide a clearer understanding of the phosphorylation of Fl,6P_2ases. By taking all necessary precautions to minimise proteolysis during the purification of these enzymes, any putative phosphorylation site should not be lost. Alternatively, rapid isolation of Fl,6P_2ase by immunoprecipitation from extracts of mouse and rabbit livers would establish whether these enzymes are phosphorylated <u>in vivo.</u> The proteolysis hypothesis would predict that the enzymes are labelled <u>in vivo</u> at a phosphorylation site very close to the C-terminal end.

The endogenous phosphate content of rat liver Fl,6P_2ase was estimated to be 0.54 moles of phosphate per subunit. The finding that samples of Fl,6P_2ase from the same batch could be phosphorylated to the extent of one mole of phosphate per subunit by cAMP-PrK implies that the endogenous phosphate was present at a site(s) distinct from the cAMP-PrK phosphorylation site. Cohen (1982) has remarked that multi-site phosphorylation is a common occurrence in enzyme regulation and it is therefore possible that Fl,6P_2ase is a substrate for other

protein kinases. Although Pilkis et al (1980) have demonstrated that rabbit skeletal muscle phosphorylase kinase did not catalyse the phosphorylation of Fl,6P_2ase, there have been no other reported studies of cyclic AMP-independent phosphorylation of the enzyme. Brand and Soling (1975) have identified a cyclic AMP-independent "PFK-kinase" which catalysed the incorporation of ^{32}P from [$\gamma-^{32}P$]ATP into a serine residue in phosphofructokinase-1 with a stoichiometry of 1 mole of phosphate per mole of subunit. This phosphorylation brought about the dissociation of a dimer comprising PFK-kinase and inactive PFK-1 subunit, and was accompanied by the association of PFK-1 subunits to form an active tetramer. Only one mole of phosphate incorporated per tetramer was necessary for full activation, and the rate of activation appeared to be considerably reduced in starved animals. The activation process could be reversed by a Mg^{2+} dependent "PFK-phosphatase". Further studies confirmed that the active and inactive enzyme predominated in the livers of fed rats or glucose-perfused livers of starved rats whereas the inactive enzyme prevailed in the livers of starved rats (Brand et al, 1976). Bearing in mind that the factors which influence the regulation of PFK-1 generally produce a reciprocal response in Fl,6P_2ase, an attractive hypothesis would be that Fl,6P_2ase might be phosphorylated by PFK-kinase. Clearly, any future study of cyclic AMP- independent phosphorylation of Fl,6P_2ase should ascertain whether the enzyme is a substrate for PFK-kinase.

The dephosphorylation of rat liver Fl,6P_2ase was catalysed by phosphoprotein phosphatases 1, 2A and 2C, a result which supports the view that these phosphatases have overlapping substrate specificities (Cohen 1982). It has been postulated that phosphatase 1, which is associated with glycogen and microsome fractions of liver, is

important in the regulation of glycogen metabolism, protein synthesis and cholesterol synthesis. Phosphatases 2A and 2C however appear to be located entirely in the liver cytosol, where phosphatase 2A is very active towards the regulatory enzymes of gluconeogenesis, fatty acid synthesis and cholesterol synthesis. Phosphatase 2C is concerned mainly with cholesterol synthesis, (Cohen, 1982). The finding that phosphatase 2A was the most active against $Fl,6P_2ase$ is consistent with this hypothesis.

The effects of the hormones glucagon and insulin on the phosphorylation of $Fl,6P_2ase$ in rat hepatocytes were examined. There existed essentially 3 approaches to isolating the phosphorylated enzyme from the cell extract. One possibility was to utilise specific elution from a procion red-sepharose column as described in section 3.2. However, the binding capacity of this resin for $Fl,6P_2ase$ in a cell extract would be very low. Moreover, $Fl,6P_2ase$, which would only bind to this resin at Treacl buffer concentrations less than 30mM, was unlikely to bind in the presence of 0.1M NaF which was necessarily included in the homogenisation medium as a phosphatase inhibitor. Procion red-sepharose chromatography therefore was not adopted to isolate the phosphorylated enzyme. An alternative approach would have been immunoprecipitation of $Fl,6P_2ase$. This method is of considerable advantage because a rapid and specific isolation of the enzyme can be achieved. Had antibodies to $Fl,6P_2ase$ been available, this method would certainly have been employed. The procedure adopted to study the phosphorylation of $Fl,6P_2ase$ <u>in vivo</u>, which is described in section 2.7(e), utilised activity staining to identify $Fl,6P_2ase$ in native polyacrylamide gels on which samples of total soluble protein from hepatocytes had been electrophoresed. Further separation, which was achieved by SDS polyacrylamide gel electrophoresis, gave reproducible

amounts of Fl,6P_2ase in each lane. The identification of a band on the SDS gel as Fl,6P_2ase was confirmed by a one dimensional peptide "fingerprint" produced by the method of Cleveland et al (1977).

The results clearly demonstrated that Fl,6P_2ase was phosphorylated <u>in vivo.</u> Although ^{32}P was detected in Fl,6P_2ase from cells treated with saline or saturating concentrations of insulin or glucagon, there appeared to be a slightly higher ^{32}P content in the enzyme isolated from glucagon-treated cells. A quantitative estimate of the phosphate content of the enzyme could be better achieved by scanning the SDS gel to measure the amount of protein, followed by slicing the gel and counting each slice for ^{32}P. This approach was recently adopted by Claus et al (1981b) who noticed an increase in the phosphate content of Fl,6P_2ase from 2.3 to 3.6 moles per tetramer following a ten minute incubation period with 10^{-8} M glucagon. This finding is therefore in good agreement with the result obtained in this study.

The questions remain as to why Fl,6P_2ase from saline or insulin-treated cells contained phosphate and also why glucagon treatment produced only a very slight increase in the phosphate content of the enzyme; several possibilities exist. It is conceivable that the phosphorylation of Fl,6P_2ase <u>in vivo</u> might not be complete after a 10 or 15 minute incubation period in the presence of hormone. This hypothesis could be tested by carrying out a time course study of the phosphorylation of the enzyme <u>in vivo</u> which would ascertain when the incorporation of ^{32}P was complete and the stoichiometry of the reaction. To explain the presence of ^{32}P in Fl,6P_2ase from cells treated with saline or insulin, the possibility exists that the enzyme is phosphorylated by a glucagon-independent kinase at a site

distinct from the cAMP-PrK phosphorylation site. The results from the alkali-labile phosphate measurement would tend to support this hypothesis. A one-dimensional peptide "fingerprint" carried out by the method of Cleveland et al (1977) when autoradiographed, should establish whether two separate phosphorylation sites exist on different peptides. Moreover, a mechanism whereby an insulin-dependent phosphorylation site is dephosphorylated and simultaneously the glucagon-dependent site is phosphorylated might be in operation. This process could be reversed by insulin treatment and therefore the phosphate content of the enzyme would seem to vary by only a small amount. This would be similar to the phosphorylation of acetyl CoA carboxylase (Brownsey and Denton, 1982). Again, an autoradiograph taken from a "Cleveland map" of samples of the phosphorylated enzyme, isolated at different time points following insulin or glucagon treatment, would indicate whether ^{32}P was being incorporated into or lost from distinct sites. Since the experiments performed in this study and in the investigation by Claus et al, (1981b) utilised cells from fed rats, the phosphorylation of $Fl,6P_2ase$ in hepatocytes from starved rats should be examined. Here again the possibility exists that a cyclic AMP-independent kinase, such as PFK-kinase, might be operating and could be investigated using the system of "Cleveland mapping" followed by autoradiography as described above.

In conclusion, the work presented and discussed in this chapter clearly provides some scope for further study of the phosphorylation of $Fl,6P_2ase$.

Figure 4.1

Time course of the phosphorylation of rat liver fructose 1,6-bisphosphatase by the catalytic subunit of cyclic AMP-dependent protein kinase from bovine liver, <u>in vitro</u>

Samples were withdrawn at the indicated times. The phosphorylation conditions and the determination of ^{32}P incorporation were as described in section 2.7(a). To measure F1,6P_2ase activity, aliquots of 10 μl of the assay mixture were placed in 90 μl of ice-cold 50 mM Treacl-KOH, pH 7.5 containing 100 mM KCl and 0.1 mM EDTA. Assays were performed immediately, as described in section 2.3(a). The symbols are as follows:

□ Incorporation of ^{32}P in the absence of F1,6P_2ase or cAMP-PrK

○ Incorporation of ^{32}P into F1,6P_2ase in the presence of cAMP-PrK

◇ F1,6P_2ase activity in the control mixture

■ F1,6P_2ase activity in the phosphorylation mixture

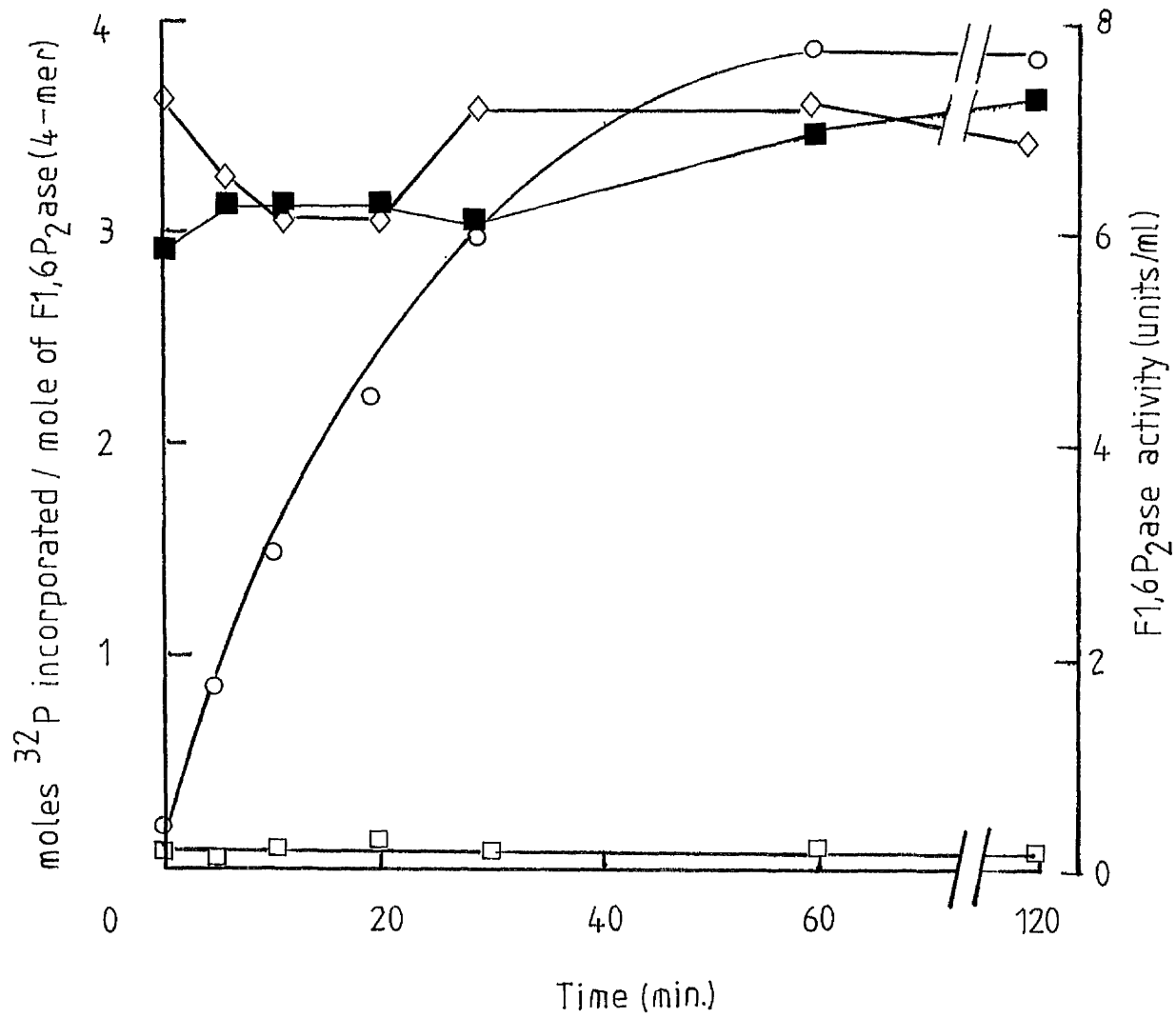

moles ^{32}P incorporated / mole of F1,6P_2ase (4-mer)
F1,6P_2ase activity (units/ml)
Time (min.)
0
20
40
60
120
1
2
3
4
6
8

Figure 4.2

Native polyacrylamide gel electrophoresis of phosphorylated rat liver fructose 1,6-bisphosphatase

Electrophoresis in 7% native polyacrylamide gels and the subsequent slicing and counting of the gels was carried out as described in sections 2.4(a) and 2.7(b) respectively.

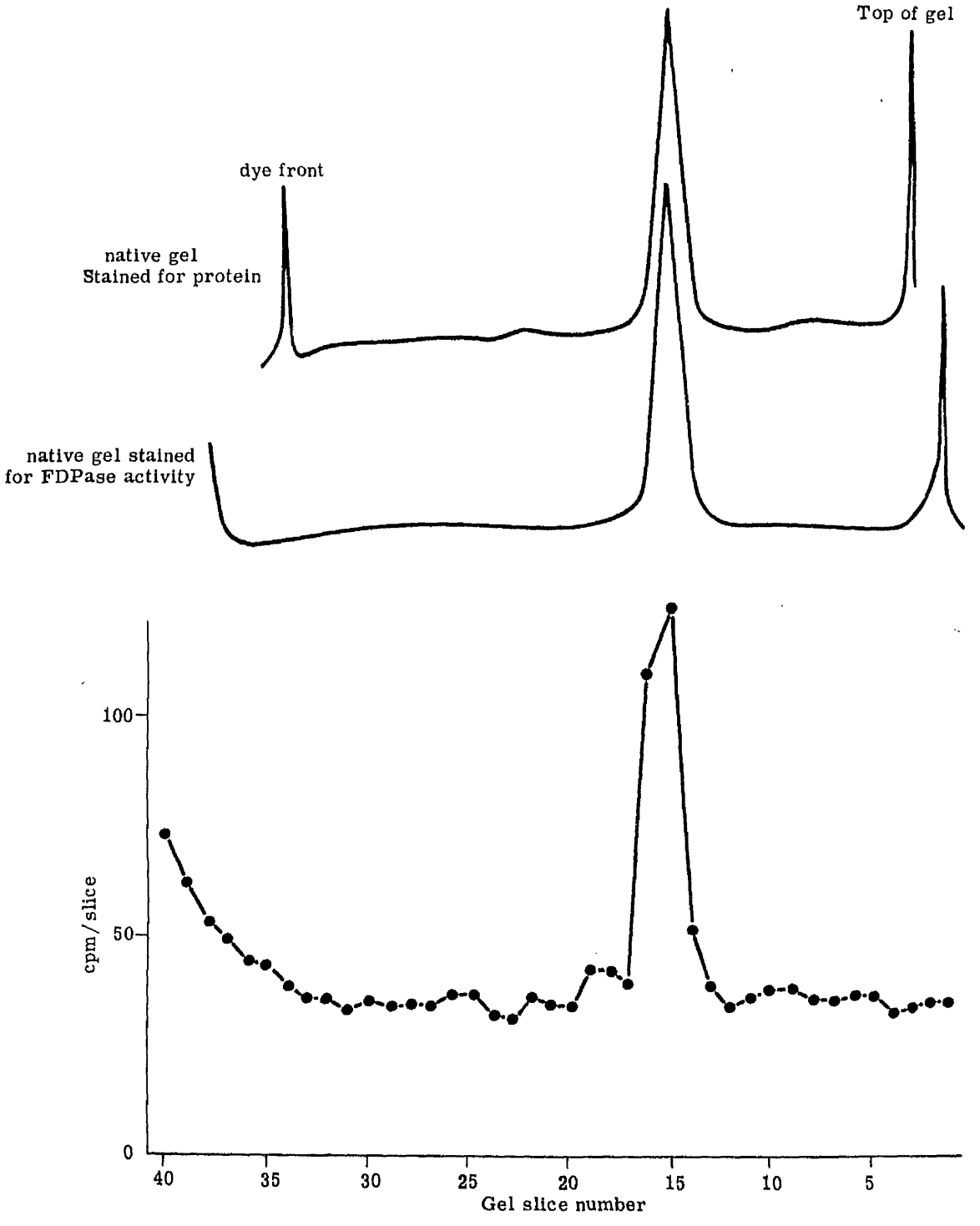

Top of gel
dye front
native gel
Stained for protein
native gel stained
for FDPase activity
100
50
0
cpm/slice
40
35
30
25
20
15
10
5
Gel slice number

Figure 4.3

Time course of the phosphorylation of rat liver fructose 1,6-bisphosphatase by the catalytic subunit of cyclic AMP-dependent protein kinase from bovine liver, in vitro

Samples were withdrawn at the indicated times and ^{32}P was determined as described in section 2.7(a). The symbols are as follows:

- □ Control in which F1,6P_2ase or cAMP-PrK had been omitted
- ○ Phosphorylation of F1,6P_2ase in the presence or absence of 200 μM AMP
- ▲ Phosphorylation of F1,6P_2ase in the presence of 100 μM F2,6P_2 or 100 μM F2,6P_2 plus 200 μM AMP
- △ Phosphorylation of F1,6P_2ase in the presence of 10 mM F1,6P_2 or 10 mM F1,6P_2 plus 200 μM AMP

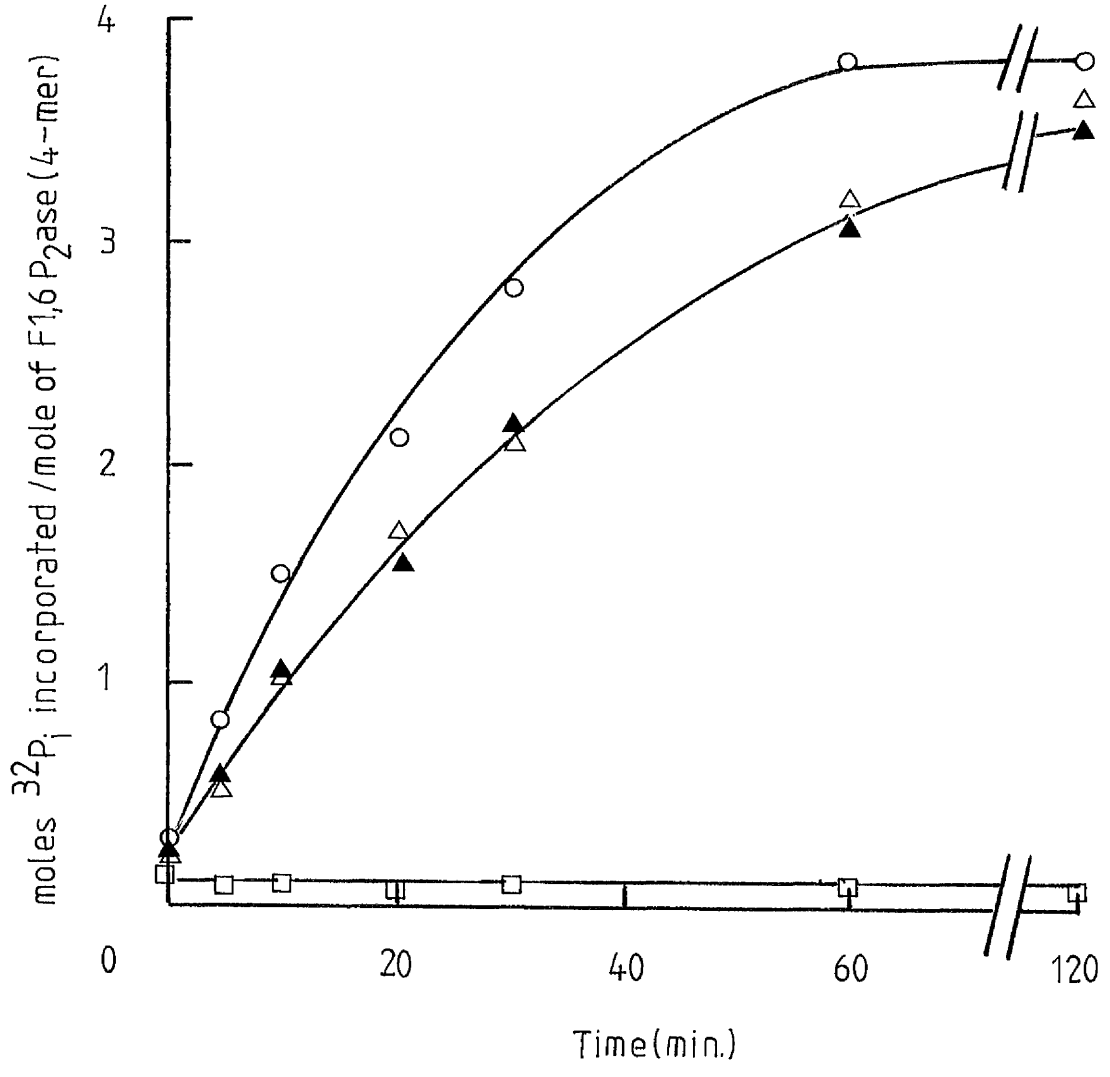

moles $^{32}P_i$ incorporated /mole of F1,6 P_2ase (4-mer)
4
3
2
1
0
20
40
60
120
Time(min.)

Figure 4.4

SDS polyacrylamide slab gel containing rat liver fructose 1,6-bisphosphatase which was phosphorylated with ^{32}P *in vivo*

The phosphorylation procedure and isolation of $F1,6P_2$ase were carried out as described in section 2.7(e).

The lanes are as follows:

1 Purified rat liver $F1,6P_2$ase

2-5 Samples from saline-treated hepatocytes

6-9 Samples from hepatocytes treated with 10^{-9} M insulin

10-13 Samples from hepatocytes treated with 10^{-7} M glucagon

14-16 Purified rat liver $F1,6P_2$ase

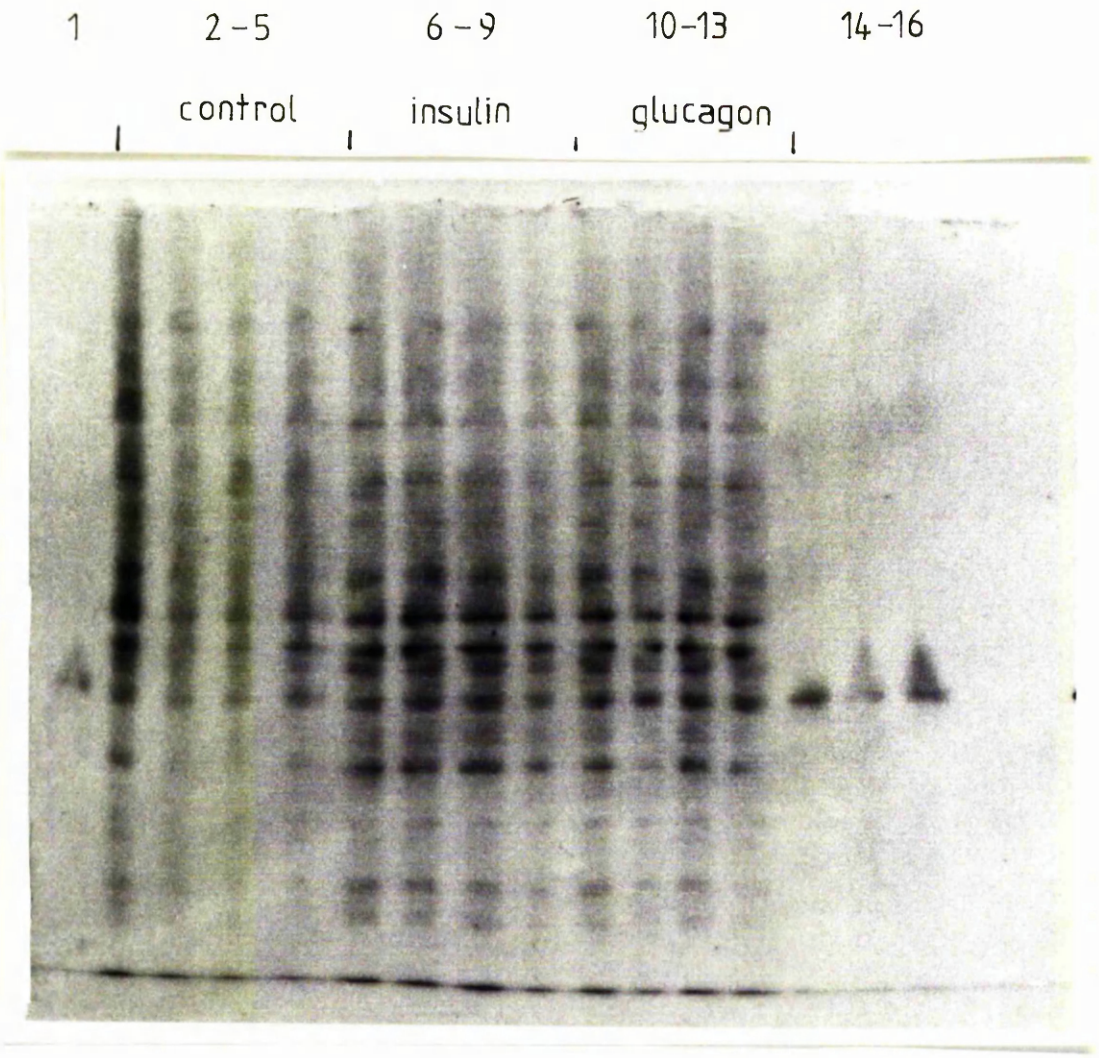

1
2 – 5
6 – 9
10–13
14–16
control
insulin
glucagon

Figure 4.5

Autoradiograph of the SDS polyacrylamide gel presented in figure 4.4

The lanes are as follows:

1-4 Samples from saline-treated hepatocytes

5-8 Samples from hepatocytes treated with 10^{-7} M insulin

9-12 Samples from hepatocytes treated with 10^{-7} M glucagon

1-4 5-8 9-12

control insulin glucagon

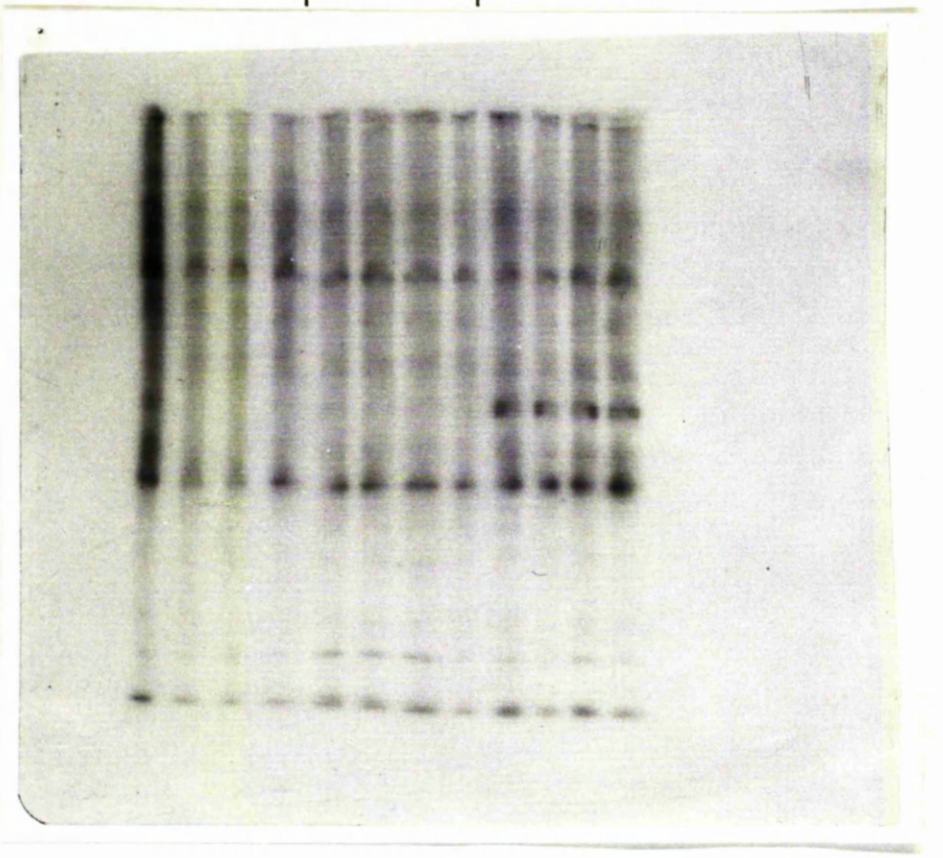


Figure 4.6

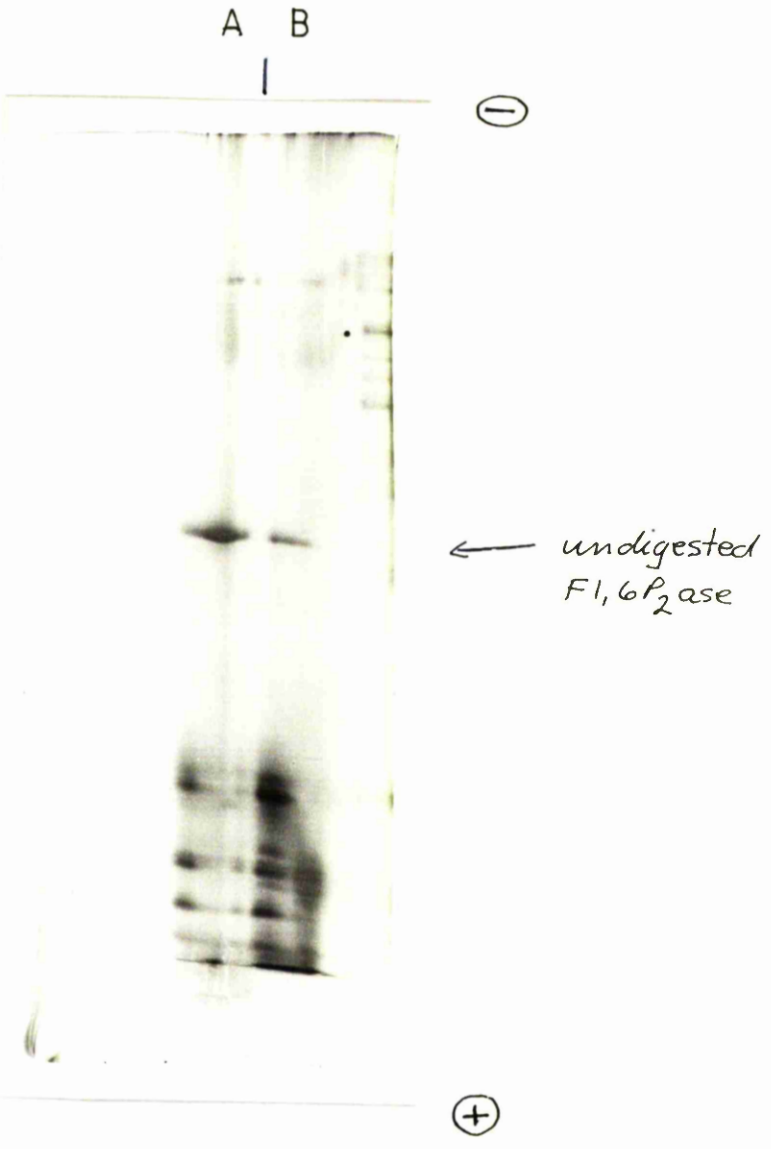
Cleveland map of purified F1,6P_2ase and F1,6P_2ase isolated from rat hepatocytes

Samples were recovered from the SDS slab gel in figure 4.4. Electrophoresis and chymotryptic digestion were performed as described in section 2.4(f). The gel was stained using the silver staining method of Wray et al. (1981).

The lanes are as follows:

A Purified rat liver F1,6P_2ase

B F1,6P_2ase isolated from hepatocytes by gel electrophoresis


A
B
⊖
undigested
F1,6P2ase
⊕

CHAPTER 5

A STUDY OF THE KINETIC AND REGULATORY PROPERTIES OF NON-PHOSPHORYLATED AND PHOSPHORYLATED FORMS OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE

5.1 Introduction

The study of the regulation of Fl,6P_2ase by phosphorylation and dephosphorylation has stimulated much interest. However, the emphasis of research in this area has been directed towards an understanding of molecular aspects such as the kinetics of phosphorylation and dephosphorylation, the kinases and phosphatases involved, the sequence of the phosphorylation site and studies of phosphorylation *in vivo*. Less attention has been focused on the regulatory significance of the phosphorylation of Fl,6P_2ase, with regard to the characterisation of non-phosphorylated and phosphorylated forms of the enzyme. Activity changes, as determined under standard assay conditions, have been discussed in the previous chapter. Under physiological conditions, however, changes in the activity of the enzyme may be the consequence of an altered affinity for the substrate or sensitivity to inhibitors or activators. The objective of the research presented in this chapter was, therefore, to perform a comparative examination of the kinetic and regulatory properties of non-phosphorylated and phosphorylated rat liver Fl,6P_2ase, with a view to producing a model of the kinetic behaviour of the enzyme.

5.2 Phosphorylation of Rat Liver Fructose 1,6-Bisphosphatase

Rat liver Fl,6P_2ase, phosphorylated by the catalytic subunit of cAMP-PrK, was prepared and stored as described in section 2.7(c). No change in the activity of the enzyme, its pH ratio, or its sensitivity to inhibition by AMP could be detected over a period of 2 months. In order to determine whether the ^{32}P remained covalently associated with the enzyme, the ^{32}P content was measured at 14 day intervals over a period of 2 months. This was accomplished by estimating the total number of counts and also the total number of acid-precipitable counts

in samples of the phosphorylated enzyme. Loss of the ^{32}P from the enzyme would cause a fall in the ratio of acid preciptable counts/ total counts. As demonstrated in table 5.1, the ratio of acid-preciptable counts/total counts remained constant over the 2 month period, indicating that the ^{32}P remained covalently associated with the enzyme.

5.3 Molecular Weight and pH Optima

To ascertain whether phosphorylation produced a change in the aggregation state of rat liver Fl,6P_2ase, the native molecular weight of the phosphorylated enzyme was estimated using the gel filtration method of Andrews (1965) as described in section 2.5(c). A standard curve of the elution volume/void volume ratio against log molecular weight is given in figure 3.8 from which a molecular weight of 156000 was obtained for phosphorylated rat liver Fl,6P_2ase. A comparison of this value with the value of 158000 which was estimated for the non-phosphorylated enzyme (chapter 3 in this study), indicates that both the non-phosphorylated and phosphorylated forms of rat liver Fl,6P_2ase are tetrameric.

An examination of the effect of pH on the activities of non-phosphorylated and phosphorylated rat liver Fl,6P_2ases was also carried out. The profiles are given in figure 5.1 and show that the two forms of the enzyme have similar pH optima in the range 7.3 - 7.5

5.4 Substrate Studies

Non-phosphorylated and phosphorylated forms of rat liver Fl, 6P_2ase each gave rise to a linear double-reciprocal plot of initial velocity against Fl, 6P_2 concentration at pH 7.2 and at a free Mg^{2+} ion concentration of 2 mM, (figure 5.2). The K_m values obtained for the non-phosphorylated and phosphorylated enzymes were 2.87 ± 0.16 μM

and 1.94 $\pm$ 0.06 μM respectively, which clearly demonstrates that the phosphorylated enzyme has a higher affinity for $Fl,6P_2$. This experiment was carried out 3 times (only one prep) and the results were always qualitatively similar. While these studies were in progress, Ekman and Dalqvist-Edberg (1981) reported a qualitatively similar finding, although the K_m values obtained by these workers were higher and varied depending on the type of buffer used in the assay.

Both non-phosphorylated and phosphorylated forms of Fl, $6P_2$ase again gave rise to linear double reciprocal plots of initial velocity against $Fl,6P_2$ concentration when assayed at several fixed concentrations of free Mg^{2+} ions (figures 5.3 and 5.5). Replots of the slopes or intercepts of the primary plots against the reciprocal of the free Mg^{2+} ion concentration were parabolic (figures 5.3 and 5.5), as were double reciprocal plots of initial velocity against the free Mg^{2+} ion concentration at fixed levels of free $Fl,6P_2$, (figures 5.4 and 5.6). Thus, in accord with the findings of Nimmo and Tipton (1975b) for the ox liver enzyme at neutral pH, the two forms of the enzyme respond cooperatively to Mg^{2+} ions.

The values for K_m and V_{max} at each free Mg^{2+} ion concentration, for both forms of the enzyme, were calculated from the data in figures 5.3 and 5.5 and are presented in table 5.2. In terms of V_{max}, non-phosphorylated and phosphorylated $Fl,6P_2$ases were activated to the same extent by increasing concentrations of free Mg^{2+} ions. An examination of the K_m values at each free Mg^{2+} ion concentration shows that the phosphorylation-dependent decrease in K_m which was observed at 2mM free Mg^{2+} ions also appeared to be present at lower concentrations of free Mg^{2+} ions. The finding that the K_m values obtained at 1 mM free Mg^{2+} ions were significantly lower than the values obtained

at 2mM free Mg^{2+} ions in the previous experiment might have resulted from the fact that different batches of enzyme were used in the two sets of experiments.

5.5 Inhibition by AMP and Fructose 2,6-Bisphosphate

The activities of non-phosphorylated and phosphorylated forms of rat liver Fl,$6P_2$ase at different levels of Fl,$6P_2$ were investigated over a range of fixed concentrations of AMP at pH 7.2 and at a free Mg^{2+} ion concentration of 2mM. The two forms of the enzyme gave rise to linear double reciprocal plots of initial velocity against Fl,$6P_2$ concentration. The lines at different AMP concentrations were parallel (figures 5.7 and 5.8). It would therefore appear that AMP acts as an uncompetitive inhibitior: the conventional explanation for this would be that AMP can only bind to the enzyme after the substrate has bound. This result is in agreement with a previously reported finding that AMP did not bind to rat liver Fl,$6P_2$ase in the absence of substrate as measured by direct binding studies (Tejwani et al, 1976). Non-phosphorylated and phosphorylated Fl,$6P_2$ases were compared in terms of the reduction in V_{max} obtained at several fixed concentrations of AMP (table 5.3). As judged by this criterion, the data indicate that phosphorylation did not produce any significant change in the sensitivity of the enzyme to inhibition by AMP.

The inhibition of rat liver Fl,$6P_2$ase by F2,$6P_2$ was examined with respect to Fl,$6P_2$ at pH 7.2 and at a free Mg^{2+} ion concentration of 2mM. Both non-phosphorylated and phosphorylated enzymes gave linear double-reciprocal plots of initial velocity against Fl,$6P_2$ concentration at several concentrations of free F2,$6P_2$, (figures 5.9 and 5.11). No activity could be detected from F2,$6P_2$ in the absence of Fl,$6P_2$. Pilkis et al (1981a) recently reported that F2,$6P_2$ behaved as a simple competitive inhibitor with respect to Fl,$6P_2$ase. However, this result

was disputed by Van Schaftingen and Hers (1981b) who demonstrated that $F2,6P_2$ interacted with $F1,6P_2$ase in a cooperative manner. The finding that the plots in figures 5.9 and 5.11 appear to intersect to the right of the 1/rate axis is inconsistent with a simple competitive type of inhibition. The shapes of these plots are discussed further in Chapter 6. Furthermore, replots of the data in the form of Dixon plots (figures 5.10 and 5.12) are parabolic which implies that the enzyme responds sigmoidally to $F2,6P_2$. The significance of these findings is also discussed in the following chapter. Since the plots in figures 5.9 and 5.11 intersected to the right of the 1/rate axis, the comparison of non-phosphorylated and phosphorylated forms of $F1,6P_2$ase with respect to inhibition by $F2,6P_2$ was based on the slopes of these plots which gave the ratio K_m/V_{max}. In this respect, the non-phosphorylated and phosphorylated enzymes were inhibited by $F2,6P_2$ with equal sensitivities (table 5.4). Although it is difficult to obtain K_i values for $F2,6P_2$ from Dixon plots which are parabolic, values of approximately 0.3 μM were estimated for both forms of the enzyme.

The inhibition of $F1,6P_2$ase by AMP with respect to $F1,6P_2$ was examined at pH 7.2 at a free Mg^{2+} ion concentration of 2 mM and in the presence of 1.5 μM free $F2,6P_2$. Non-phosphorylated $F1,6P_2$ase produced linear double-reciprocal plots of initial velocity against $F1,6P_2$ concentration at several concentrations of free AMP, which intersected on the left of the 1/rate axis (figure 5.13). A qualitatively similar response was obtained with the phosphorylated enzyme, (figure 5.14). The quantitative comparison of non-phosphorylated and phosphorylated $F1,6P_2$ases which was based on the K_m/V_{max} ratios obtained from the slopes of the double reciprocal plots, showed that the sensitivity to inhibition by AMP in the presence of $F2,6P_2$ remained unaltered by phosphorylation. When the inhibition of $F1,6P_2$ase

by AMP, with respect to $F1,6P_2$, was compared in the absence and presence of $F2,6P_2$ (figures 5.7 and 5.13), one distinctive feature was that AMP did not behave as an uncompetitive inhibitor in the presence of $F2,6P_2$. In fact, the inhibition by AMP appeared to be non-competitive though it was difficult to be sure. This is examined more closely in the latter part of this chapter (section 5.7). Replots of the slopes of figures 5.13 and 5.14 were parabolic (not illustrated) and therefore it was difficult to ascertain the values for K_i. Another peculiar feature of the comparison between figures 5.9 and 5.13 concerns the behaviour of the enzyme in the absence of AMP. In figure 5.9 the line at 1.47 μM $F2,6P_2$ clearly gives a negative value for V_{max} whereas this is not the case for the corresponding line in figure 5.13 at 1.5 μM $F2,6P_2$. The enzyme concentration in the assay was about 4-fold greater in the latter case and it is possible that some of the abnormal kinetic behaviour depends on enzyme concentration.

An examination of the inhibition of $F1,6P_2$ase at several concentrations of $F2,6P_2$ in the presence of 25 μM free AMP, at pH 7.2 and at a free Mg^{2+} ion concentration of 2mM, was carried out. Double-reciprocal plots of initial velocity against $F1,6P_2$ concentration were linear and intersected to the left of the 1/rate axis, (figures 5.15 and 5.16). Phosphorylation did not alter the sensitivity of the enzyme to inhibition by $F2,6P_2$ in the presence of AMP as judged by the comparison of the K_m and V_{max} values calculated from each of the double-reciprocal plots, (table 5.6). Once again, a striking difference was noticed when comparing the types of inhibition given by $F2,6P_2$ in the absence and presence of 25 μM AMP (figures 5.9 and 5.15): in the presence of AMP the intersection of the plots on the right of the 1/rate axis (as seen in figure 5.9), was no longer apparent. The lines obtained at finite levels of $F2,6P_2$ appear to intersect at or

near one point and so $F2,6P_2$ appeared to produce mixed inhibition. Replots of the slopes of figures 5.15 and 5.16 against the concentration of free $F2,6P_2$ were linear. From these replots, values of 0.12 μM and 0.14 μM were obtained for K_i for the non-phosphorylated and phosphorylated enzymes respectively.

5.6 The Kinetic Interactions between Mg^{2+} ions, AMP and Fructose 2,6-Bisphosphate

The results of the previous section demonstrated the ability of $F2,6P_2$ to influence the inhibition of $F1,6P_2$ase by AMP and conversely the ability of AMP to affect inhibition by $F2,6P_2$. In this section, the interactions of AMP, $F2,6P_2$ and Mg^{2+} ions were further investigated and in addition possible phosphorylation - dependent changes in the values of the Hill coefficients and apparent affinities for these ligands were examined.

The cooperative response of non-phophorylated and phosphorylated forms of rat liver $F1,6P_2$ase to AMP was examined at several concentrations of free Mg^{2+} ions. The results, which are presented as Hill plots in figures 5.17 and 5.18, demonstrate that an increase in the free Mg^{2+} ion concentration produced an increase in the Hill coefficient, h, with a corresponding increase in the apparent K_i for AMP. A similar finding was first reported by Nimmo and Tipton (1975c) for ox liver $F1,6P_2$ase. The data also indicate that phosphorylation did not appear to alter significantly the values for h and the apparent K_i for AMP (table 5.7).

When the sigmoid response of $F1,6P_2$ase to free Mg^{2+} ions was investigated at different free AMP concentrations it was found that AMP caused a decrease in h and an increase in the apparent K_a for Mg^{2+} ions, (figures 5.19 and 5.20). In contrast, Nimmo and Tipton (1975c) demonstrated that AMP caused an increase in h with respect to

Mg^{2+} ions for ox liver $Fl,6P_2$ase. The explanation for this finding is discussed in Chapter 7, (section 7.1). Once again, there was no significant difference between the responses of non-phosphorylated and phosphorylated forms of the enzyme as judged by the values for h and the apparent K_i for Mg^{2+} ions, (table 5.8).

The kinetic interactions between Mg^{2+} ions and $F2,6P_2$ were investigated. Increasing concentrations of free Mg^{2+} ions had little effect either on h, or on the apparent K_i for $F2,6P_2$, (figures 5.21 and 5.22). Again there was no significant difference between the phosphorylated and non-phosphorylated forms of the enzyme. The values of h obtained from the Hill plots were all greater than 1.0 which in accord with the results presented in section 5.5, indicated that the inhibition by $F2,6P_2$ was not simple.

An examination of the effect of $F2,6P_2$ on the cooperative response of non-phosphorylated $Fl,6P_2$ase to Mg^{2+} ions demonstrated that increasing concentrations of free $F2,6P_2$ decreased h and increased the apparent K_a for Mg^{2+} (figure 5.23). The results obtained with the phosphorylated enzyme were qualitatively and quantitatively very similar (figure 5.24 and table 5.10. The significance of the $F2,6P_2$ - dependent decrease in h is discussed in Chapter 7 (section 7.1).

The interactions between AMP and $F2,6P_2$ were studied at a fixed free Mg^{2+} ion concentration of 2mM. The results indicated that increasing concentrations of free $F2,6P_2$ caused a considerable decrease in both h and the apparent K_i for AMP, (figure 5.25). In contrast, when the effect of AMP on the inhibition of $Fl,6P_2$ase by $F2,6P_2$ was examined, AMP caused a decrease in the apparent K_i for $F2,6P_2$ but did not alter h, (figure 5.27). Similar observations were made with the phosphorylated enzyme (figures 5.26 and 5.28 : tables 5.11 and 5.12).

5.7 The Binding of AMP by Rat Liver Fructose 1,6-Bisphosphatase

The data presented in figure 5.7 demonstrated that AMP was an uncompetitive inhibitor of $Fl,6P_2$ase with respect to $Fl,6P_2$. However, when the inhibition by AMP was examined in the presence of $F2,6P_2$ plots which were not characteristic of uncompetitive inhibition were generated (figure 5.13). These findings immediately suggested that AMP was unable to bind to $Fl,6P_2$ase except in the presence of $Fl,6P_2$ or $F2,6P_2$. To test this hypothesis, the binding of AMP to rat liver $Fl,6P_2$ase was investigated in the absence and presence of $Fl,6P_2$ or $F2,6P_2$ using the equilibrium dialysis technique as described in section 2.9(b). The results were expressed as Scatchard plots of the number of moles of AMP bound per mole of enzyme (expressed at the tetramer) (r) against r/AMP ,(figure 5.29). In the absence of either $Fl,6P_2$ or $F2,6P_2$, no binding of AMP could be detected at concentrations of up to 50 μM. However, AMP bound to the enzyme in the presence of either 100 μM $Fl,6P_2$ or 10 μM $F2,6P_2$: the slopes of the plots corresponded to dissociation constants of the enzyme . AMP complex of 2.4 μM and 3.7 μM respectively and in each case the vertical intercept corresponded to a maximal binding of approximately 2 moles of enzyme per mole of AMP. The values for the dissociation constants compare well with the K_i values obtained from the Hill plots and the stoichiometry of binding of 2 moles of AMP per mole of enzyme is in agreement with the findings of Tejwani et al (1976) for rat liver $Fl,6P_2$ase and Nimmo and Tipton (1975c) for the ox liver enzyme. As yet, there have been no other reports of the effect of $F2,6P_2$ on the binding of AMP by rat liver $Fl,6P_2$ase.

5.8 Discussion : The Kinetic Significance of Phosphorylation

A thorough examination of the kinetic and regulatory properties of non-phosphorylated and phosphorylated forms of rat liver Fl,6P_2ase has been carried out. The results have clearly demonstrated that phosphorylation reduced the K_m by a factor of 1.5 to 2.0. It is therefore reasonable to propose that this increase in the affinity for Fl,6P_2 is responsible, at least in part, for the activation of the enzyme by cAMP - PrK. A similar result was observed in a recent study by Ekman and Dalqvist-Edberg (1981) who reported K_m values for the non-phosphorylated and phosphorylated enzymes of 20 μM and 11 μM respectively when assayed in 10 mM potassium phosphate buffer at pH 7.5. Values of 8 μM and 3 μM respectively were obtained when the buffer was 10mM Tris-HCl at pH 7.5. Given that cells do contain some inorganic phosphate, which might be expected to affect Fl,6P_2ase, it is arguable that the K_m values obtained using the phosphate-buffered system are the most significant in physiological terms. Moreover, the relative increase in activity at <u>in vivo</u> Fl,6P_2 levels of 7 to 67 μM (Van Schaftingen et al, 1980a) would be greater if the prevailing K_m values approximated those observed in the phosphate buffered system rather than the lower values observed in the other two buffer systems.

Although **phosphorylation** caused a significant decrease in the K_m, phosphorylation-dependent changes were not observed in the sensitivity of the enzyme to Mg^{2+} ions, AMP, F2,6P_2 or combinations of these allosteric ligands. In a similar study, using purified pig kidney Fl,6P_2ase, Mendicino et al (1978) were unable to detect changes in the degree of inhibition by AMP or by high levels of Fl,6P_2. However, Marikofer-Zwez et al (1981) have recently communicated that, following intraperitoneal injection of glucagon, the activity of Fl,6P_2ase in isolated rat liver cytosol was increased by 44% and this

was accompanied by an increase in the K_i for $F2,6P_2$ from 0.1 μM to 0.2 μM. Since liver extracts were used in these experiments, the possibility exists that the $F2,6P_2$ in the assay would be hydrolysed by fructose 2,6-bisphosphatase. Glucagon treatment would increase the rate of hydrolysis of $F2,6P_2$ byactivating $F2,6P_2$ase (El - Maghrabi et al, 1982) and therefore the inhibition of $F1,6P_2$ase from glucagon-treated rats by $F2,6P_2$ might appear to be less when compared with the controls. This emphasises the desirability of performing comparative studies with purified enzymes. However, assuming that phosphorylation does increase the K_i for $F2,6P_2$, it is possible that the change was obscured under the assay conditions employed in this study (section 2.3(a)). Bearing in mind the effect of the composition of the assay mixture on the affinity of the enzyme for $F1,6P_2$ alterations in the sensitivity to inhibitors might be perceptible under assay conditions which more accurately simulate the <u>in vivo</u> environment. For this reason a comparative re-examination of the inhibition of non-phosphorylated and phosphorylated forms of $F1,6P_2$ase should be performed in an assay system at physiological ionic strength and phosphate concentration. One other possibility which is worth considering is the effect of a second phosphorylation such as has been postulated in Chapter 4. Any changes in the properties of $F1,6P_2$ase in response to cyclic AMP-dependent phosphorylation might also be dependent on the phosphorylation state of a second phosphorylation site. This hypothesis clearly merits some investigation.

A model of the cooperative interactions of rat liver $F1,6P_2$ase, based on the kinetic data presented in this chapter and in the following chapter, is presented and discussed in Chapter 7 (section 7.1).

Figure 5.1

pH optima of non-phosphorylated and phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.3(a); 50 mM imidazole-HCl buffer was used in the pH range 6.0-7.4, 50 mM Tris-HCl buffer in the pH range 7.4-9.0 and 50 mM glycine-KOH buffer in the pH range 9.0-10.0. The symbols are:

○ non-phosphorylated rat liver F1,6P_2ase

□ phosphorylated rat liver F1,6P_2ase

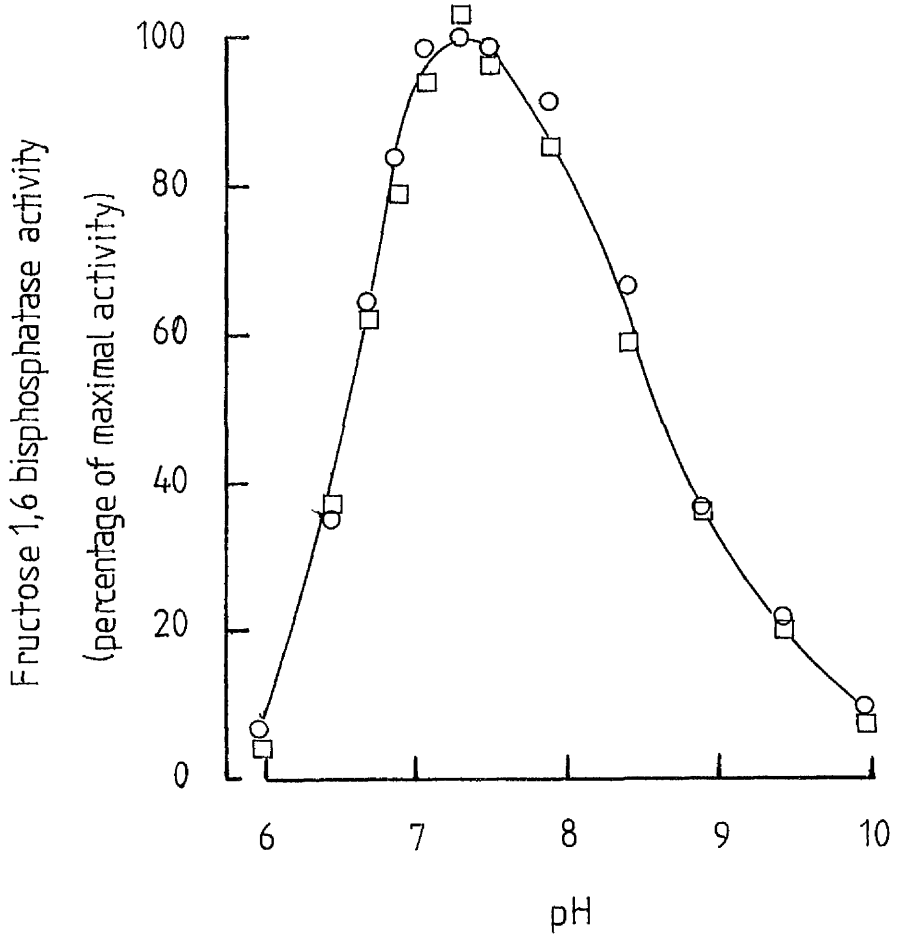

Fructose 1,6 bisphosphatase activity
(percentage of maximal activity)
100
80
60
40
20
0
6
7
8
9
10
pH

Figure 5.2

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at 2 mM free Mg^{2+} for non-phosphorylated and phosphorylated rat liver fructose 1,6-bisphosphatase

The assays were performed as described in section 2.9(a).

The symbols are:

□ non-phosphorylated F1,6P_2ase

○ phosphorylated F1,6P_2ase

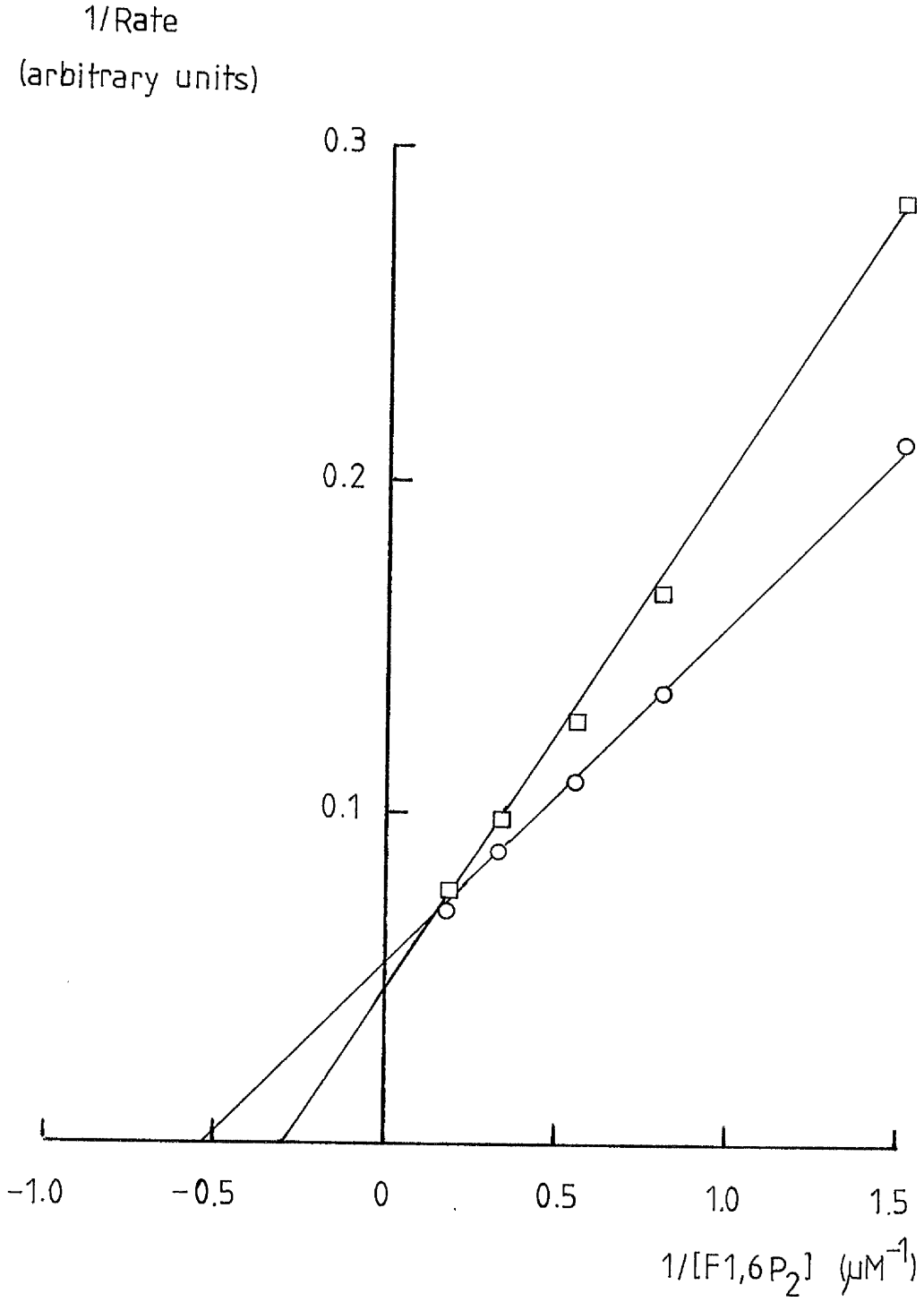
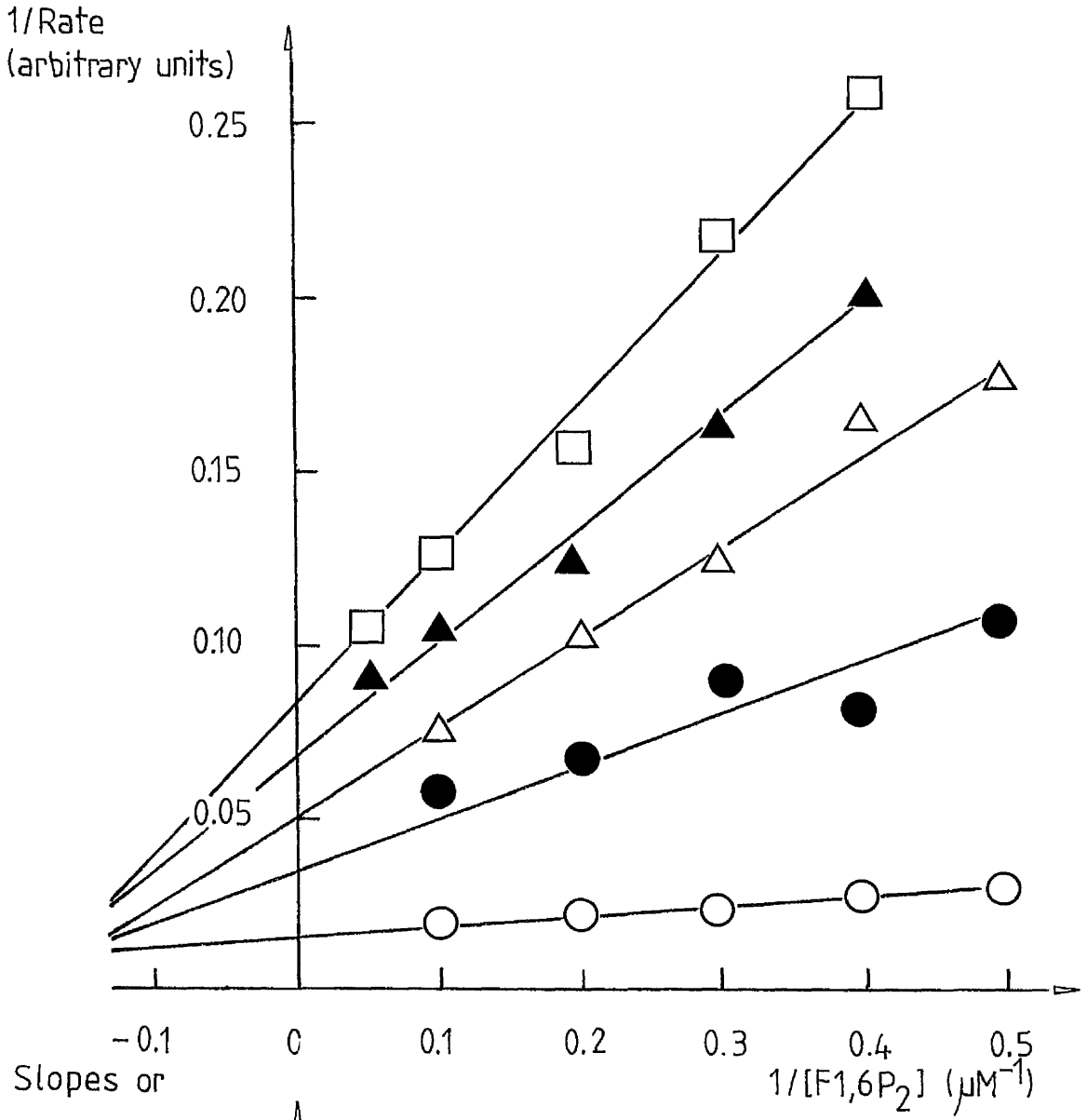

1/Rate
(arbitrary units)
0.3
0.2
0.1
-1.0
-0.5
0
0.5
1.0
1.5
1/[F1,6P2] (μM-1)

Figure 5.3

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at a series of fixed concentrations of free Mg^{2+} ions for non-phosphorylated rat liver fructose 1,6-bisphosphatase

The assays were performed as described in section 2.9(a), and the concentrations of free Mg^{2+} ions were: 1.0 mM (○), 0.33 mM (●), 0.25 mM (△), 0.20 mM (▲) and 0.17 mM (□). Inset: the variation of the slopes (◇) and intercepts (◆) as a function of the free Mg^{2+} ion concentration.


1/Rate
(arbitrary units)
0.25
0.20
0.15
0.10
0.05
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[F1,6P2] (μM-1)

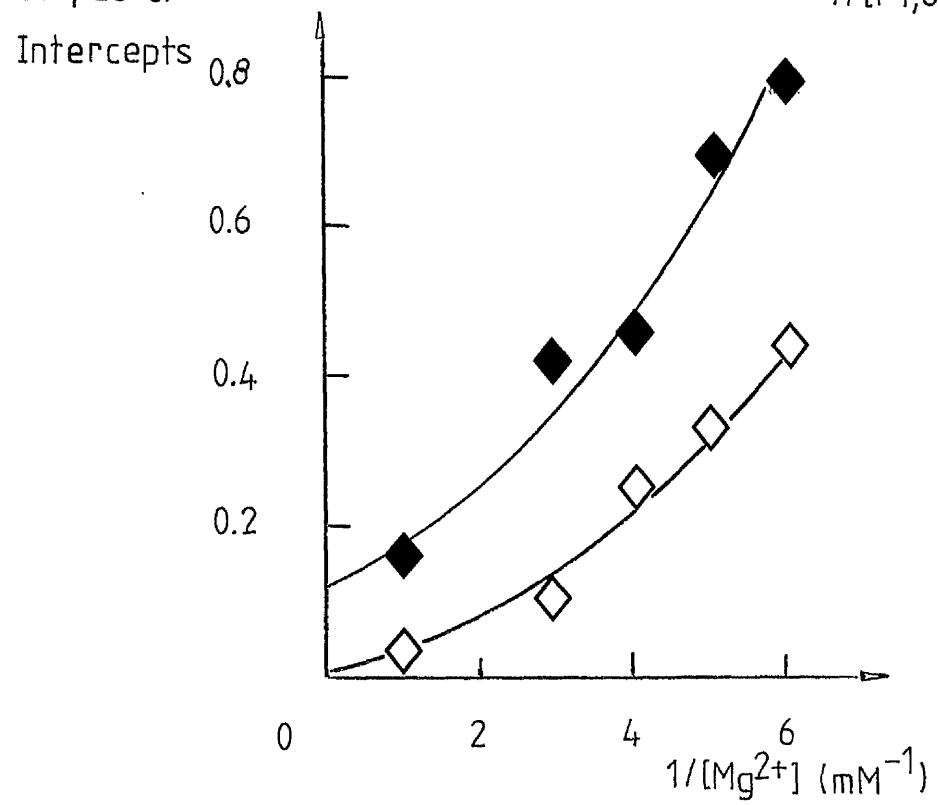

Slopes or
Intercepts
0.8
0.6
0.4
0.2
0
2
4
6
1/[Mg2+] (mM-1)

Figure 5.4

Double-reciprocal plots of initial velocity against free Mg^{2+} ion concentration at pH 7.2 and at a series of fixed concentrations of free fructose 1,6-bisphosphate for non-phosphorylated rat liver fructose 1, 6-bisphosphatase

The data of figure 5.3 were replotted against the reciprocal of the free Mg^{2+} ion concentration. The free fructose 1,6-bisphosphate concentrations were: 10.0 μM (○), 5.0 μM (●), 3.3 μM (△) and 2.5 μM (▲).

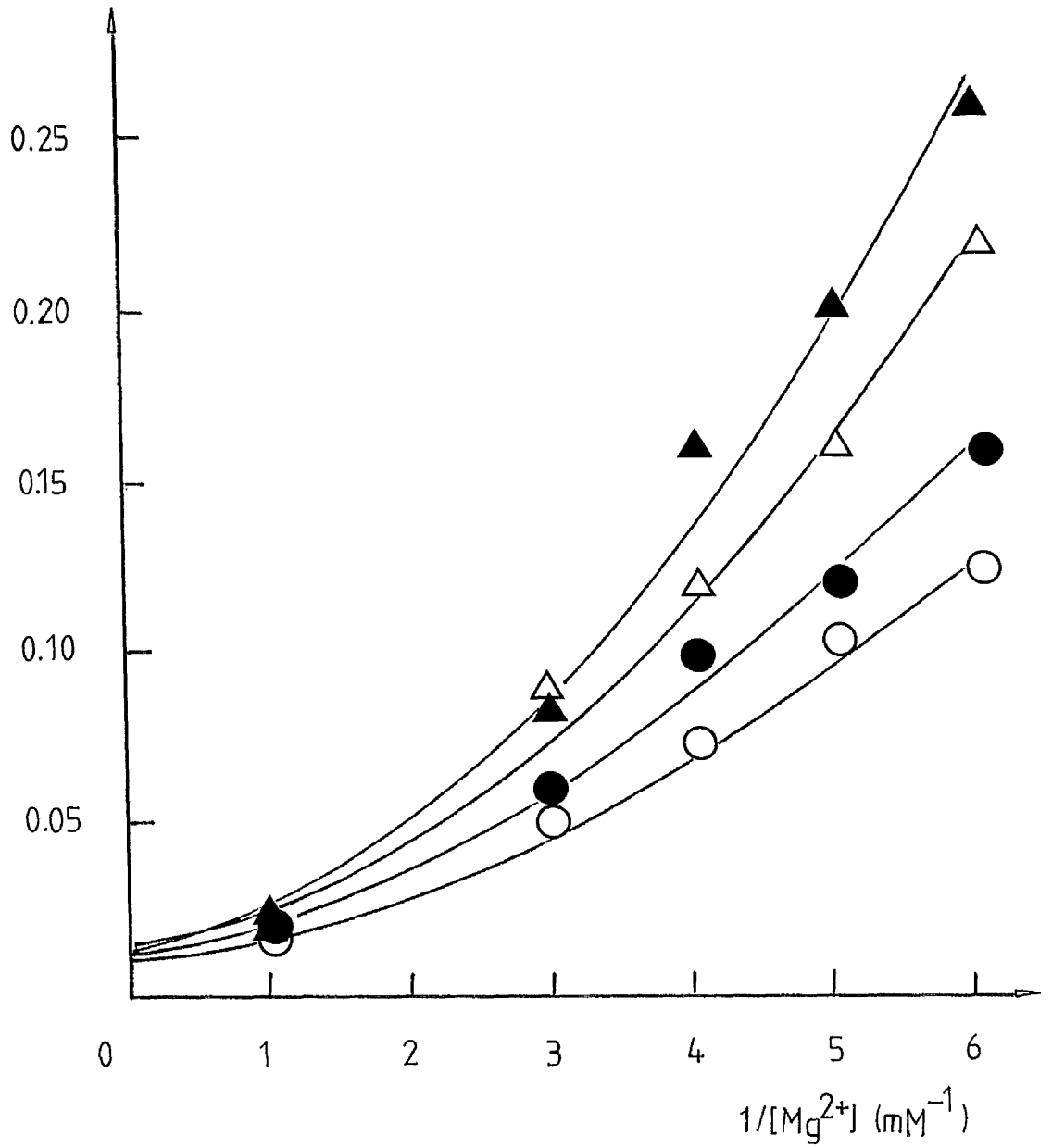
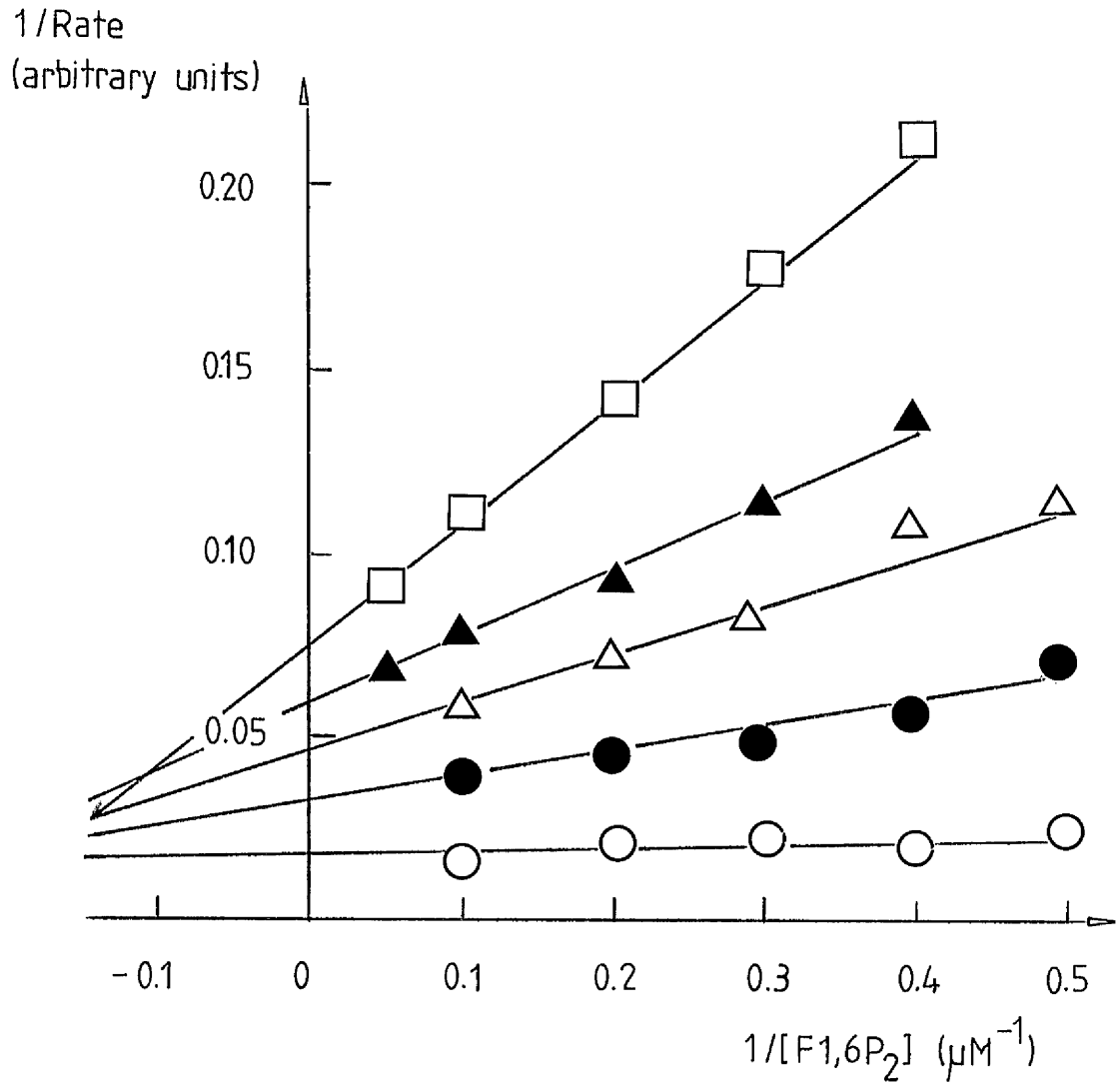

1/Rate
(arbitrary units)
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
5
6
1/[Mg2+] (mM-1)

Figure 5.5

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentrations at pH 7.2 and at a series of fixed concentrations of free Mg^{2+} ions for phosphorylated rat liver fructose 1,6-bisphosphatase

The assays were performed as described in section 2.9(a), and the concentrations of free Mg^{2+} ions were: 1.0 mM (○), 0.33 mM (●), 0.25 mM (△), 0.20 mM (▲) and 0.17 mM (□). Inset: The variation of the slopes (◇) and intercepts (◆) as a function of the free Mg^{2+} ion concentration.


1/Rate
(arbitrary units)
0.20
0.15
0.10
0.05
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[F1,6P2] (μM-1)

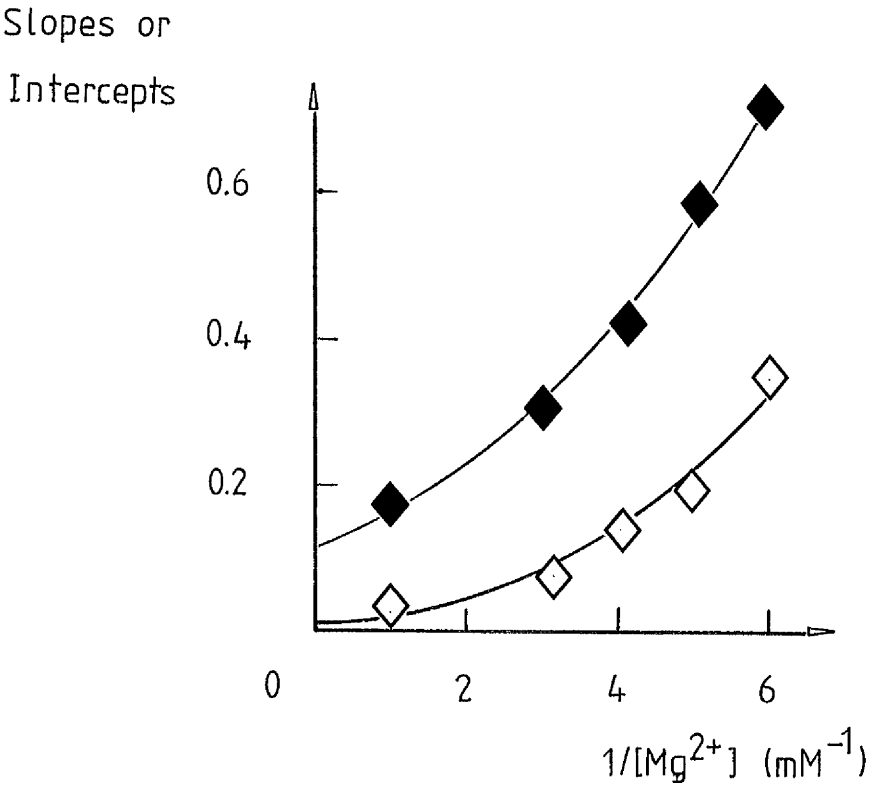

Slopes or
Intercepts
0.6
0.4
0.2
0
2
4
6
1/[Mg2+] (mM-1)

Figure 5.6

Double-reciprocal plots of initial velocity against free Mg^{2+} ion concentration at pH 7.2 and at a series of fixed concentrations of free fructose 1,6-bisphosphate for phosphorylated rat liver fructose 1,6-bisphosphatase

The data of figure 5.5 were replotted against the reciprocal of the free Mg^{2+} ion concentration. The free fructose 1,6-bisphosphate concentrations were: 10.0 μM (○), 5.0 μM (●), 3.3 μM (△) and 2.5 μM (▲).

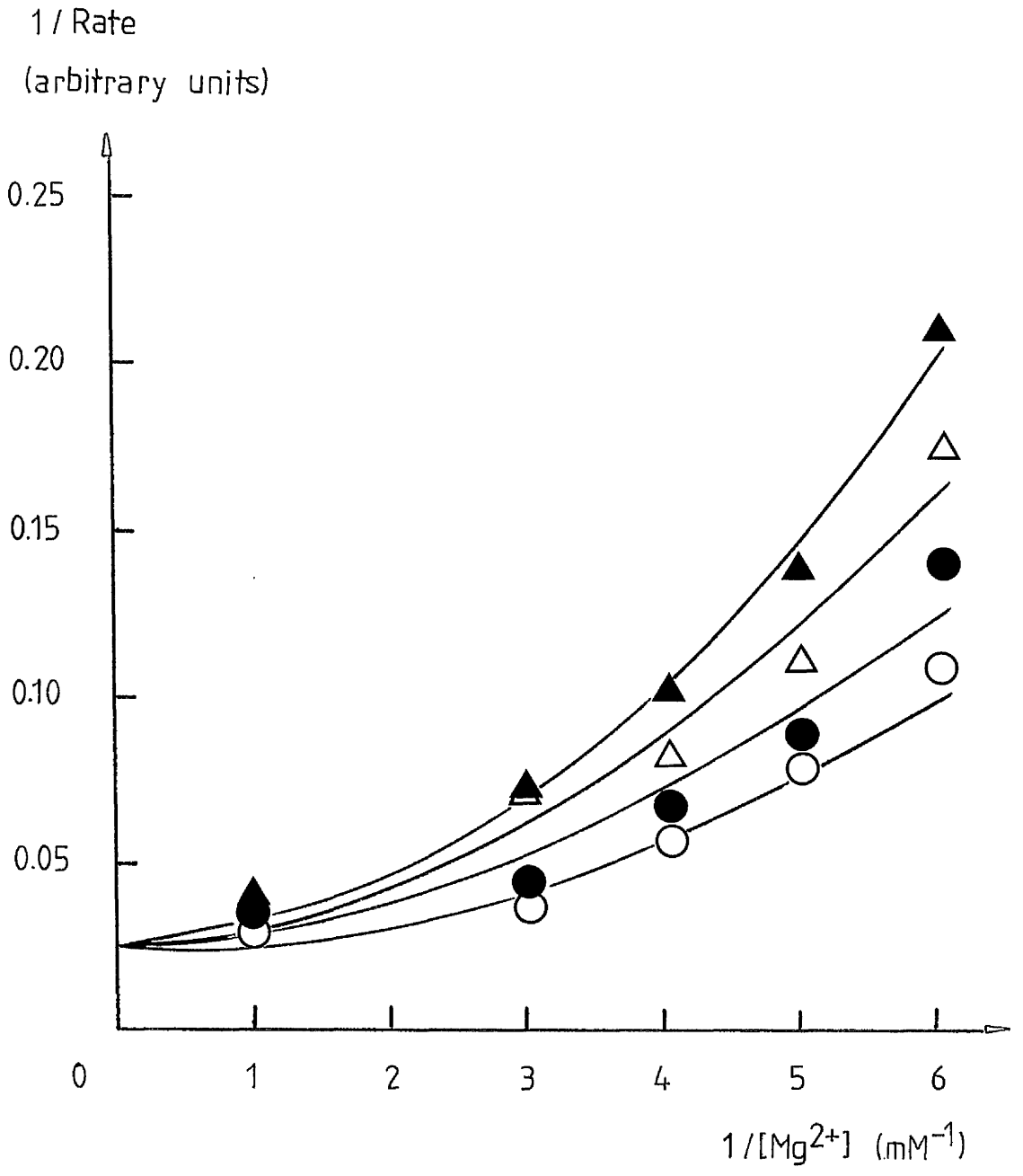

1 / Rate
(arbitrary units)
0.25
0.20
0.15
0.10
0.05
0
1
2
3
4
5
6
1/[Mg2+] (mM-1)

Figure 5.7

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at a series of fixed concentrations of free AMP for non-phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM free Mg^{2+} ions. The concentrations of free AMP were: 0 (○), 10 μM (●), 20 μM (△) and 30 μM (▲).

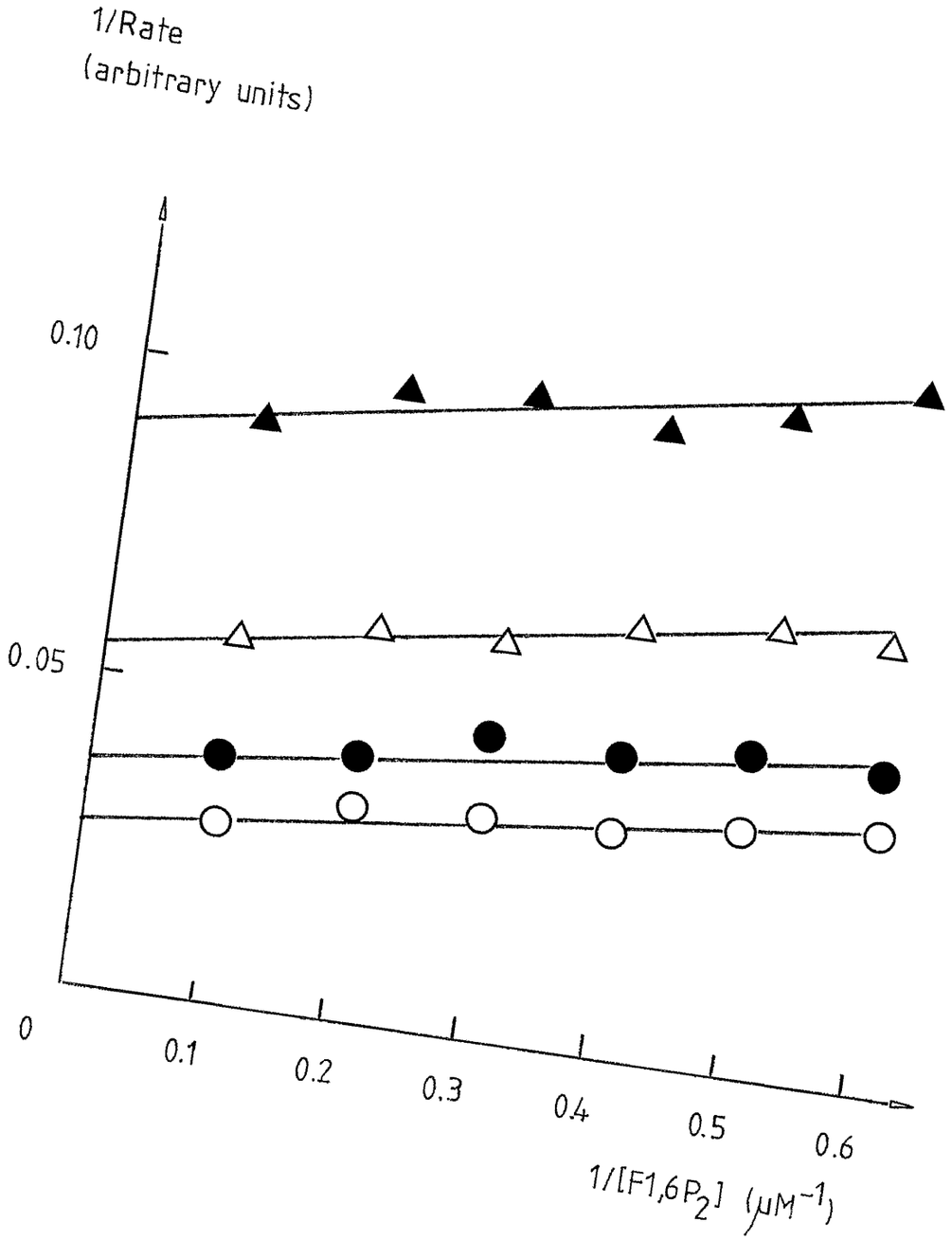

1/Rate
(arbitrary units)
0.10
0.05
0
0.1
0.2
0.3
0.4
0.5
0.6
1/[F1,6P2] (μM-1)

Figure 5.8

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at a series of fixed concentrations of free AMP for phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM free Mg^{2+} ions. The concentrations of free AMP were: 0 (○), 10 μM (●), 20 μM (△) and 30 μM (▲).

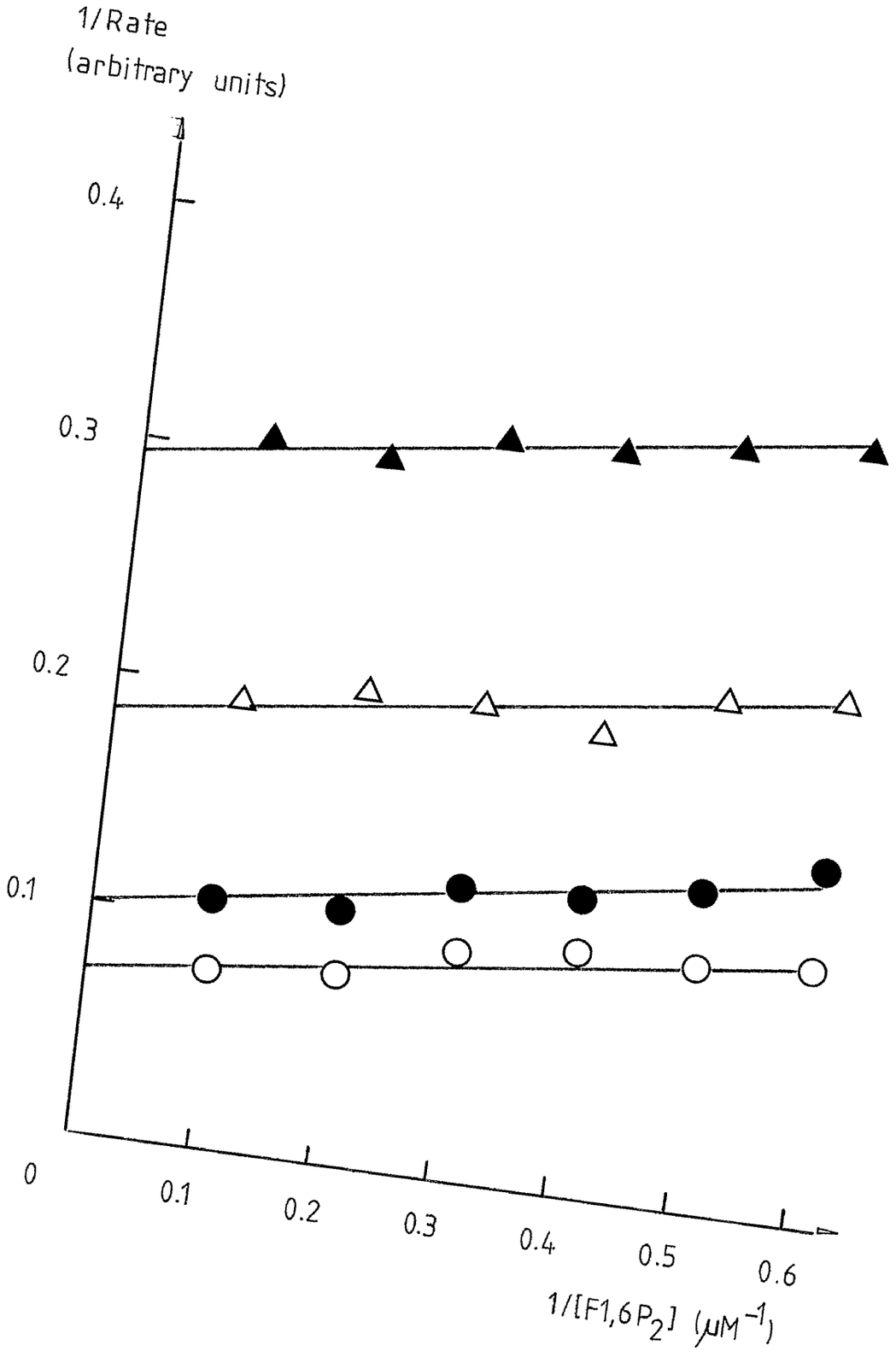

1/Rate
(arbitrary units)
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
1/[F1,6P2] (μM-1)

Figure 5.9

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at a series of concentrations of free fructose 2,6-bisphosphate for non-phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM free Mg^{2+} ions. The concentrations of free fructose 2,6-bisphosphate were: 0 (△), 0.5 μM (▲), 0.29 μM (◇), 0.59 μM (◆) and 1.47 μM (□).

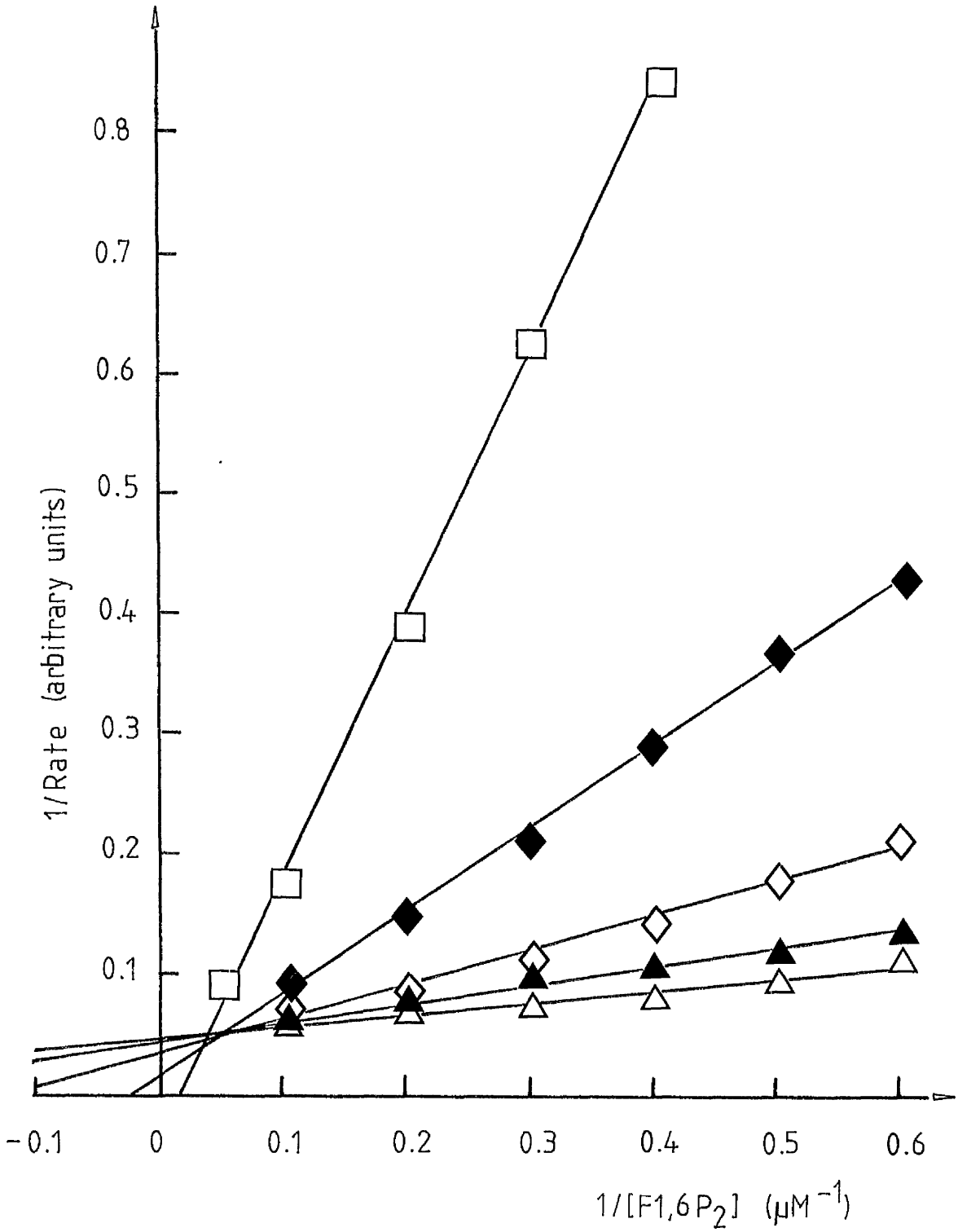

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/Rate (arbitrary units)
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
1/[F1,6P2] (μM-1)

Figure 5.10

Dixon plots for the inhibition of non-phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate

The data from figure 5.9 were replotted against the fructose 2,6-bisphosphate concentration. The free fructose 1,6-bisphosphate concentrations were: 10.0 μM (△), 5.0 μM (▲), 3.3 μM (◇), 2.5 μM (◆), 2.0 μM (□) and 1.67 μM (■).

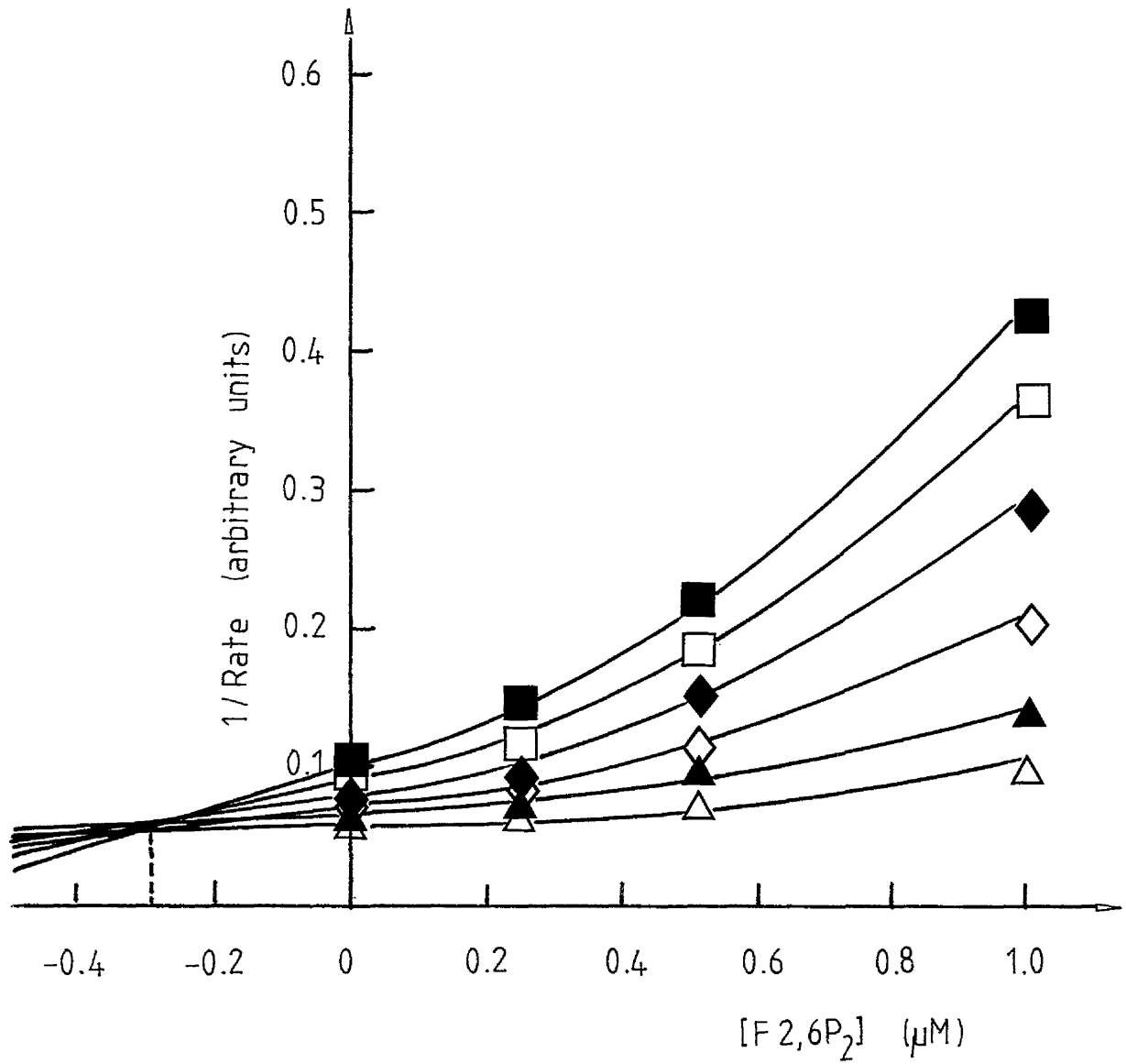

0.6
0.5
0.4
0.3
0.2
0.1
1 / Rate (arbitrary units)
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
[F 2,6P2] (μM)

Figure 5.11

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2 and at a series of concentrations of free fructose 2,6-bisphosphate for phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM free Mg^{2+} ions. The concentrations of free fructose 2,6-bisphosphate were: 0 (△), 0.15 μM (▲), 0.29 μM (◇), 0.59 μM (◆) and 1.47 μM (□).

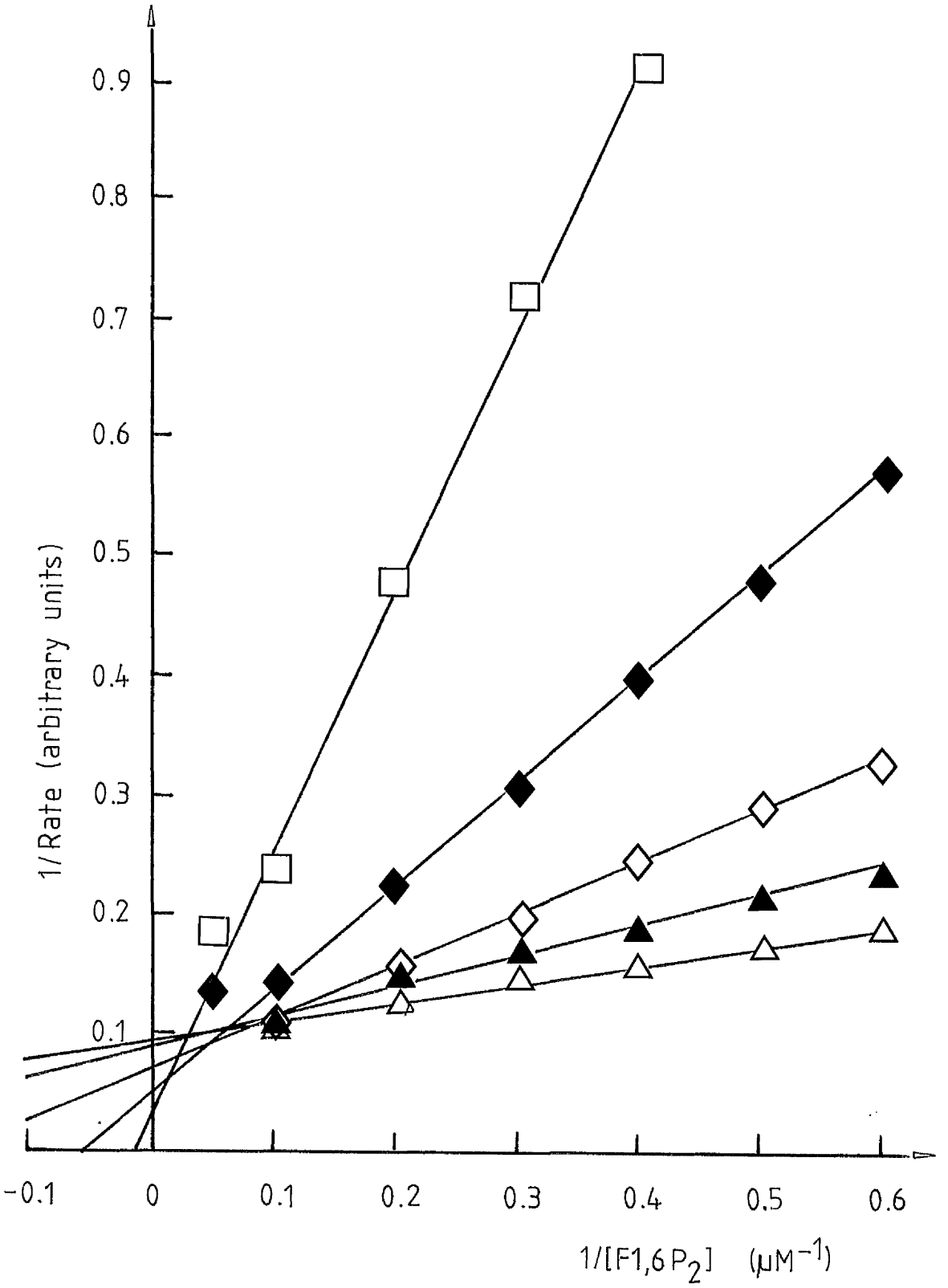

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/Rate (arbitrary units)
-0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
1/[F1,6P2] (μM-1)

Figure 5.12

Dixon plots for the inhibition of phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate

The data from figure 5.11 were replotted against the fructose 2,6-bisphosphate concentration. The free fructose 1,6-bisphosphate concentrations were: 10.0 μM (△), 5.0 μM (▲), 3.3 μM (◇), 2.5 μM (◆), 2.0 μM (□) and 1.67 μM (■).

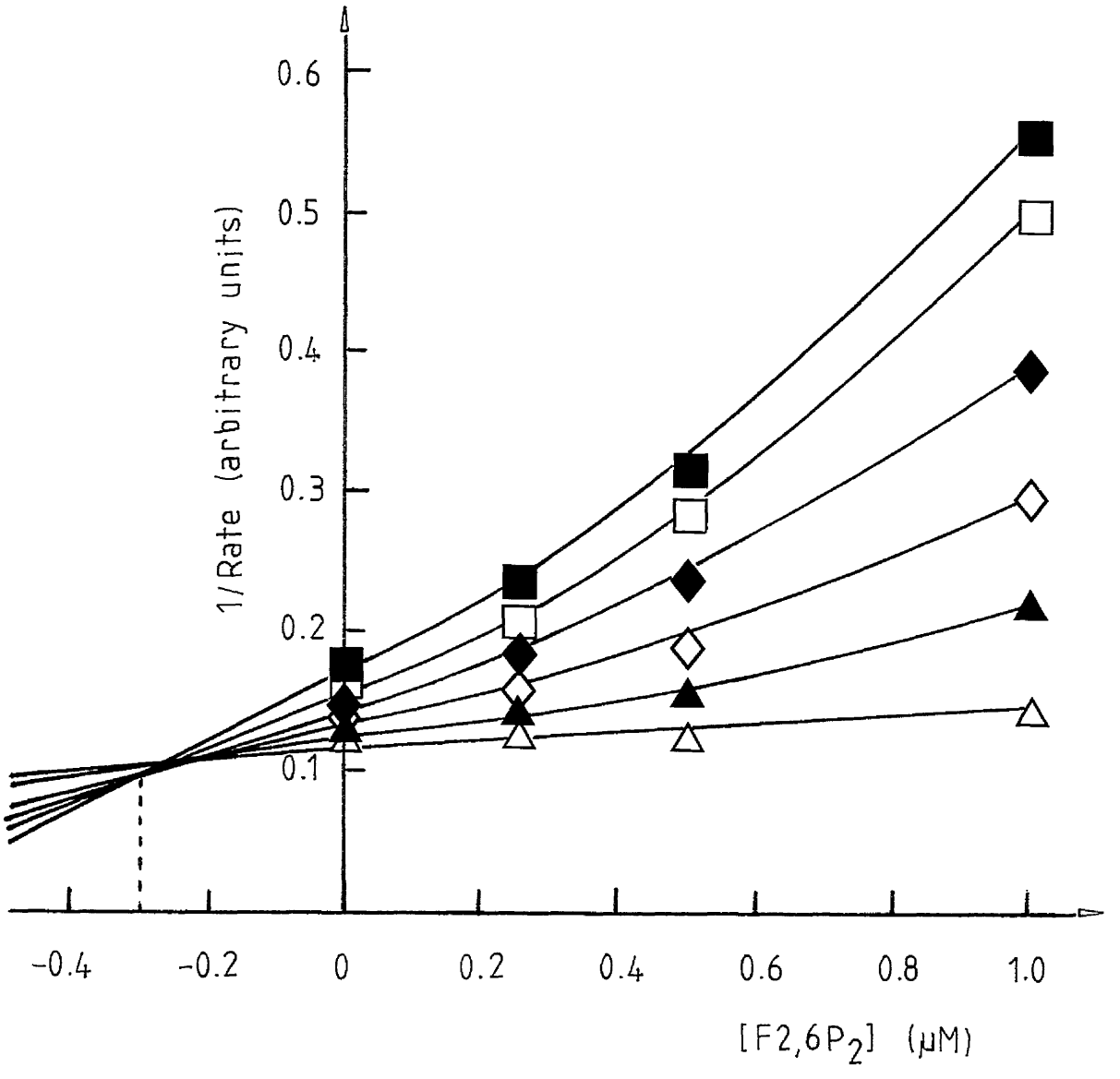

0.6
0.5
0.4
0.3
0.2
0.1
1/Rate (arbitrary units)
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
[F2,6P2] (μM)

Figure 5.13

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2, at 1.5 μM free fructose 2,6-bisphosphate and at a series of fixed concentrations of free AMP for non-phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM Mg^{2+} ions. The concentrations of free AMP were: 0 (△), 2.5 μM (▲), 5.0 μM (◇) and 10.0 μM (◆).

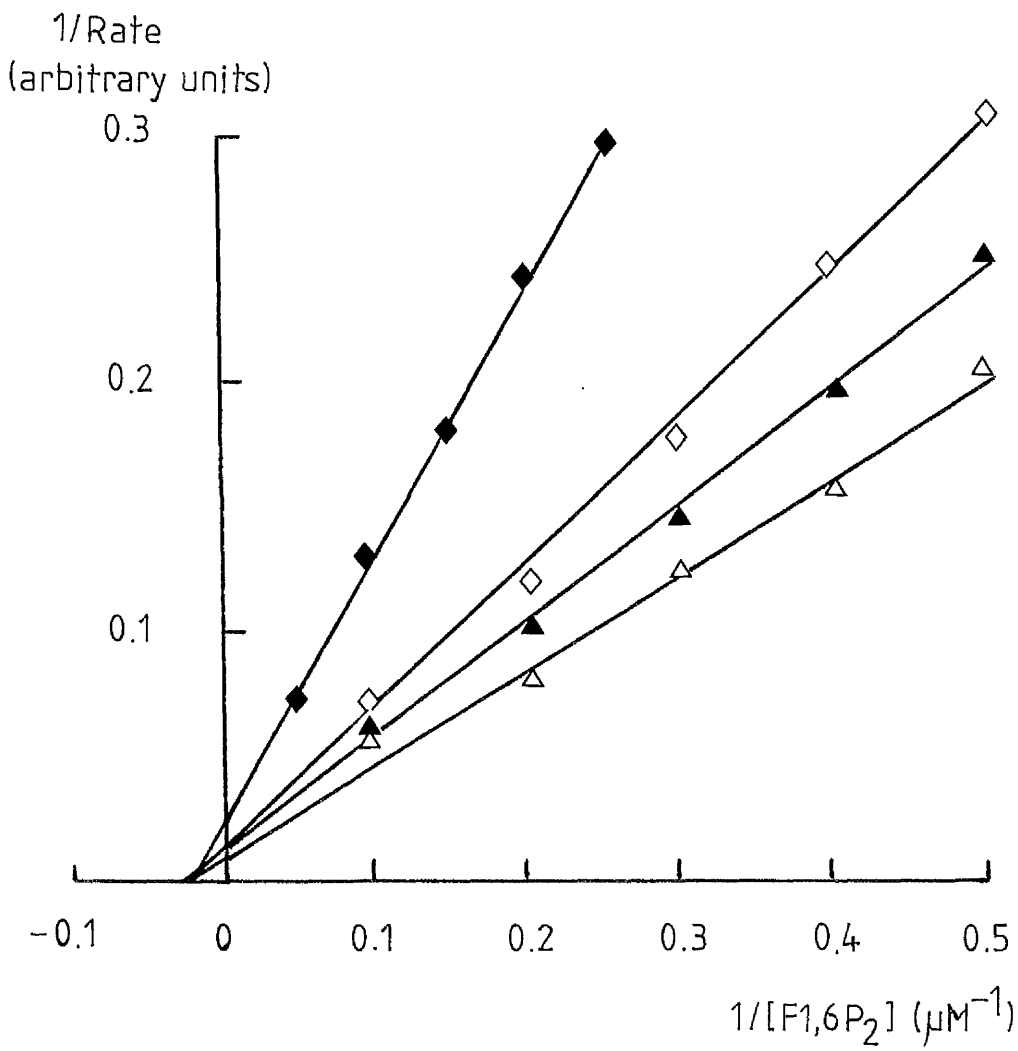

1/Rate
(arbitrary units)
0.3
0.2
0.1
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[F1,6P2] (μM-1)

Figure 5.14

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2, at 1.5 μM free fructose 2,6-bisphosphate and at a series of fixed concentrations of free AMP for phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM Mg^{2+} ions. The concentrations of free AMP were: 0 (△), 2.5 μM (▲), 5.0 μM (◇) and 10.0 μM (◆).

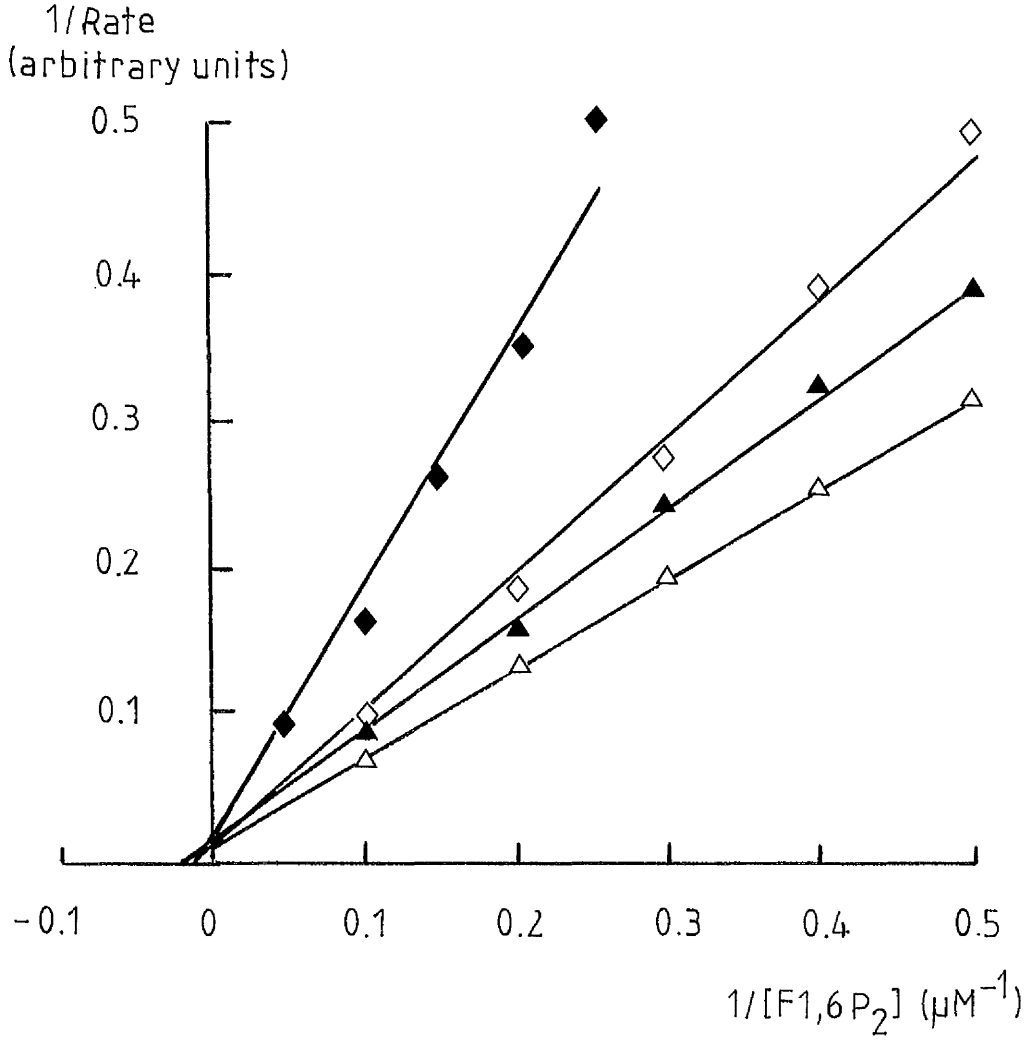

1/Rate
(arbitrary units)
0.5
0.4
0.3
0.2
0.1
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[F1,6P2] (μM-1)

Figure 5.15

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2, at 25 μM free AMP and at a series of fixed concentrations of free fructose 2,6-bisphosphate for non-phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM Mg^{2+} ions. The concentrations of free fructose 2,6-bisphosphate were: 0 (O), 0.15 μM (▲), 0.29 μM (△), 0.59 μM (■) and 1.47 μM (□).

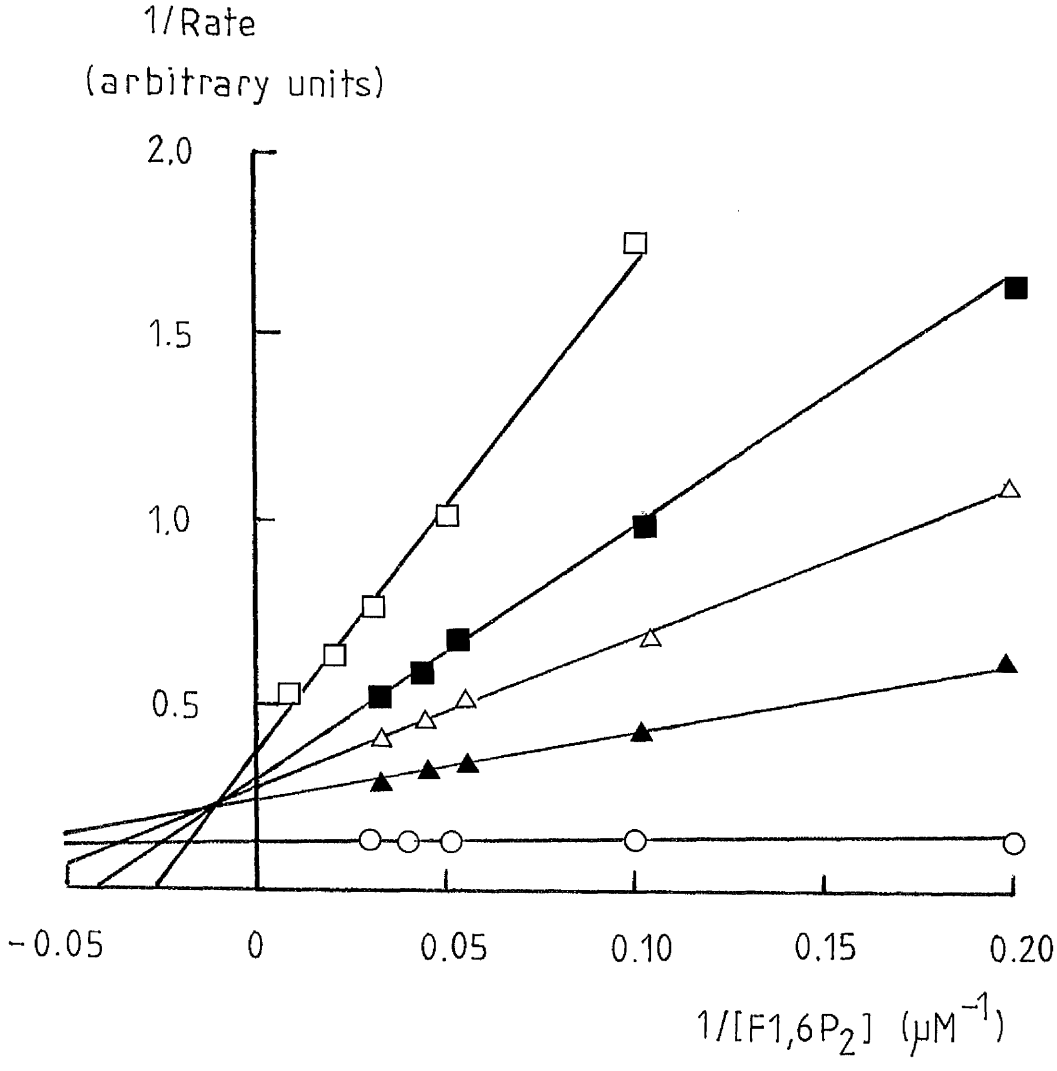

1/Rate
(arbitrary units)
2.0
1.5
1.0
0.5
-0.05
0
0.05
0.10
0.15
0.20
1/[F1,6P2] (μM-1)

Figure 5.16

Double-reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at pH 7.2, at 25 μM free AMP and at a series of fixed concentrations of free fructose 2,6-bisphosphate for phosphorylated rat liver fructose 1,6-bisphosphatase

The enzyme was assayed as described in section 2.9(a), with 2 mM Mg^{2+} ions. The concentrations of free fructose 2,6-bisphosphate were: 0 (0), 0.15 μM (▲), 0.29 μM (△), 0.59 μM (■) and 1.47 μM (□).

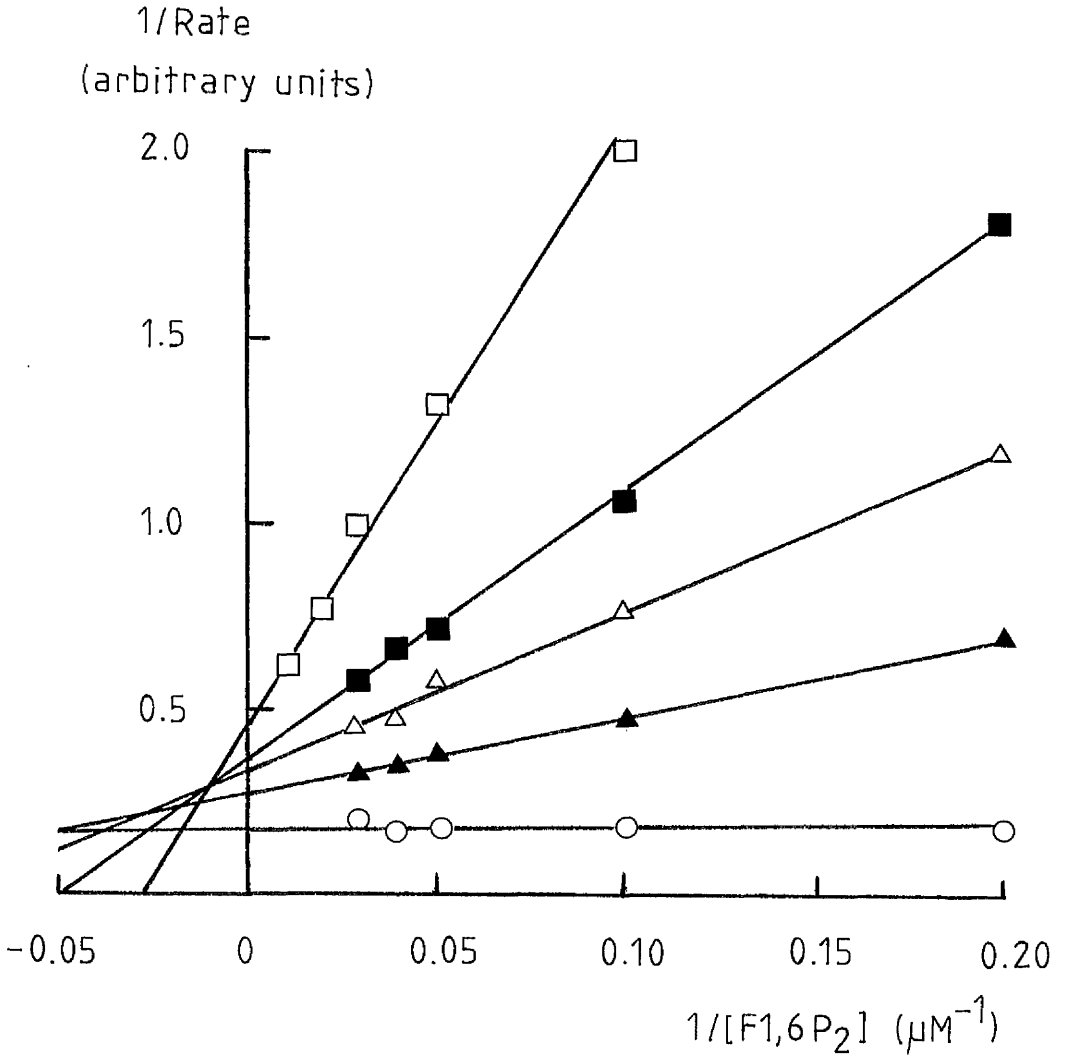

1/Rate
(arbitrary units)
2.0
1.5
1.0
0.5
-0.05
0
0.05
0.10
0.15
0.20
1/[F1,6P2] (μM-1)

Figure 5.17

Hill plots of the inhibition of non-phosphorylated rat liver fructose 1,6-bisphosphatase by AMP at a series of fixed concentrations of free Mg^{2+} ions

The concentration of fructose 1,6-bisphosphate was 100 μM and the concentrations of free AMP were expressed in μM. The concentrations of free Mg^{2+} ions were: 0.17 mM (□), 0.25 mM (▲), 0.67 mM (△), 1.50 mM (●) and 3.00 mM (○). The assays were performed as described in section 2.9(a).

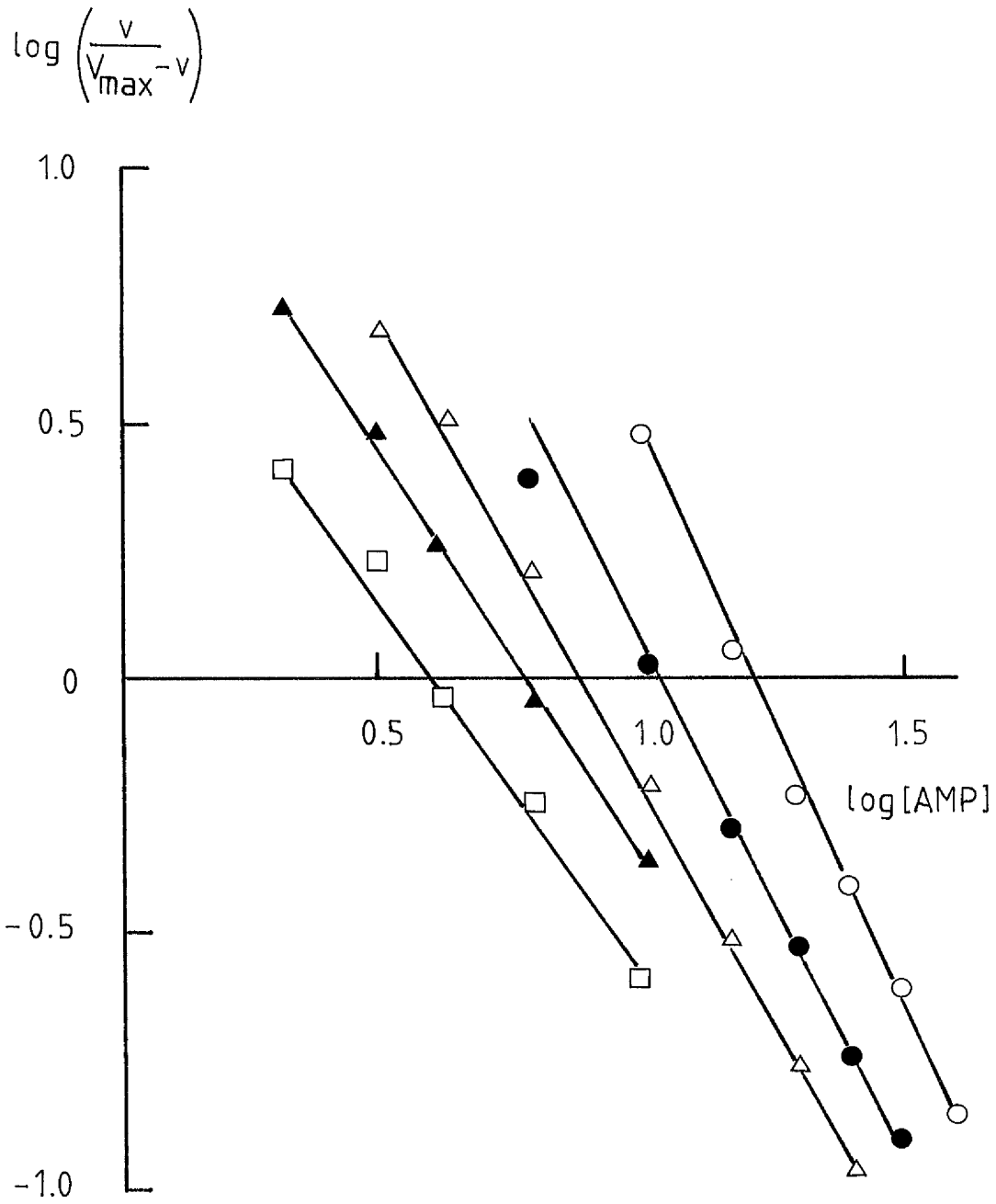

log (v / (Vmax - v))
1.0
0.5
0
-0.5
-1.0
0.5
1.0
1.5
log [AMP]

Figure 5.18

Hill plots of the inhibition of phosphorylated rat liver fructose 1,6-bisphosphatase by AMP at a series of fixed concentrations of free Mg^{2+} ions

The concentration of fructose 1,6-bisphosphate was 100 μM and the concentrations of free AMP were expressed in μM. The concentrations of free Mg^{2+} ions were:

0.17 mM (□), 0.25 mM (▲), 0.67 mM (△), 1.50 mM (●) and 3.00 mM (○). The assays were performed as described in section 2.9(a).

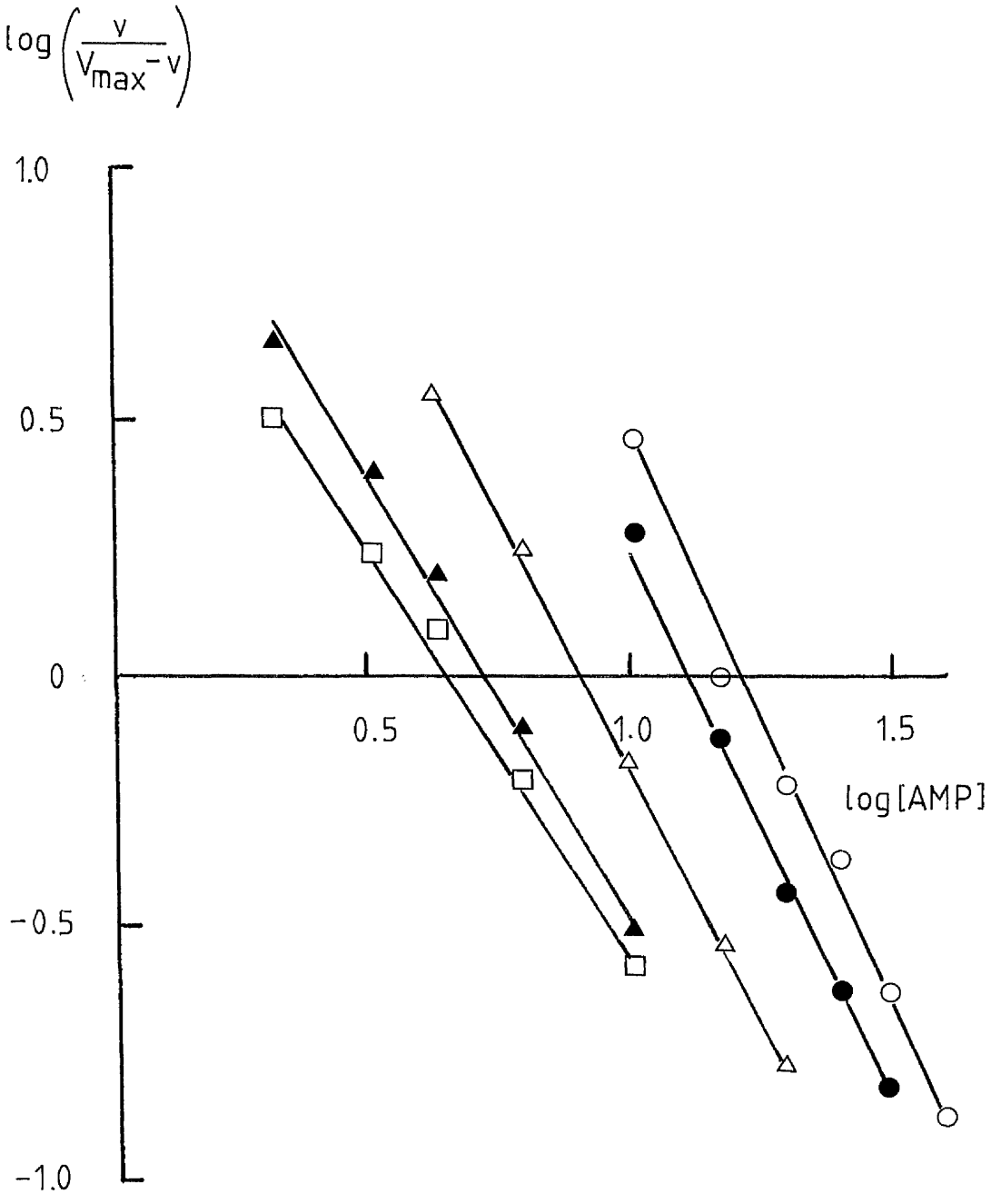

log (v / (Vmax - v))
1.0
0.5
0
-0.5
-1.0
0.5
1.0
1.5
log [AMP]

Figure 5.19

Hill plots of the activation of non-phosphorylated rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions at a series of fixed concentrations of free AMP

The concentration of fructose 1,6-bisphosphate was 100 μM and the concentrations of free Mg^{2+} ions were expressed in mM. To estimate values for V_{max}, the data were plotted as double-reciprocal plots and the intercept on the 1/rate axis was calculated using a computer program which performed a "least-squares to a polynomial" fit (Bevington, 1969). The concentrations of free AMP were: 0 (○), 10 μM (▲), 20 μM (△) and 30 μM (●). The assays were performed as described in section 2.9(a).

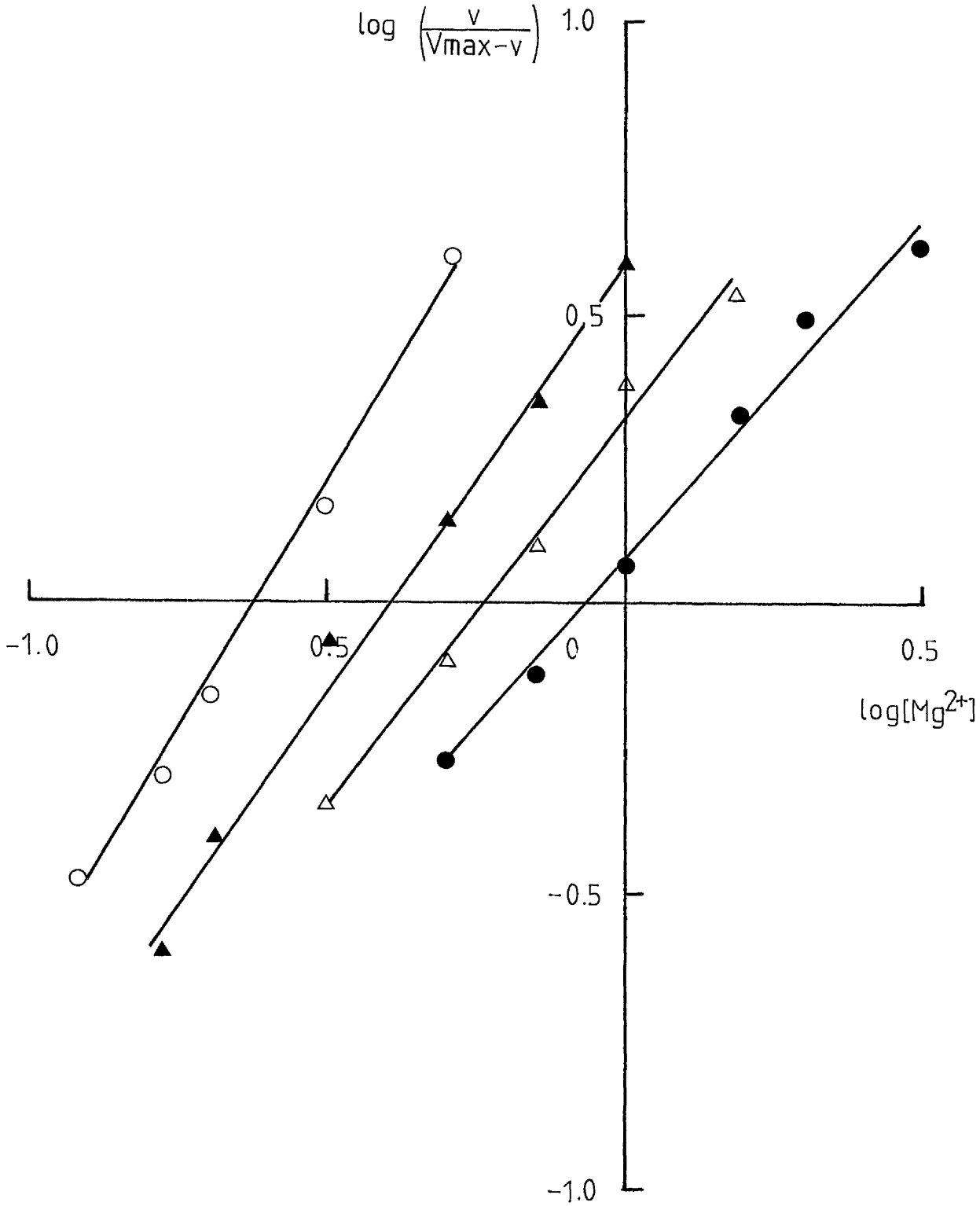

log (v / (Vmax-v))
1.0
0.5
-0.5
-1.0
-1.0
0.5
0
0.5
log[Mg2+]

Figure 5.20

Hill plots of the activation of phosphorylated rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions at a series of fixed concentrations of free AMP

The concentration of fructose 1,6-bisphosphate was 100 μM and the concentrations of free Mg^{2+} ions were expressed in mM. The values for V_{max} were calculated as described in the legend to figure 5.19. The concentrations of free AMP were: 0 (○), 10 μM (●), 20 μM (△) and 30 μM (▲). The assays were performed as described in section 2.9(a).

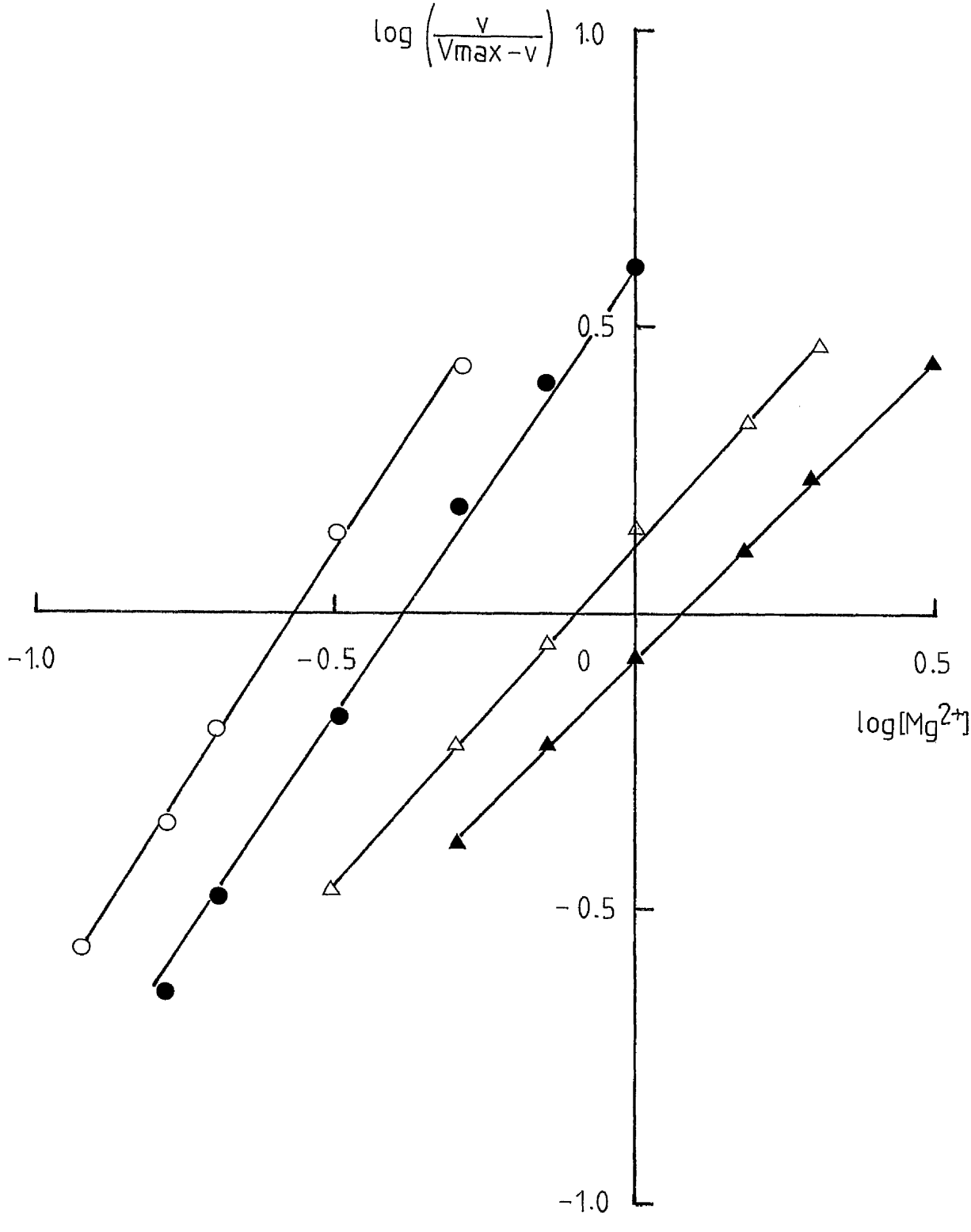

log (v / (Vmax − v))
1.0
0.5
−0.5
−1.0
−1.0
−0.5
0
0.5
log[Mg2+]

Figure 5.21

Hill plots of the inhibition of non-phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate at a series of fixed concentrations of free Mg^{2+} ions

The fructose 1,6-bisphosphate concentration was 25 μM and the concentrations of free fructose 2,6-bisphosphate were expressed in μM. The concentrations of free Mg^{2+} ions were: 0.17 mM (■), 0.67 mM (□) and 3.00 mM (◆). The assays were performed as described in section 2.9(a).

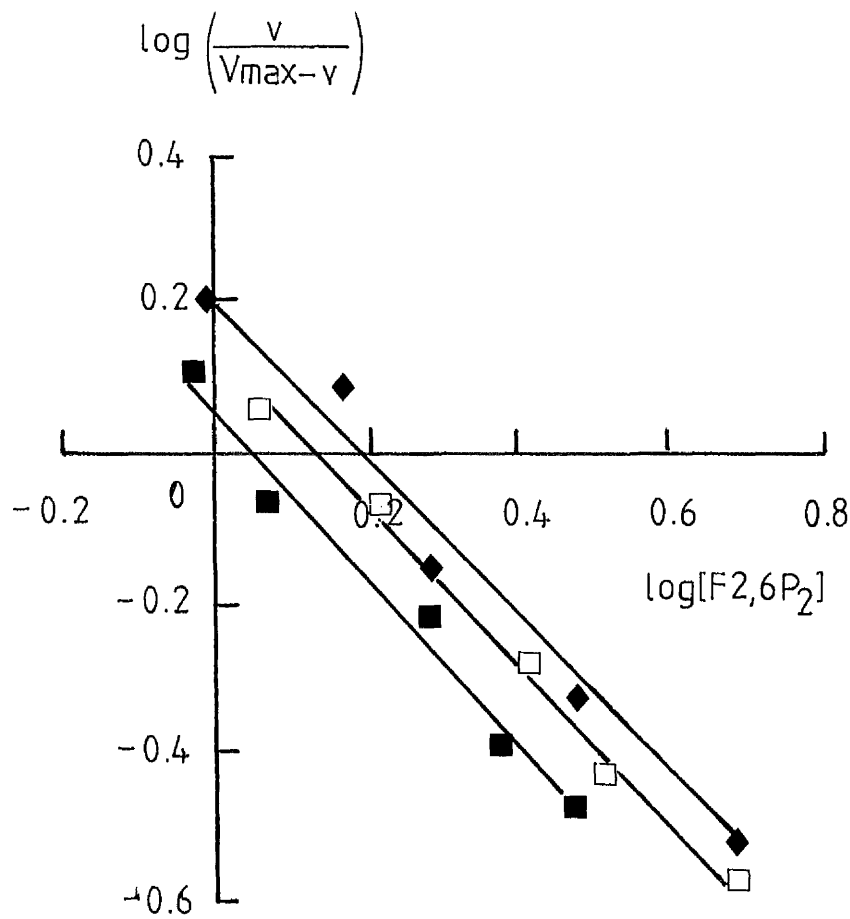

log (v / (Vmax−v))
0.4
0.2
−0.2
−0.4
−0.6
−0.2
0
0.2
0.4
0.6
0.8
log[F2,6P2]

Figure 5.22

Hill plots of the inhibition of phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate at a series of fixed concentrations of free Mg^{2+} ions

The fructose 1,6-bisphosphate concentration was 25 μM and the concentrations of free fructose 2,6-bisphosphate were expressed in μM. The concentrations of free Mg^{2+} ions were: 0.17 mM (■), 0.67 mM (□) and 3.00 mM (◆). The assays were performed as described in section 2.9(a).

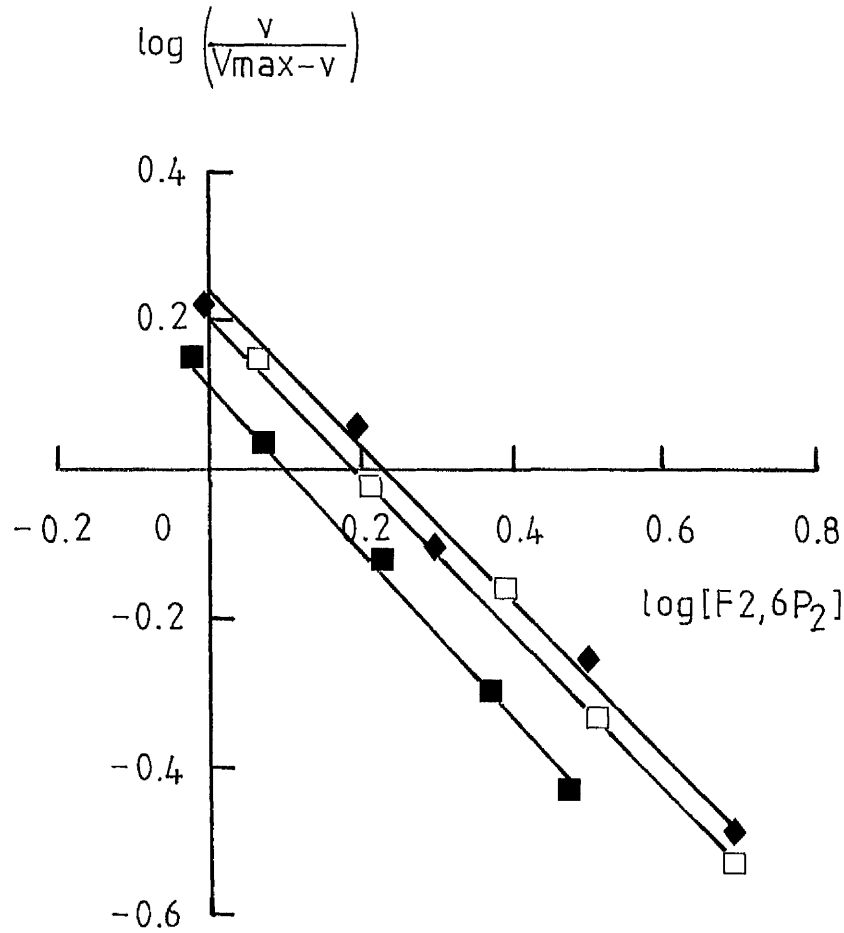

log (v / (Vmax-v))
0.4
0.2
-0.2
-0.4
-0.6
-0.2
0
0.2
0.4
0.6
0.8
log[F2,6P2]

Figure 5.23

Hill plots of the activation of non-phosphorylated rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions at a series of fixed concentrations of free fructose 2,6-bisphosphate

The concentration of free fructose 1,6-bisphosphate was 25 μM and the concentrations of free Mg^{2+} ions were expressed in mM. The values for V_{max} were estimated as described in the legend to figure 5.19. The concentrations of free fructose 2,6-bisphosphate were: 0 (○), 0.9 μM (●), 1.7 μM (△) and 2.2 μM (▲). The assays were performed as described in section 2.9(a).

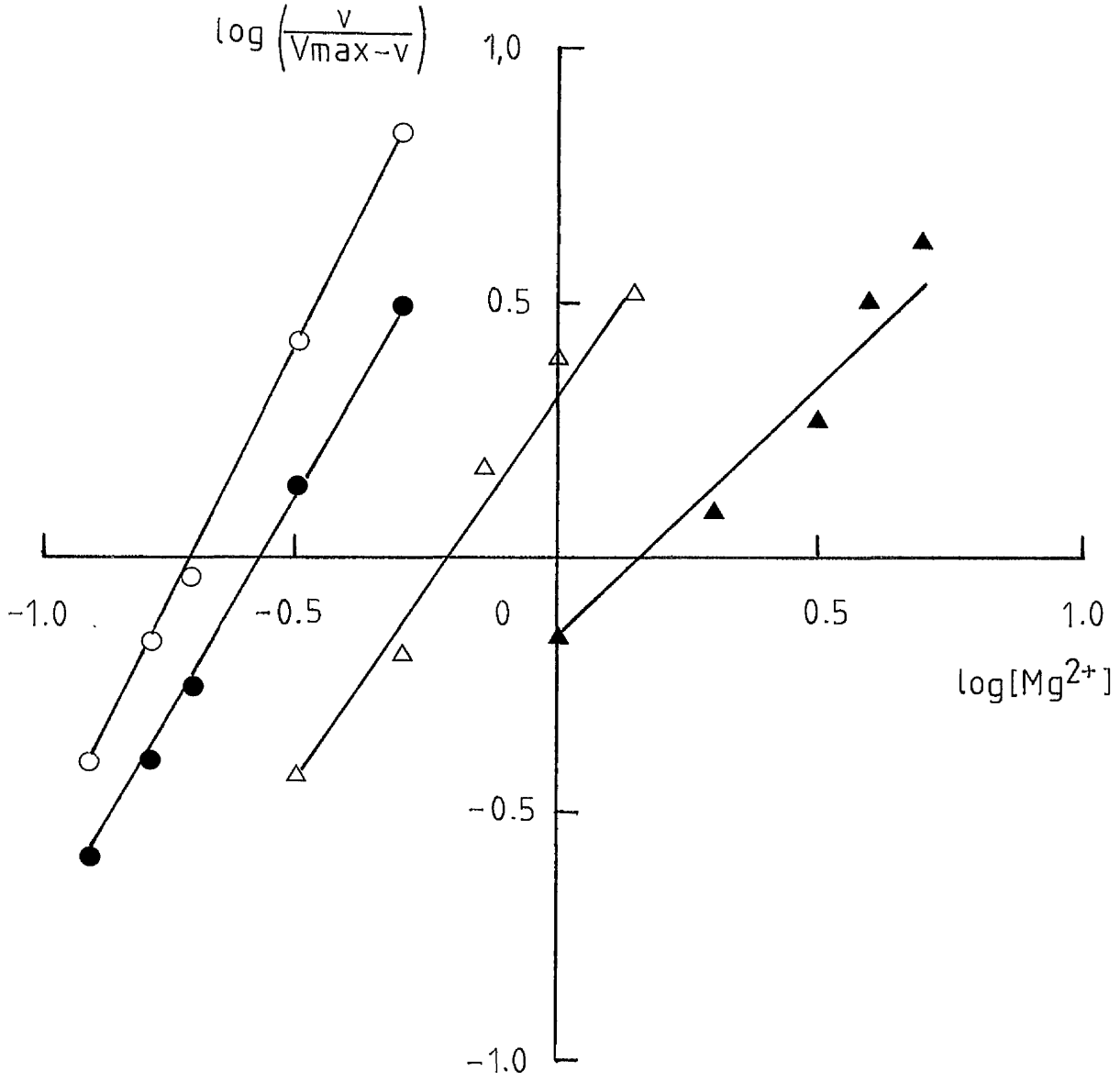

log (v / (Vmax-v))
1,0
0.5
-0.5
-1.0
-1.0
-0.5
0
0.5
1.0
log[Mg2+]

Figure 5.24

Hill plots of the activation of phosphorylated rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions at a series of fixed concentrations of free fructose 2,6-bisphosphate

The concentration of fructose 1,6-bisphosphate was 25 μM and the concentrations of free Mg^{2+} were expressed in mM. The values for V_{max} were calculated as described in the legend to figure 5.19. The concentrations of free fructose 2,6-bisphosphate were 0 (○), 0.9 μM (●), 1.7 μM (△) and 2.2 μM (▲). The assays were performed as described in section 2.9(a).

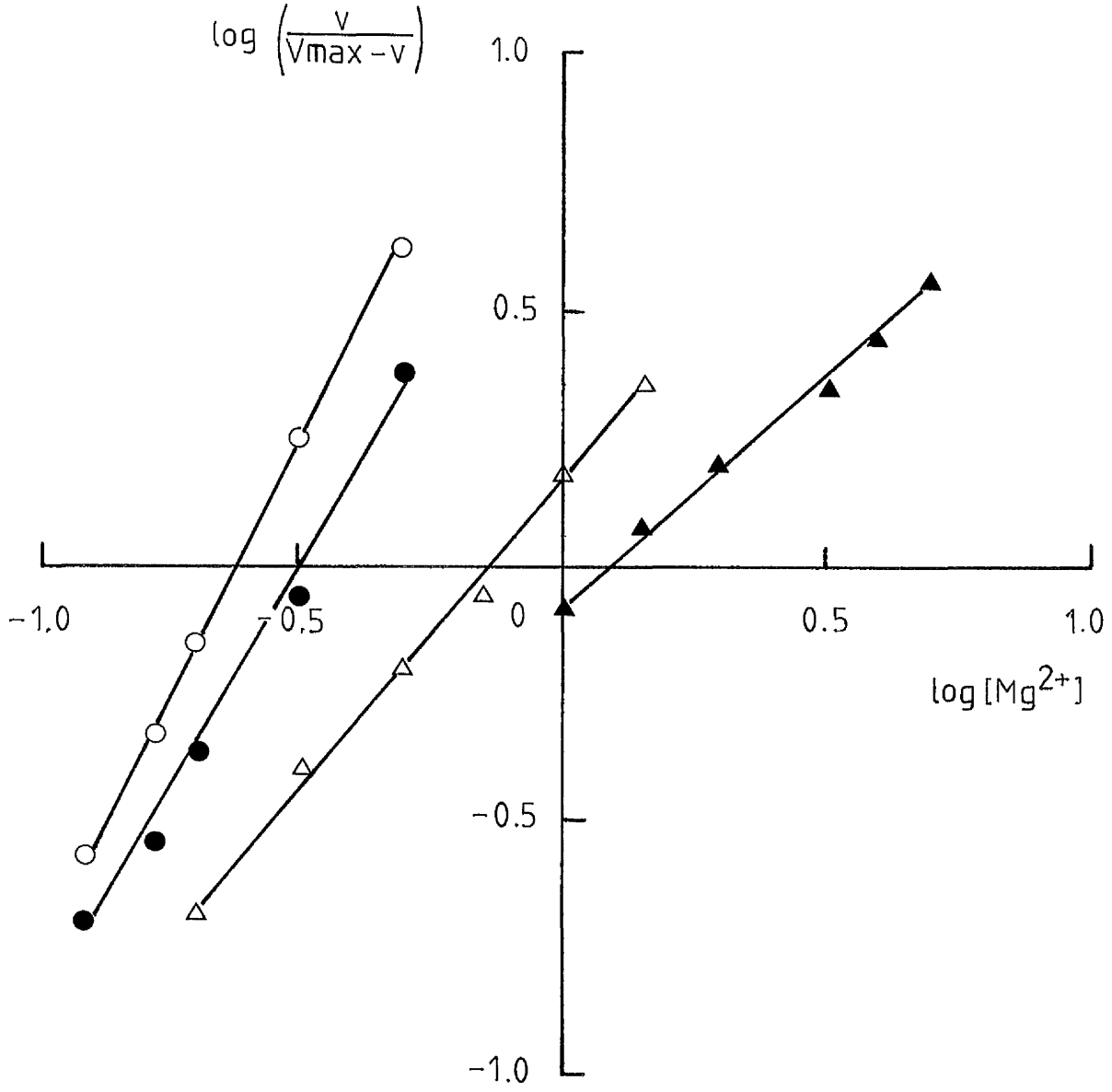

log (V / (Vmax - v))
1.0
0.5
-0.5
-1.0
-1.0
-0.5
0
0.5
1.0
log [Mg2+]

Figure 5.25

Hill plots of the inhibition of non-phosphorylated rat liver fructose 1,6-bisphosphatase by AMP at a series of fixed concentrations of free fructose 2,6-bisphosphate

The concentration of fructose 1,6-bisphosphate was 25 μM and the concentration of free Mg^{2+} ions was 2 mM. The concentrations of free AMP were expressed in μM and the concentrations of free fructose 2,6-bisphosphate were: 0 (◇), 0.59 μM (◆), 1.47 μM (□) and 2.94 μM (■). The assays were performed as described in section 2.9(a).

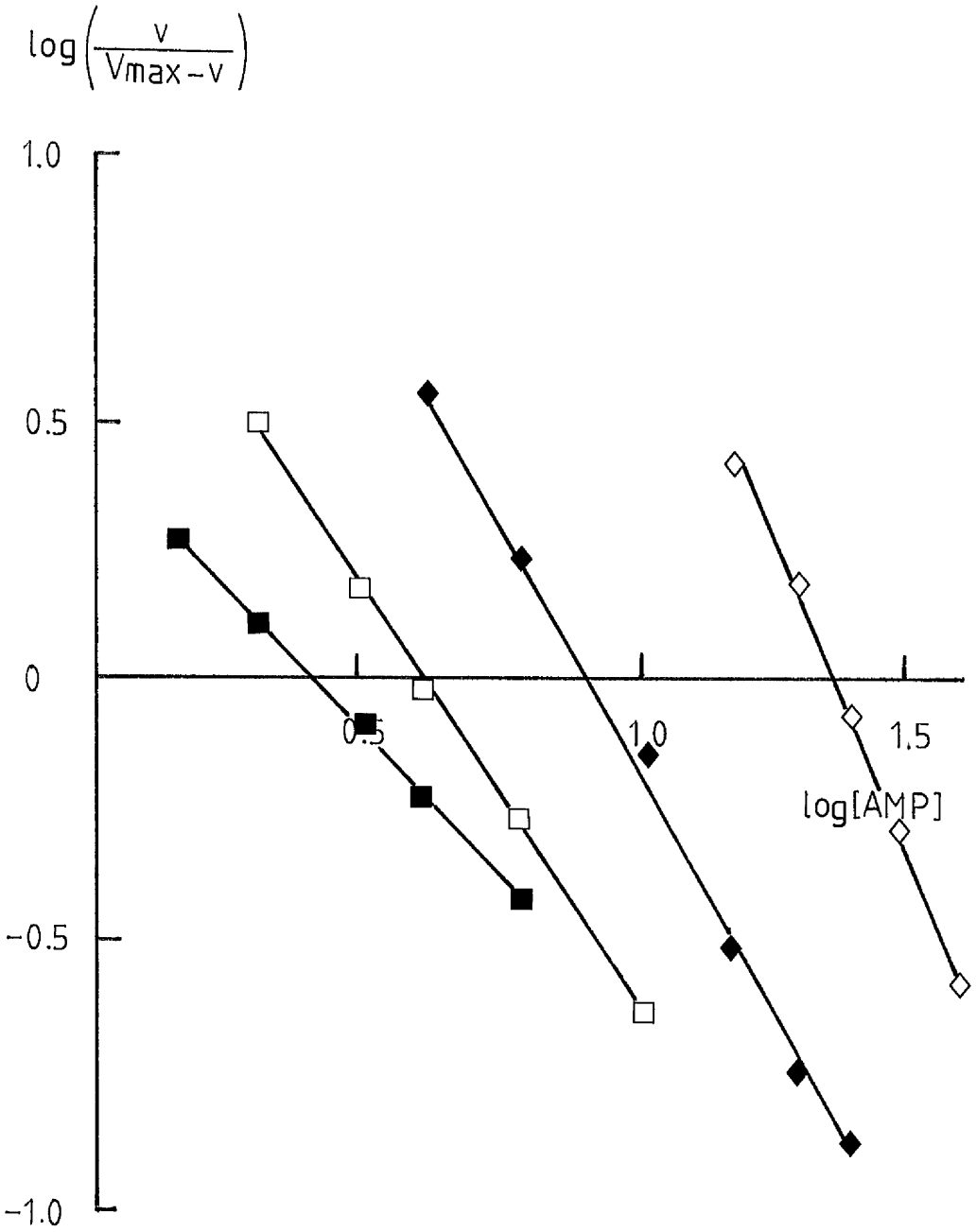

log(v / (Vmax - v))
1.0
0.5
0
-0.5
-1.0
0.5
1.0
1.5
log[AMP]

Figure 5.26

Hill plots of the inhibition of phosphorylated rat liver fructose 1,6-bisphosphatase by AMP at a series of fixed concentrations of free fructose 2,6-bisphosphate

The concentration of fructose 1,6-bisphosphate was 25 μM and the concentration of free Mg^{2+} ions was 2 mM. The concentrations of free AMP were expressed in μM and the concentrations of free fructose 2,6-bisphosphate were: 0 (◇), 0.59 μM (◆), 1.47 μM (□) and 2.94 μM (■).

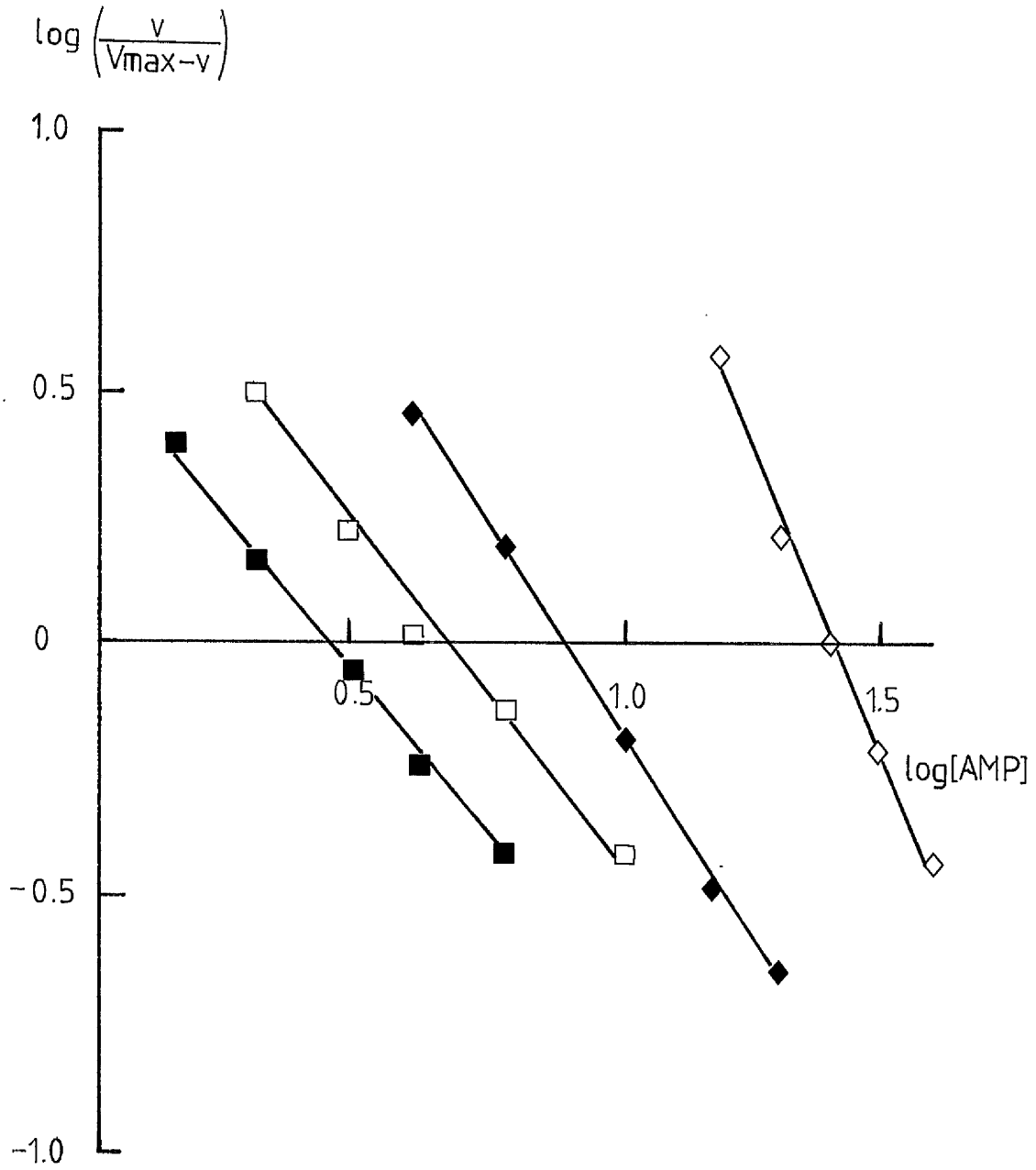

log (v / (Vmax−v))
1.0
0.5
0
−0.5
−1.0
0.5
1.0
1.5
log[AMP]

Figure 5.27

Hill plots of the inhibition of non-phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate at a series of concentrations of free AMP

The concentration of fructose 1,6-bisphosphate was 25 μM and the concentration of free Mg^{2+} ions was 2 mM. The concentrations of free fructose 2,6-bisphosphate were expressed in μM and the concentrations of free AMP were: 0 (◇), 10 μM (◆), 20 μM (□) and 30 μM (■). The assays were performed as described in section 2.9(a).

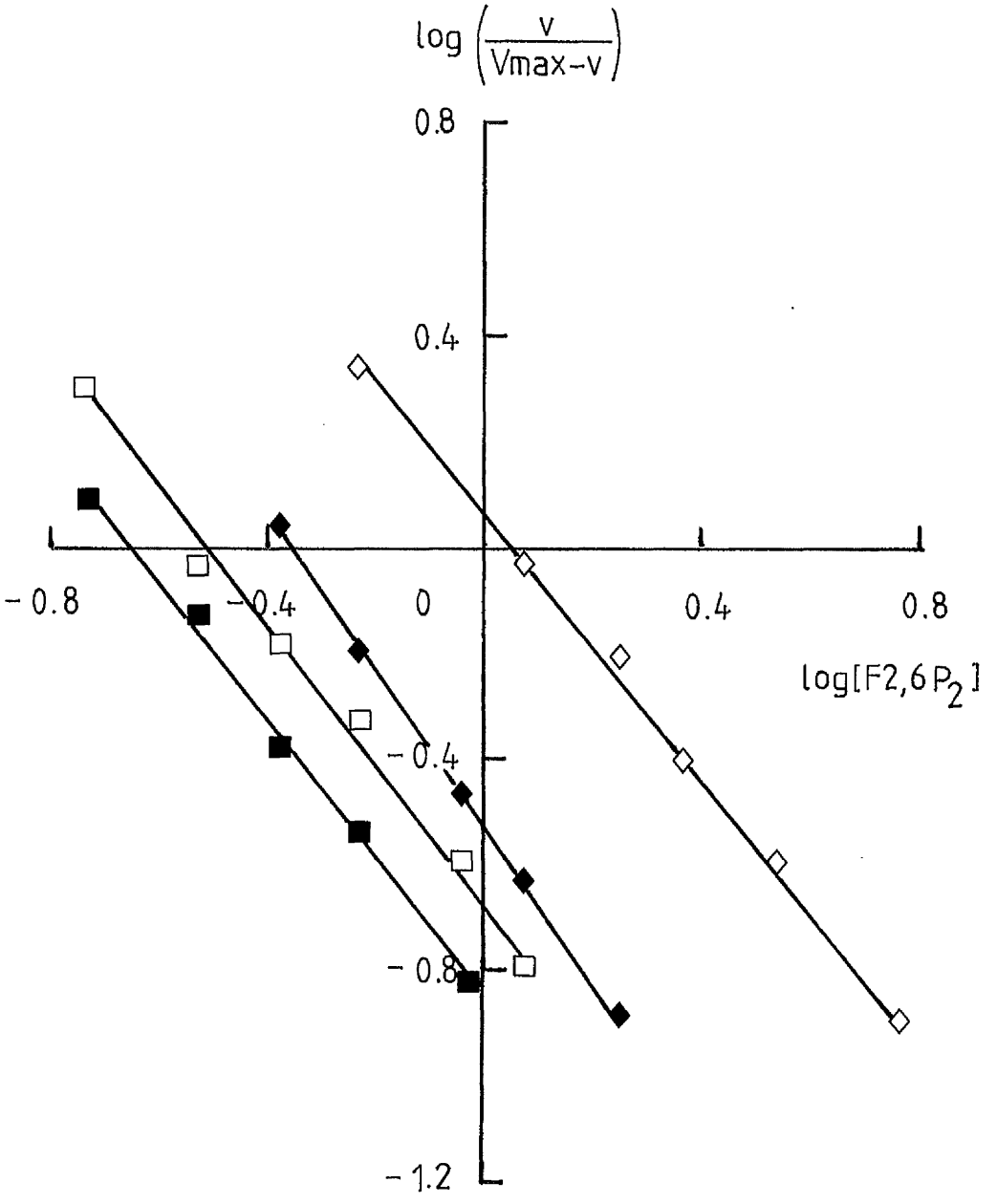

log (v / Vmax-v)
0.8
0.4
-0.8
-0.4
0
0.4
0.8
log[F2,6P2]
-0.4
-0.8
-1.2

Figure 5.28

Hill plots of the inhibition of phosphorylated rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate at a series of concentrations of free AMP

The concentration of fructose 1,6-bisphosphate was 25 μM and the concentration of free Mg^{2+} ions was 2 mM. The concentrations of free fructose 2,6-bisphosphate were expressed in μM and the concentrations of free AMP were: 0 (◇), 10 μM (◆), 20 μM (□) and 30 μM (■). The assays were performed as described in section 2.9(a).

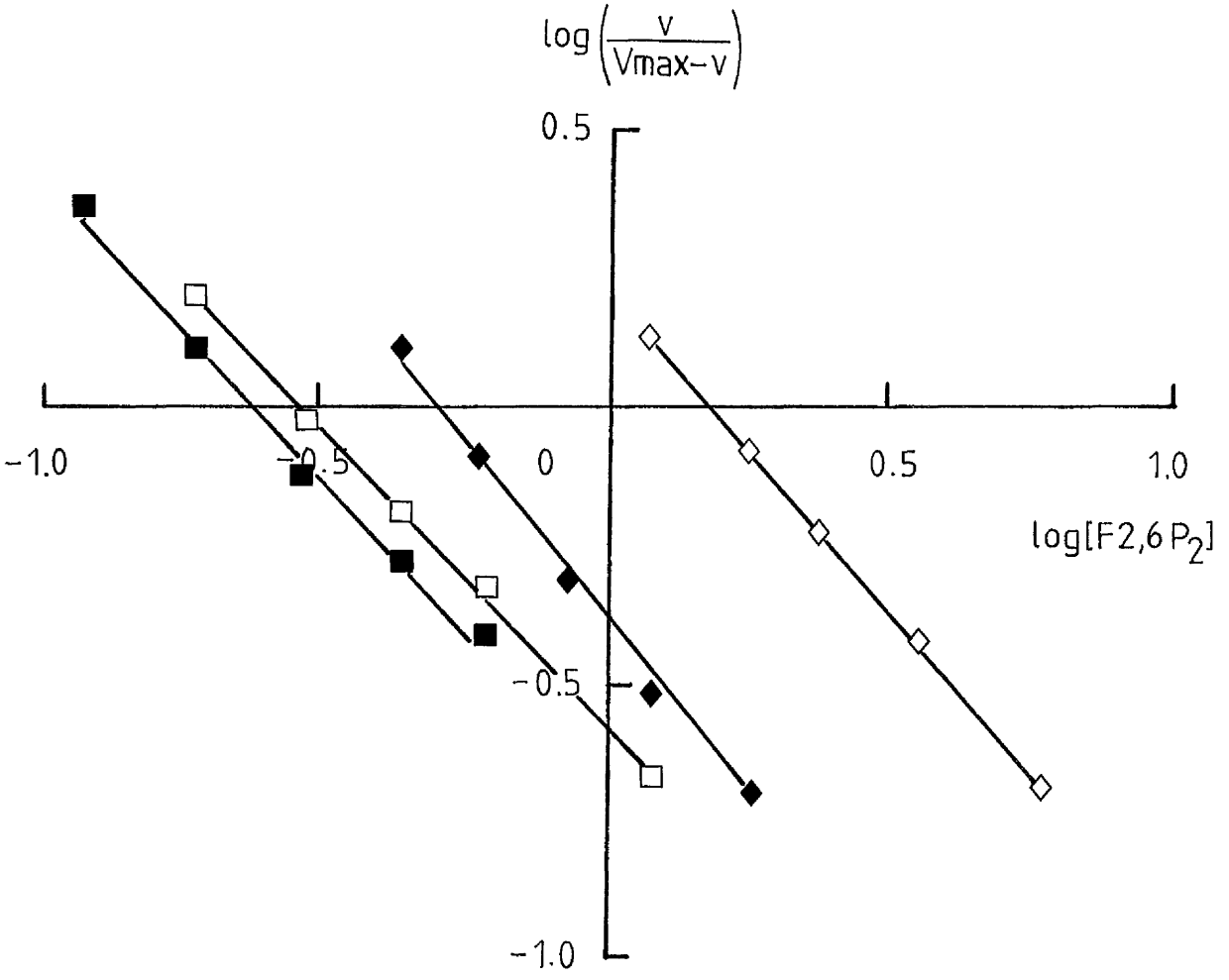

log (v / (Vmax−v))
0.5
−1.0
−0.5
0
0.5
1.0
log[F2,6 P2]
−0.5
−1.0

Figure 5.29

The binding of AMP by rat liver fructose 1,6-bisphosphatase

The binding of AMP to the enzyme was studied as described in section 2.9(b). The equilibration buffer comprised 50 mM Treacl-KOH buffer, pH 7.2, 100 mM KC1, 10 μM EDTA, 0.1 mM DTT and either 100 μM $F1,6P_2$ (△) or 10 μM $F2,6P_2$ (▲).

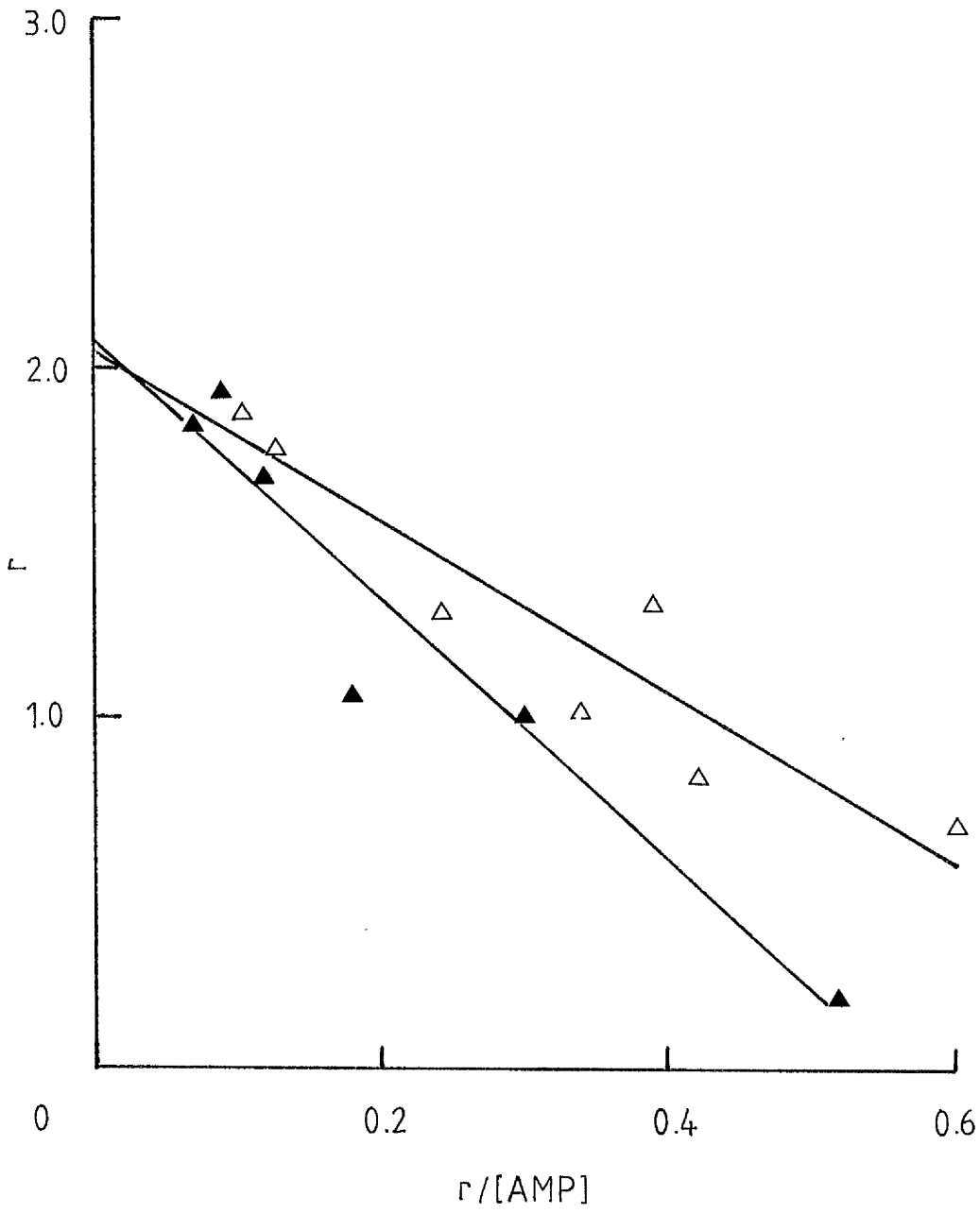

3.0
2.0
1.0
r
0
0.2
0.4
0.6
r/[AMP]

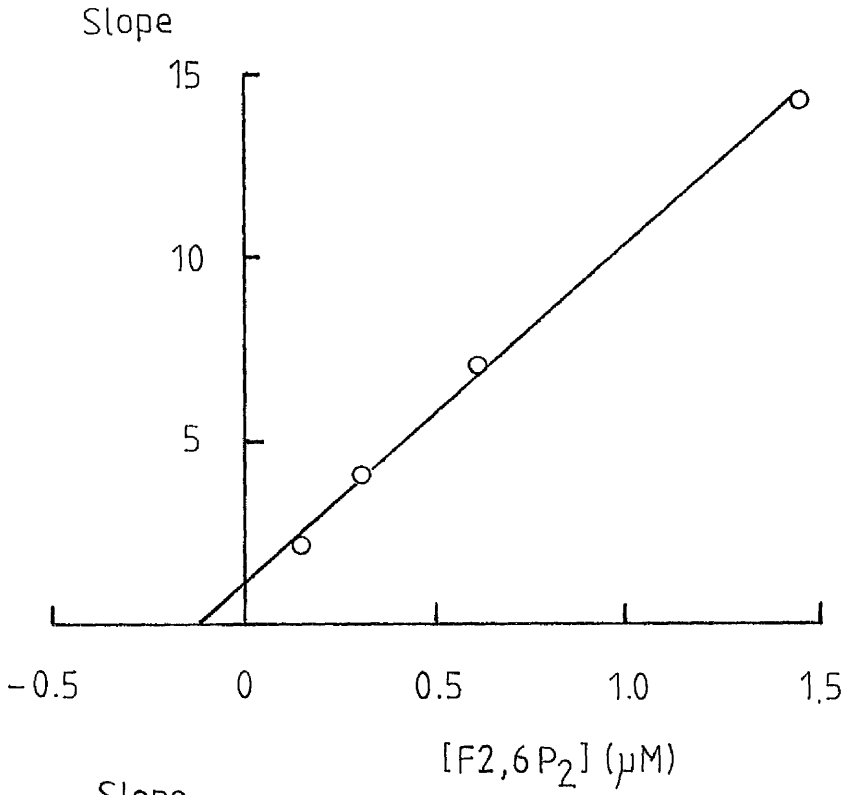
Figure 5.30

Replots of the slopes of the double reciprocal plots in figures 5.15 and 5.16 against the concentration of free fructose 2,6-bisphosphate for non-phosphorylated and phosphorylated rat liver fructose 1,6-bisphosphatase

Details of the primary plots are given in the legends to figures 5.15 and 5.16. The symbols are:

○ non-phosphorylated F1,6P_2ase

□ phosphorylated F1,6P_2ase


Slope
15
10
5
-0.5
0
0.5
1.0
1.5
[F2,6P2] (μM)

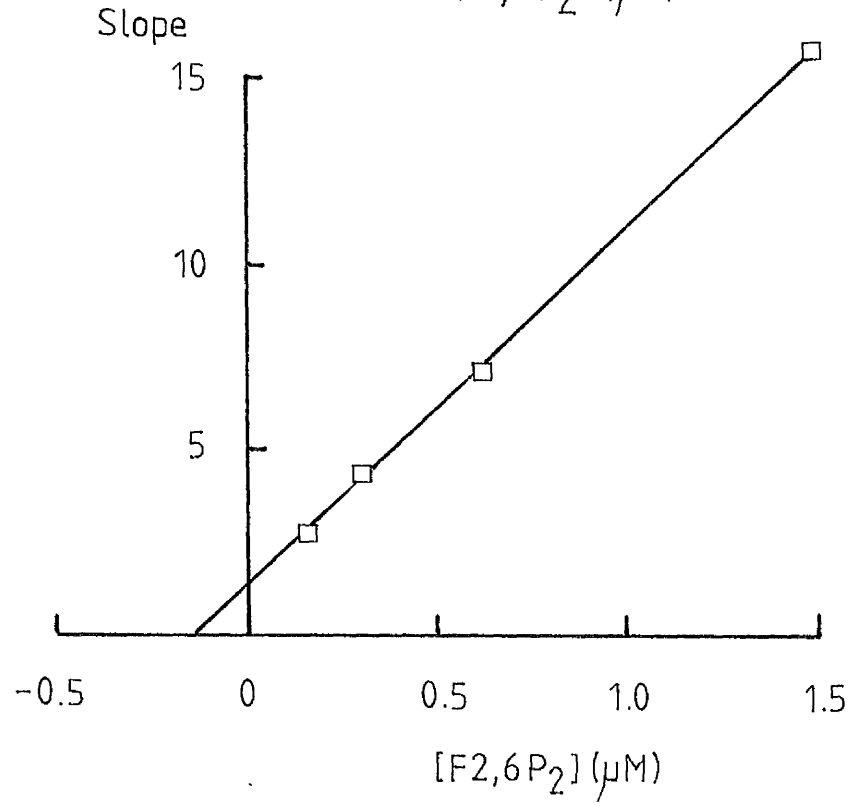

Slope
15
10
5
-0.5
0
0.5
1.0
1.5
[F2,6P2] (μM)

Table 5.1

The ^{32}P content of phosphorylated rat liver fructose 1,6-bisphosphatase.

The enzyme was phosphorylated and stored as described in section 2.7(c). Aliquots of 10μl were withdrawn, placed in 2 ml of scintillant (4 g diphenyloxazole/litre in triton:toluene, 1:2 (V/V) and counted for ^{32}P. Samples of enzyme, which had been acid-precipitated from 10μl aliquots as described in section 2.7(a) were also counted for ^{32}P.

Time since phosphorylation	acid-precipitable counts / total counts
days	
0	0.98
14	0.97
28	0.89
42	0.96
56	1.02

Table 5.2

Comparison of the activation of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by free Mg^{2+} ions.

The values were calculated as described in section 2.9(a) from the data in figures 5.3 and 5.5, and are given with the corresponding standard errors. The values of V_{max} are expressed as a percentage of the values at 1 mM free Mg^{2+}.

free Mg^{2+} ions	V_{max}		K_m	
	Non-phos.	Phos.	Non-phos.	Phos.
mM	%	%	μM	μM
0.17	20.7 ± 2.0	21.8 ± 2.2	5.5 ± 0.7	4.7 ± 0.3
0.20	24.8 ± 2.9	28.7 ± 1.5	4.8 ± 0.7	3.3 ± 0.3
0.25	35.8 ± 7.8	39.1 ± 5.7	5.9 ± 1.5	3.6 ± 0.5
0.33	38.0 ± 4.7	56.0 ± 6.4	2.6 ± 0.5	2.6 ± 0.5
1.00	100 ± 9.7	100 ± 5.4	1.4 ± 0.3	0.7 ± 0.2

Table 5.3

Comparison of the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by free AMP.

The values were calculated as described in section 2.9(a) from the data in figures 5.7 and 5.8, and are given with the corresponding standard errors. The values of V_{max} are expressed as a percentage of the values at zero AMP concentration.

free AMP	V_{max}	
	Non-phos.	Phos.
μM	%	%
0	100 ± 5	100 ± 6
10	76 ± 6	76 ± 6
20	49 ± 6	38 ± 5
30	31 ± 4	25 ± 4

Table 5.4

Comparison of the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by free fructose 2,6-bisphosphate.

The values were calculated as described in section 2.9(a) from the data in figures 5.9 and 5.11, and are given with the corresponding standard errors. Since the lines in figures 5.9 and 5.11 appear to intersect to the right of the 1/rate axis, the comparison is made on the criterion of slope which gives the ratio K_m/V_{max}. The data are presented as multiples of the values at zero fructose 2,6-bisphosphate concentration. These values were different for the two forms of the enzyme and so to facilitate comparison they have been assigned arbitrary values of 1.

free fructose 2,6-bisphosphate	Slope	
	Non-phos.	Phos.
μM	arbitrary units	arbitrary units
0	1.0 $\pm$ 0.2	1.0 $\pm$ 0.1
0.15	1.2 $\pm$ 0.1	1.7 $\pm$ 0.2
0.29	2.7 $\pm$ 0.3	3.0 $\pm$ 0.3
0.59	6.1 $\pm$ 0.7	6.3 $\pm$ 0.6
1.47	19.7 $\pm$ 2.2	16.5 $\pm$ 0.9

Table 5.5

Comparison of the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by free AMP in the presence of 1.5μM free fructose 2,6-bisphosphate.

The values were calculated as described in section 1.9(a) from the data in figures 5.13 and 5.14, and are given with the corresponding standard errors. Since the lines in figures 5.13 and 5.14 appear to intersect close to the origin, the comparison is made on the criterion of slope which gives the ratio $K_m/V_{max.}$ The data are printed as multiples of the values at zero AMP concentration. These values were different for the two forms of the enzyme and so to facilitate comparison they have been assigned arbitrary values of 1.

free AMP	Slope	
	Non-phos.	Phos.
μM	arbitrary units	arbitrary units
0	1.0 $\pm$ 0.6	1.0 $\pm$ 0.03
1.0	1.26 $\pm$ 0.08	1.25 $\pm$ 0.04
2.5	1.59 $\pm$ 0.08	1.73 $\pm$ 0.17
10.0	2.95 $\pm$ 0.06	3.28 $\pm$ 0.24

Table 5.6

Comparison of the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by free fructose 2,6-bisphosphate in the presence of 25 μM free AMP.

The values were calculated as described in section 2.9(a) from the data in figures 5.15 and 5.16, and are given with the corresponding standard errors. The values of V_{max} are expressed as a percentage of the values at zero fructose 2,6-bisphosphate concentration.

free fructose 2,6-bisphosphate	V_{max}		K_m for $F1,6P_2$	
	Non-phos.	Phos.	Non-phos.	Phos.
μM	%	%	μM	μM
0	100 ± 9	100 ± 5	1.1 ± 0.1	1.0 ± 0.3
0.15	60 ± 4	64 ± 3	8.6 ± 0.3	9.0 ± 0.2
0.29	47 ± 4	50 ± 7	14.1 ± 0.6	13.7 ± 1.0
0.59	41 ± 3	44 ± 3	19.8 ± 1.0	19.7 ± 0.6
1.47	39 ± 3	32 ± 7	39.8 ± 2.4	31.5 ± 2.2

Table 5.7

Comparison of the effect of the concentration of free Mg^{2+} ions on the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by AMP.

The data were calculated from the Hill plots in figures 5.17 and 5.18 as described in section 2.9(a). The Hill coefficient is designated h and K_i represents the free AMP concentration necessary to give 50% inhibition. Statistical analyses of all the Hill plot data were carried out using the Bevington (1969) computer program as described in section 2.9(a). This program did not calculate standard errors.

free Mg^{2+} ions	h		Apparent K_i	
	Non-phos.	Phos.	Non-phos.	Phos.
mM			μM	μM
0.17	1.44	1.56	3.9	4.5
0.25	1.63	1.71	5.9	5.1
0.67	1.84	1.89	7.6	8.1
1.50	2.19	2.11	10.8	12.8
3.00	2.22	2.24	16.3	16.3

Table 5.8

Comparison of the effect of the concentration of free AMP on the activation of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions.

The data were calculated as described in section 2.9(a) from the Hill plots in figures 5.19 and 5.20. The Hill coefficient is designated h and K_a represents the free Mg^{2+} ion concentration necessary to attain 50% V_{max}.

free AMP	h		Apparent K_a	
	Non-phos.	Phos.	Non-phos.	Phos.
μM			mM	mM
0	1.71	1.65	0.24	0.27
10	1.46	1.54	0.40	0.41
20	1.34	1.17	0.58	0.78
30	1.15	1.03	0.85	1.17

Table 5.9

Comparison of the effect of the concentration of free Mg^{+2} ions on the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate.

The data were calculated as described in section 2.9(a) from the Hill plots in figures 5.21 and 5.22. The Hill coefficient is designated h and K_i represents the free fructose 2,6-bisphosphate concentration necessary for 50% inhibition.

free Mg^{2+} ions	h		Apparent K_i	
	Non-phos.	Phos.	Non-phos.	Phos.
mM			μM	μM
0.17	1.10	1.14	1.12	1.26
0.67	1.11	1.06	1.38	1.55
3.00	1.13	1.04	1.55	1.70

Table 5.10

Comparison of the effect of the concentration of free fructose 2,6-bisphosphate on the activation of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by Mg^{2+} ions.

The data were calculated as described in section 2.9(a) from the Hill plots in figures 5.23 and 5.24. The Hill coefficient is designated h and K_a represents the free Mg^{2+} ion concentration necessary to attain 50% V_{max}.

free fructose 2,6-bisphosphate	h		Apparent K_a	
	Non-phos.	Phos.	Non-phos.	Phos.
μM			μM	μM
0	2.00	1.94	0.19	0.24
0.9	1.72	1.72	0.28	0.52
1.7	1.44	1.21	0.60	0.71
2.2	1.00	0.92	1.42	1.24

Table 5.11

Comparison of the effect of the concentration of free fructose 2,6-bisphosphate on the inhibition of non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by AMP.

The data were calculated as described in section 2.9(a) from the Hill plots in figures 5.25 and 5.26. The Hill coefficient is designated h and K_i represents the free AMP concentration necessary for 50% inhibition.

free fructose 2,6-bisphosphate	h		Apparent K_i	
	Non-phos.	Phos.	Non-Phos.	Phos.
μM			μM	μM
0	2.43	2.45	22.91	25.90
0.59	1.79	1.57	7.94	7.82
1.47	1.56	1.27	3.98	4.71
2.94	1.08	1.19	2.51	2.80

Table 5.12

Comparison of the effect of the concentration of free AMP on the inhibition on non-phosphorylated and phosphorylated forms of rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate.

The data were calculated as described in section 2.9(a) from the Hill plots in figures 5.27 and 5.28. The Hill coefficient is designated h and K_i represents the free fructose 2,6-bisphosphate concentration necessary for 50% inhibition.

free AMP	h		Apparent K_i	
	Non-phos.	Phos.	Non-phos.	Phos.
μM			μM	μM
0	1.26	1.16	1.13	1.49
10	1.47	1.28	0.44	0.49
20	1.34	1.12	0.31	0.37
30	1.30	1.09	0.23	0.24

CHAPTER 6

A STUDY OF THE INHIBITION OF RAT LIVER FRUCTOSE 1,6-BISPHOSPHATASE BY FRUCTOSE 2,6-BISPHOSPHATE

6.1 Introduction

Two groups have recently reported that $F2,6P_2$ is a potent inhibitor of $F1,6P_2$ase. Pilkis et al (1981) demonstrated that $F2,6P_2$ behaved as a competitive inhibitor with respect to $F1,6P_2$, a result which seemed plausible owing to the structural similarity of these two metabolites. Shortly afterwards, however, Van Schaftingen and Hers (1981b) argued that the inhibition of $F1,6P_2$ase by $F2,6P_2$ was more complex since $F2,6P_2$ appeared to change the substrate saturation curve from hyperbolic to sigmoidal. Moreover, both groups were able to show that the inhibition by $F2,6P_2$ was synergistic with inhibition by AMP. In chapter 5 of this study, it was shown that in the presence of $F2,6P_2$ double reciprocal plots of rate against $F1,6P_2$ concentration intersected in the top right quadrant; this was clearly uncharacteristic of simple competitive inhibition. Parabolic Dixon plots were also obtained which implied that $F1,6P_2$ase responded sigmoidally to $F2,6P_2$. Furthermore, the data indicated that $F2,6P_2$ was able to influence the inhibition given by AMP. Taken together, these findings clearly emphasise the complex nature of the interaction of $F2,6P_2$ with $F1,6P_2$ase. It was therefore the objective of the work presented in this chapter to provide a more extensive characterisation of the inhibition of rat liver $F1,6P_2$ase by $F2,6P_2$.

6.2 The Properties of Inhibition by Fructose 2,6-Bisphosphate

In the previous chapter, it was shown that, at several fixed concentrations of free $F2,6P_2$, a series of linear double reciprocal plots of initial velocity against $F1,6P_2$ concentration intersected to the right of the 1/rate axis (figure 5.9 and 5.11). Extrapolation of these plots to the 1/rate axis would appear to indicate that increasing concentrations of $F2,6P_2$ caused an increase in V_{max} and in

some cases a negative value for V_{max} would be obtained, which is clearly impossible. In order to understand this behaviour, the inhibition of purified rat liver Fl,6P_2ase by F2,6P_2 was examined over a range of concentrations of Fl,6P_2 at which the active site would normally be fully saturated. A series of parabolic double reciprocal plots of initial velocity against Fl,6P_2 concentration, which intersected on the 1/rate axis, were obtained (figure 6.1) and this result suggested that two types of inhibition could be in operation. The finding that V_{max} remained unaltered implied that the inhibition was partly competitive in nature. The data in figure 5.10 shows that Fl,6P_2 ase responds cooperatively to F2,6P_2. A comparison of figure 6.1 with figure 5.10 shows that F2,6P_2 also appears to induce cooperativity with respect to Fl,6P_2. In this respect when the data were replotted in the form of initial velocity against Fl,6P_2 concentration the curves were hyperbolic in the absence of F2,6P_2, but became increasingly sigmoidal as the F2,6P_2 concentration was increased, (figure 6.2); it was also observed that F2,6P_2 abolished the inhibition given by high concentrations of substrate. Figure 6.2 also shows that AMP greatly increased the sensitivity of the enzyme to inhibition by F2,6P_2. At 20 μM Fl,6P_2, 25 μM AMP reduced the activity to 37% of the value obtained in its absence; 1.5 μM F2,6P_2 decreased the activity to 58%. However, in the presence of both 25 μM AMP and 1.5 μM F2,6P_2, the activity was less than 3% of the activity observed in the absence of both these inhibitors. It would therefore appear that AMP and F2,6P_2 act synergistically to inhibit rat liver Fl,6P_2ase. It is difficult to discern whether the substrate saturation curves are sigmoidal in the presence of both AMP and F2,6P_2. However, when the same data were plotted in the double-reciprocal form, (figure 5.15),

the plots were linear indicating that there was no cooperativity with respect to $Fl,6P_2$. It is therefore possible that high concentrations of $F2,6P_2$, such as those used in the absence of AMP, are required to induce cooperativity with respect to $Fl,6P_2$.

6.3 The Second Site Hypothesis

The results presented so far in this and the previous chapter are consistent with the inhibition of rat liver $Fl,6P_2$ase by $F2,6P_2$ being brought about by two different types of interaction. It is reasonable, therefore to postulate that $F2,6P_2$ could interact with two distinct sites on the enzyme. One of these sites would be the catalytic site, for which $F2,6P_2$ would compete with $Fl,6P_2$. However, the cooperative effects of $F2,6P_2$ and the synergistic interactions between $F2,6P_2$ and AMP are inconsistent with simple competitive inhibition alone but they could be expected to be mediated through the binding of $F2,6P_2$ to an allosteric site. As discussed in Chapter 1, Nimmo and Tipton (1975b) demonstrated that the blocking of a reactive thiol group in ox liver $Fl,6P_2$ase using p-chloromercuribenzoate abolished the sensitivity of the enzyme to inhibition by high levels of substrate, but did not affect the catalytic activity. This result and the finding that inhibitory, but not non-inhibitory, concentrations of $Fl,6P_2$ provided protection against thiol reagents, suggested that the inhibition was an allosteric effect. However, the partial nature of the inhibition and the very low affinity of the allosteric site for $Fl,6P_2$ indicated that the inhibition was not significant in regulatory terms. Bearing this in mind, it is possible that this low affinity site for $Fl,6P_2$ could actually be the allosteric site for $F2,6P_2$. This site was thought of as an inhibitory site for $Fl,6P_2$ simply because at the time the existence of $F2,6P_2$ was unknown. The finding that $F2,6P_2$ abolished the inhibition given by high concentrations

of $Fl,6P_2$ (figure 6.2) provides clear support for this hypothesis.

In order to test the "second site" hypothesis, it was first necessary to determine whether there was a reactive thiol group at the allosteric site for $Fl,6P_2$ on rat liver $Fl,6P_2$ase and if so, whether blocking this thiol group would influence the inhibition given by high concentrations of $Fl,6P_2$. It would then be easier to dissect the complex effects of inhibition by $F2,6P_2$ into their component parts.

6.4 The Reaction of Rat Liver Fructose 1,6-Bisphosphatase with Thiol-Group Reagents

In order to ascertain the number of reactive thiol groups in rat liver $Fl,6P_2$ase, a 0.2 mg/ml solution of the enzyme in 20 mM Treacl-KOH buffer, pH 7.5, containing 40 mM KCl was incubated at 25°C in the presence of a large excess of 5,5' - dithio-bis (2-nitrobenzoate) (DTNB). The change in absorbance was measured at 412 nm and was consistent with the rapid reaction of 0.8 thiol groups per subunit. When the experiment was repeated in the presence of 8M urea to unfold the enzyme, the absorbance change was consistent with the rapid reaction of 7.0 thiol groups per subunit. These results are in fairly good agreement with the presence of 9 residues of cysteine per subunit as reported in chapter 3 of this study, and suggest that one thiol group per subunit is highly reactive towards DTNB. Following the rapid reaction of rat liver $Fl,6P_2$ase with DTNB, the activity of the enzyme was reduced by approximately 50% and continued to fall even after the thiol-blocking reaction had been terminated by the addition of excess DTT. For this reason a kinetic study of the DTNB-treated enzyme was not carried out.

In order to investigate the effect of blocking the reactive thiol group, rat liver $Fl,6P_2$ase was incubated in the presence of N-ethylmaleimide (NEM). The results show that, under the conditions

Table 6.1

The protection of rat liver fructose 1,6-bisphosphatase against inactivation by N-ethylmaleimide

Samples of 50 μl, each containing 0.125 mg/ml $F1,6P_2$ase in 50 mM Treacl-KOH, pH 7.5 plus 100 mM KC1, were incubated at $0^{o}C$ for 20 minutes in the presence of 25 μM NEM. Further additions are detailed below. The enzyme was assayed as described in section 2.3(a) and the activity after 20 minutes of incubation with NEM is expressed as a percentage of the value at zero time.

Additions		$F1,6P_2$ase activity
NEM	Others	
		%
-	-	100
+	-	55
+	0.2 mM $F1,6P_2$	58
+	0.2 mM AMP	50
+	0.2 mM $F1,6P_2$ + 0.2 mM AMP	54
+	5 mM $F1,6P_2$	94
+	5 mM F6P	65
+	5 mM P_i	61
+	5 mM Mg $C1_2$	52
+	10 μM $F2,6P_2$	98
+	10 μM $F2,6P_2$ + 0.2 mM AMP	98
+	100 μM $F2,6P_2$	102
+	4 μM $F2,6P_2$	87
+	4 μM $F2,6P_2$ + 0.2 mM AMP	84

described in the legend to figure 6.3, the reaction appeared to be complete within 20 minutes with a corresponding decrease in enzyme activity of 50-55%. Under the same conditions, but in the absence of NEM, the enzyme activity remained constant. When the reaction was performed in the presence of 5mM $Fl,6P_2$, the loss of activity was prevented to a significant extent. However, the presence of 100μM $F2,6P_2$ totally protected the enzyme against inactivation by NEM. These results suggest that the reactive thiol group is situated at or near a site on the enzyme which binds $Fl,6P_2$, but has a considerably greater affinity for $F2,6P_2$.

The protection of rat liver $Fl,6P_2$ase against reaction with NEM was examined further (table 6.1). The results show that high concentrations of $Fl,6P_2$, but not low concentrations, protected the enzyme fairly well against modification. This result implied that the target for modification was a low affinity site for $Fl,6P_2$. When $F2,6P_2$ was included at concentrations of 10μM or greater, the enzyme was completely protected from reaction with NEM. Even concentrations of $F2,6P_2$ as low as 4 μM afforded considerable protection, which suggested that the site containing the reactive thiol had a high affinity for $F2,6P_2$. AMP alone did not appear to alter the extent of modification and did not influence the effects of $Fl,6P_2$ or $F2,6P_2$ on the reaction with NEM.

This is at first sight surprising since AMP appears to increase the affinity of the enzyme for $F2,6P_2$ (figures 5.27 and 6.2) and for inhibitory levels of $Fl,6P_2$ (figure 6.2). However, in order to be certain that AMP has no effect it would be necessary to examine the rates of inactivation by NEM at a number of different concentrations of $Fl,6P_2$ and $F2,6P_2$. When other ligands were included Mg^{2+} ions gave

no protection, although F6P and P_i at high concentrations each appeared to give some slight protection against modification.

6.5 The Effect of the Modification of Rat Liver Fructose 1,6-Bisphosphatase by NEM on the Inhibition given by Fructose 2,6-Bisphosphate and High Concentrations of Fructose 1,6-Bisphosphate

To determine the effect of reaction with NEM on the inhibition given by $F2,6P_2$ and high concentrations of $F1,6P_2$, a sample of rat liver $F1,6P_2$ase was treated with NEM under the conditions described in the legend to figure 6.3. There were no additions of protective ligands and the reaction was terminated by the addition of excess DTT after 30 minutes when it had been ascertained that the decrease in enzyme activity had been completed. The enzyme was stable after this treatment.

A comparison of untreated and NEM-treated $F1,6P_2$ase with respect to inhibition by high levels of $F1,6P_2$ was performed and the results are given in figure 6.4. The values for K_i were determined as follows : the equation for a non-competitive inhibitor is

$$v = \frac{V_{max}\, S \,/\, (1 + I/K_i)}{S + K_m}$$

where v is the initial velocity, V_{max} is the maximum velocity, S is the substrate concentration, K_m is the Michaelis constant, I is the inhibitor concentration and K_i is the dissociation constant for the inhibitor. Since S is large relative to K_m at all the inhibitory concentrations of $F1,6P_2$ used, the above equation reduces to :

$$v = V_{max} \,/\, (1 + I/K_i)$$

which can be rearranged to give

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{1}{V_{max}K_i} \cdot I$$

Thus K_i can be obtained from the slope of a Dixon plot (i.e. the reciprocal of the initial velocity against the inhibitor concentration).

The values of K_i for the inhibition of untreated and NEM-treated $Fl,6P_2$ ase by high concentrations of $Fl,6P_2$ were found to be 150 μM and 850 μM respectively. This result shows that blocking the reactive thiol group with NEM did not abolish high substrate inhibition, but did cause a 5-6 fold reduction in the affinity of the allosteric site for $Fl,6P_2$.

The inhibition of NEM-treated $Fl,6P_2$ase by $F2,6P_2$ was examined. At several fixed concentrations of free $F2,6P_2$, a series of double reciprocal plots of initial velocity against $Fl,6P_2$ concentration were obtained (figure 6.5). These plots appeared to intersect at a point on the 1/rate axis which suggested that $F2,6P_2$ behaved as a simple competitive inhibitor with respect to NEM-treated $Fl,6P_2$ase. Moreover, a replot of the slopes of the double-reciprocal plots against the concentration of free $F2,6P_2$ was linear which indicated that the cooperative effects of $F2,6P_2$ had been abolished. This replot gave a value for the K_i of $F2,6P_2$ as a competitive inhibitor of 0.8 μM.

6.6. Discussion

It has been shown that approximately one reactive thiol group per subunit of rat liver $Fl,6P_2$ase can be modified by incubation with DTNB. Protection experiments against reaction with NEM have suggested that this thiol group is exposed at a site which has a low affinity for $Fl,6P_2$ but a very high affinity for $F2,6P_2$. The finding that modification with NEM caused a reduction in the sensitivity of the enzyme to high substrate inhibition has indicated that the target of the thiol group reagents is the allosteric site for $Fl,6P_2$. However, in contrast to the findings of Nimmo and Tipton (1975b) for the ox liver enzyme, treatment of rat liver $Fl,6P_2$ase with a thiol group reagent decreased the catalytic activity and did not completely

abolish high substrate inhibition. It is therefore possible that the thiol group is not actually located within the allosteric site. Rather reaction of the group may restrict conformational changes which accompany and are necessary for the binding of $Fl,6P_2$ or $F2,6P_2$ to the enzyme; conversely the binding of $Fl,6P_2$ or $F2,6P_2$ to the allosteric site would, by way of conformational change, conceal the thiol group. Nevertheless, the K_i for $Fl,6P_2$ was considerably reduced and a qualitative change in the inhibition given by $F2,6P_2$ was observed. One might at first think that the affinity of the enzyme for $F2,6P_2$ would also be reduced by a factor of 5-6. However, the structures of $Fl,6P_2$ and $F2,6P_2$ are obviously different so it could be that the reaction of the thiol group with NEM prevents binding of $F2,6P_2$ completely. The results in figure 5.6 demonstrated that $F2,6P_2$ was a competitive inhibitor in these conditions. Moreover, blocking the allosteric site for $Fl,6P_2$ abolished the cooperative interactions of $F2,6P_2$ and this in turn implies that these interactions are mediated by way of the allosteric site. Figure 6.5 also gives a value of 0.8 μM for the K_i for inhibition at the catalytic site. This value does not necessarily apply to the unmodified enzyme since the loss of enzyme activity upon NEM treatment could reflect a change in the shape of the catalytic site.

The interaction of $F2,6P_2$ with the catalytic site and an allosteric site on rat liver $Fl,6P_2$ has also been examined by Pilkis et al (1981b). Although this group have not identified the allosteric site as the site for high substrate inhibition by $Fl,6P_2$, they have demonstrated that high concentrations of $Fl,6P_2$ can mimic the synergistic effects of $F2,6P_2$ on AMP inhibition. In studies of the protection against acetylation of tyrosine residues at the catalytic site and the allosteric site for AMP, they have shown that inclusion of AMP during

the aceylation reaction protected the sensitivity of the enzyme to inhibition by AMP and that inclusion of $Fl,6P_2$ protected the catalytic activity. Since $F2,6P_2$ protected both the catalytic activity and the sensitivity to inhibition by AMP, they have argued that the allosteric sites for AMP and $F2,6P_2$ are closely associated. However, they have not ascertained how acetylation at either site might influence the inhibition of the enzyme by $F2,6P_2$. Nevertheless, if the catalytic site can be selectively blocked by acetylation without affecting the interactions at the allosteric sites, and vice versa, the possibility then exists for further investigation of the interaction of $F2,6P_2$ with $Fl,6P_2$ase by direct binding studies. While the studies in this thesis were in progress, it was estimated that chemical synthesis of [U-^{14}C] $F2,6P_2$, having a suitably high specific activity to allow one to detect its binding to $Fl,6P_2$ase, would cost in excess of £1500. However, with the recent discovery and characterisation of fructose 6-phosphate 2-kinase (Hers and Van Schaftingen, 1982), it should now be possible to prepare enzymatically [2-^{32}P] F2,6P or [U-^{14}C] $F2,6P_2$ at no great expense and this would make binding studies a feasible proposition.

Figure 6.1

The effect of increasing concentrations of free fructose 2,6-bisphosphate on rat liver fructose 1,6-bisphosphatase at saturating concentrations of free fructose 1,6-bisphosphate, expressed as double reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration, at pH 7.2

The assays were carried out as described in section 2.9(a) and the free Mg^{2+} ion concentration was 2 mM. The concentrations of free fructose 2,6-bisphosphate were: 0 (●), 1.5 μM (△), 2.9 μM (▲), 5.9 μM (□) and 11.8 μM (■).

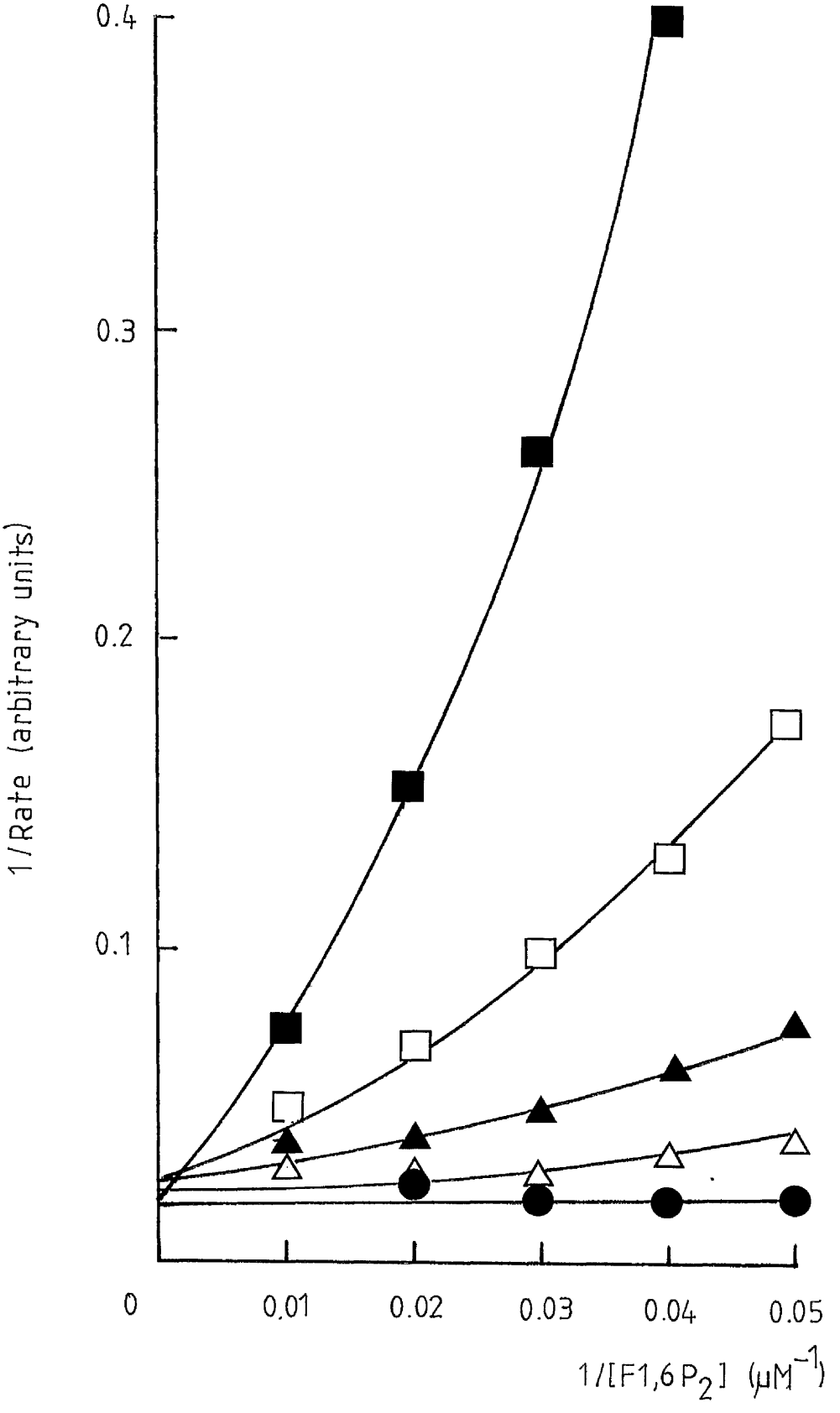

0.4
0.3
0.2
0.1
0
0.01
0.02
0.03
0.04
0.05
1/Rate (arbitrary units)
1/[F1,6P2] (μM-1)

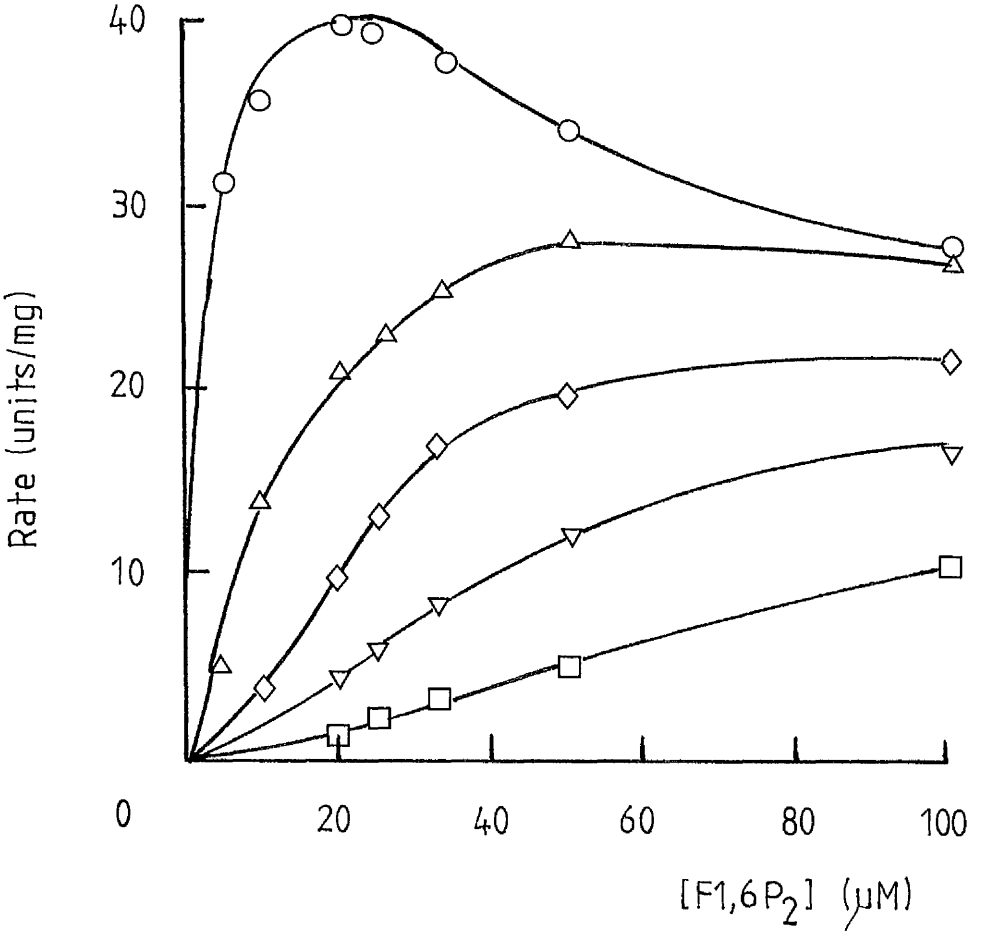
Figure 6.2

The inhibition of rat liver fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate in the absence and presence of 25 μM AMP, expressed as plots of initial velocity against free fructose 1,6-bisphosphate concentration, at pH 7.2

The assays were carried out as described in section 2.9(a), and the concentration of free Mg^{2+} ions was 2 mM.

Top graph: in the absence of AMP. The concentrations of free $F2,6P_2$ were: 0 (○), 1.5 μM (△), 2.9 μM (◇), 5.9 μM (▽) and 11.8 μM (□).

Bottom graph: in the presence of 25 μM AMP. The concentrations of free $F2,6P_2$ were: 0 (●), 0.15 μM (▲), 0.29 μM (◆), 0.59 μM (▼) and 1.50 μM (■).


40
30
20
10
0
Rate (units/mg)
20
40
60
80
100
[F1,6P2] (μM)

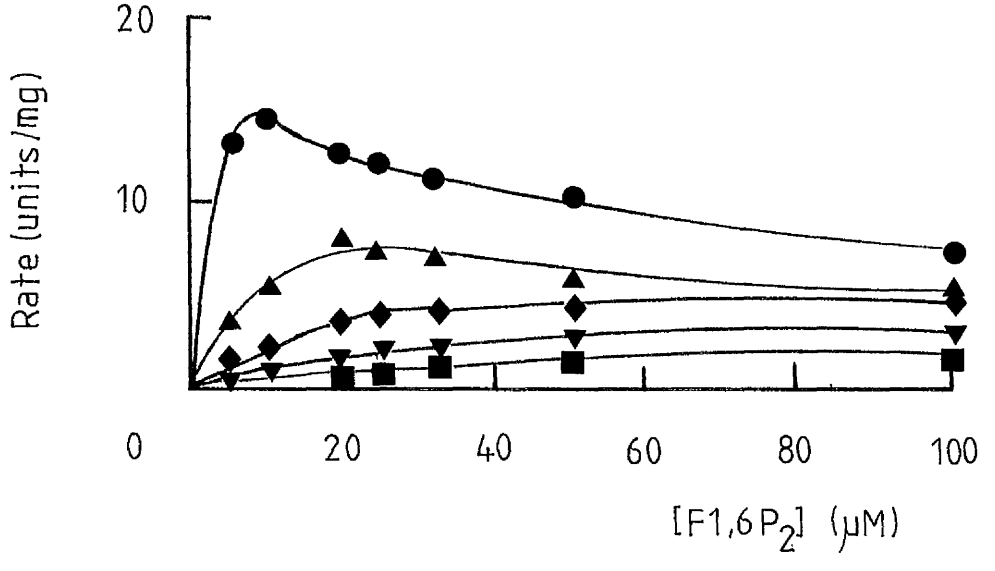

20
10
0
Rate (units/mg)
20
40
60
80
100
[F1,6P2] (μM)

Figure 6.3

The effect on the activity of rat liver fructose 1,6-bisphosphatase of treatment with N-ethylmaleimide

The enzyme was assayed as described in section 2.3(a). For treatment with NEM, 50 μl samples containing 0.125 mg/ml $F1,6P_2$ase in 50 mM Treacl-KOH, pH 7.5 containing 100 mM KC1 were incubated at $0^{o}C$ in the absence or presence of 25 μM NEM and with the additions outlined below:

no NEM (●), with NEM but no other additions (O), with NEM plus 5 mM $F1,6P_2$ (△) and with NEM plus 100 μM $F2,6P_2$ (▲).

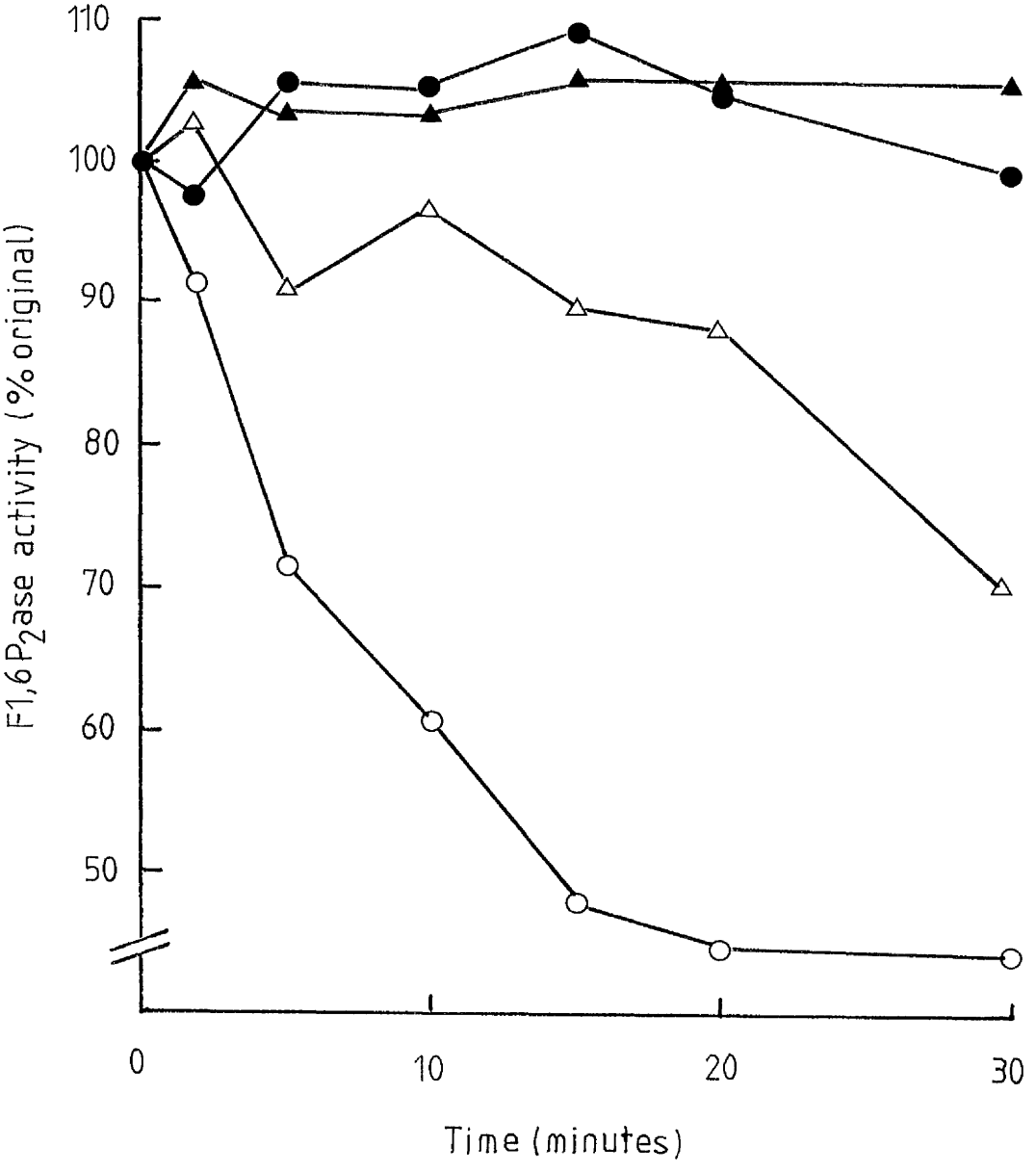

110
100
90
80
70
60
50
F1,6P2ase activity (% original)
0
10
20
30
Time (minutes)

Figure 6.4

Dixon plots of the inhibition of untreated and NEM-treated rat liver fructose 1,6-bisphosphatase by high concentrations of fructose 1,6-bisphosphate

The assays were carried out as described in section 2.3(a). Rat liver F1,6P_2ase was modified using NEM as described in the text and in the legend to figure 6.3. The symbols represent: untreated F1,6P_2ase (O) and NEM-treated F1,6P_2ase (□).

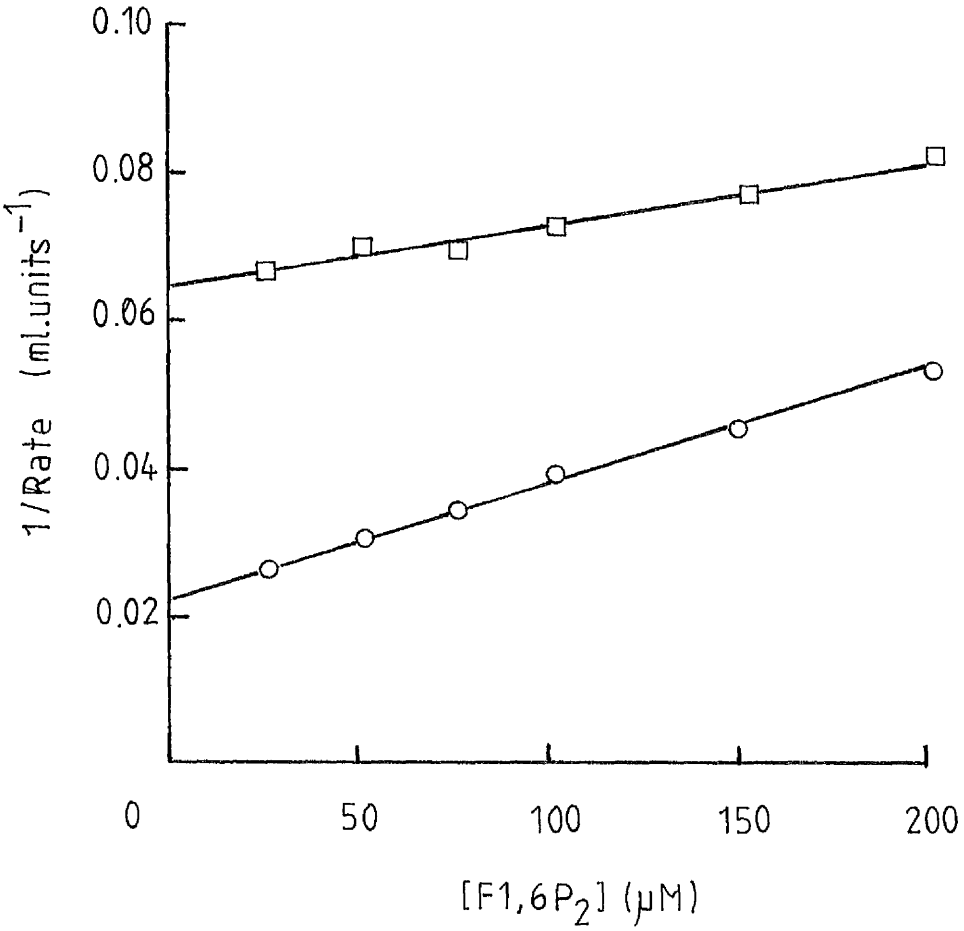
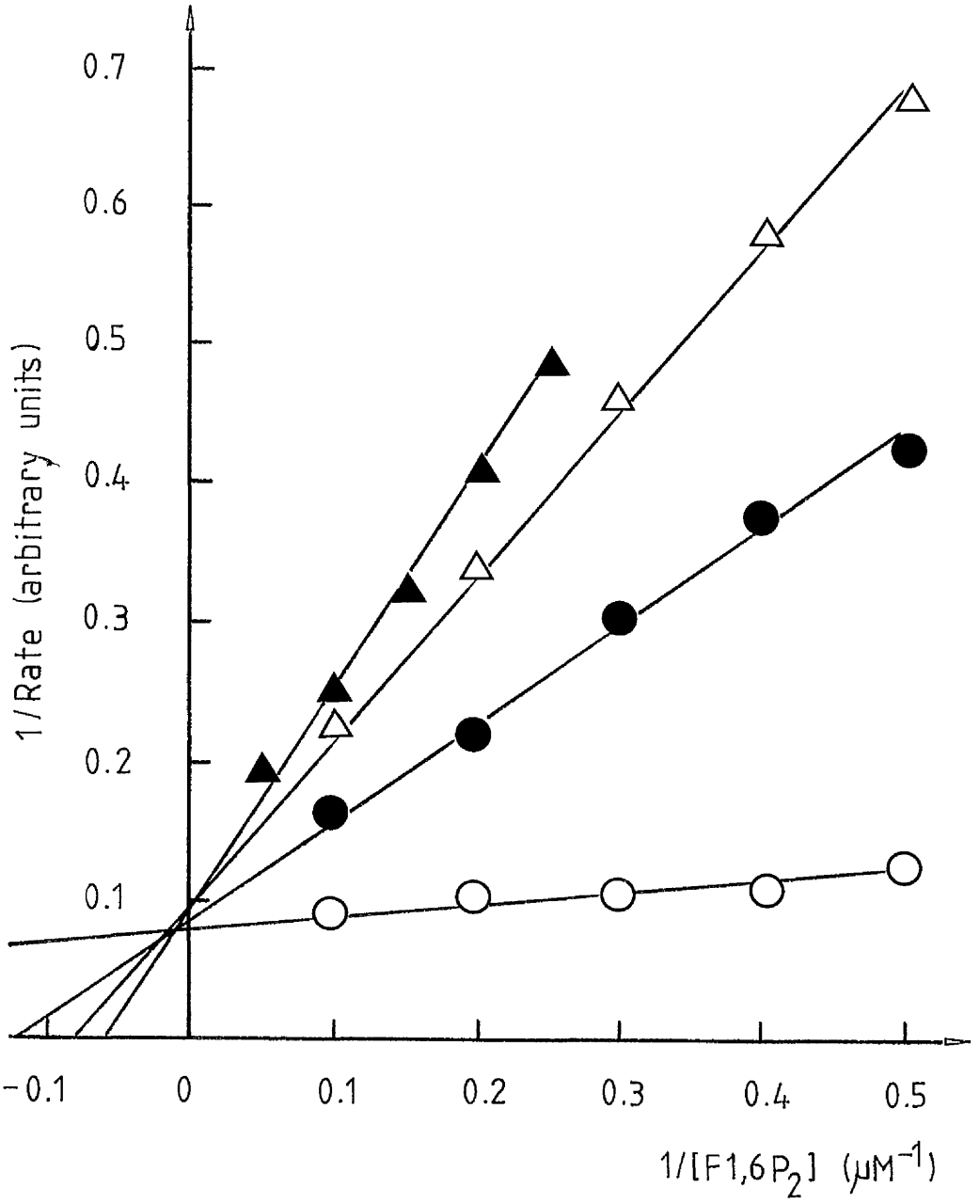
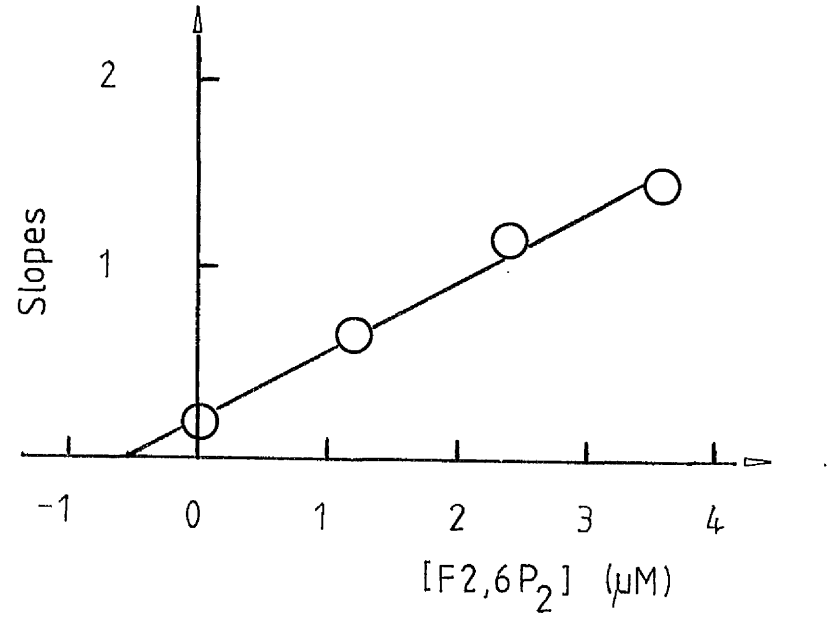

0.10
0.08
0.06
0.04
0.02
1/Rate (ml.units^{-1})
0
50
100
150
200
[F1,6P$_2$] (μM)

Figure 6.5

Double reciprocal plots of initial velocity against free fructose 1,6-bisphosphate concentration at a series of fixed concentrations of free fructose 2,6-bisphosphate for NEM-treated rat liver fructose 1,6-bisphosphatase

The assays were performed as described in section 2.9(a). Rat liver F1,6P_2ase was modified using NEM as described in the text and in the legend to figure 6.3. The free Mg^{2+} ion concentration was 2 mM and the concentrations of free fructose 2,6-bisphosphate were: 0 (O), 1.2 μM (●), 2.4 μM (△) and 3.5 μM (▲). Inset: the variation of the slopes of the double reciprocal plots against the concentration of free F2,6P_2.


0.7
0.6
0.5
0.4
0.3
0.2
0.1
1/Rate (arbitrary units)
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/[F1,6P2] (μM-1)

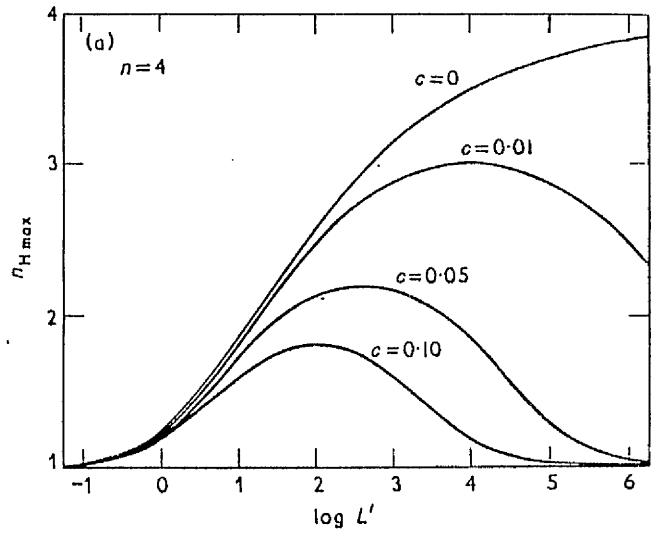

2
1
Slopes
-1
0
1
2
3
4
[F2,6P2] (μM)

CHAPTER 7

GENERAL DISCUSSION

Figure 7.1

Variation of the Hill constant (n_H) with L', the apparent allosteric constant



From Rubin, M.M. and Changeux, J.-P. (1966) J. Mol. Biol. 21 265 - 274.

7.1 A Model for the Allosteric Transitions of Rat Liver Fructose 1,6-Bisphosphatase

The general "concerted transition" model for allosteric proteins defined by Monod et al (1965) proposes that an oligomeric protein can exist in either of two conformational states (by convention termed R and T) which are in equilibrium and which differ in their affinities for ligands. In this system a homotropic ligand binds to the protein, pulling the equilibrium over towards the form which has the higher affinity for that ligand and thereby allows other such ligands to bind with greater ease. This model is described mathematically for a protein with n sites :

$$\bar{Y} = \frac{(1+\alpha)^{n-1} + L\alpha c\,(1+\alpha c)^{n-1}}{(1+\alpha)^{n} + L\,(1+\alpha c)^{n}}$$

where $\bar{Y}$ is the fraction of the protein that is bound with ligand, L, the allosteric constant, is $[T]/[R]$, c is K_R/K_T where K_R and K_T are the microscopic dissocation constants of the ligand for the R and T states respectively and α is the ratio $[\text{ligand}]/K_R$. To account for heterotropic interactions, the allosteric constant, L is replaced in the previous equation by L' such that

$$L' = L \cdot \left(\frac{1+\beta d}{1+\beta} \cdot \frac{1+\gamma e}{1+\gamma}\right)^{n}$$

where inhibitors ($K_R/K_T = d > 1$) or activators ($K_R/K_T = e < 1$) are present at concentrations β and γ relative to their respective values of K_R. Thus activators displace the equilibrium in favour of the R state whereas inhibitors pull the equilibrium over towards the T state. The Hill coefficient, h, is a function of the value of c and this relationship is demonstrated in figure 7.1. When c = 0 the ligand binds exclusively to one conformational state, but when c = 1

Table 7.1

The allosteric interactions of Mg^{2+} ions, AMP and fructose 2,6-bisphosphate with respect to rat liver fructose 1,6-bisphosphatase

The conclusions are drawn from tables 5.7 to 5.12.

Ligand	Effect on binding of other ligands		
	Mg^{2+}	AMP	$F2,6P_2$
Mg^{2+}	-	increase in h increase in K_i	no effect on h small increase in K_i
AMP	decrease in h increase in K_a	-	no effect on h decrease in K_i
$F2,6P_2$	decrease in h increase in K_a	decrease in h decrease in K_i	-

$K_R = K_T$ and the binding is not cooperative. Values of c which lie between 0 and 1 indicate non-exclusive binding, that is the ligand binds significantly but with unequal affinity to both conformational states of the protein.

The only previous unified model for the allosteric properties of any mammalian $Fl,6P_2$ase is that of Nimmo and Tipton (1975c), in which the cooperative interactions of ox liver $Fl,6P_2$ase are interpreted within the framework of the concerted transition two-state system described above. The enzyme is composed of two identical proformers, each containing one site for AMP and one site for the activating cation. Each protomer can exist in either of two conformational states, one of which binds the activating cation but not AMP, and the other which binds AMP well but the activating cation very poorly. The substrate, $Fl,6P_2$, binds equally well to both conformational states.

The allosteric interactions of Mg^{2+} ions, AMP and $F2,6P_2$ with respect to rat liver $Fl,6P_2$ase are summarised in table 7.1 and it can be seen that some of these observations are consistent with the previous model. For example, the response of the enzyme to $Fl,6P_2$ is normally hyperbolic while the responses to AMP and Mg^{2+} ions are sigmoidal. AMP and Mg^{2+} ions reduce the affinity of the enzyme for each other and Mg^{2+} ions increase the value of h for AMP. However, the finding that AMP reduces the value of h for Mg^{2+} ions rather than increases it appears to be inconsistent with the previous model. Nevertheless, this could be accommodated by modifying the model slightly such that the effects of non-exclusive binding of Mg^{2+} ions are more pronounced. This could, for example, be caused by an increase in the value of c (e.g. see figure 7.1). Another difference is that in this work inhibition by AMP is uncompetitive with respect to $Fl,6P_2$ (e.g.

Figure 7.2.

The allosteric transitions of rat liver fructose 1,6-bisphosphatase.

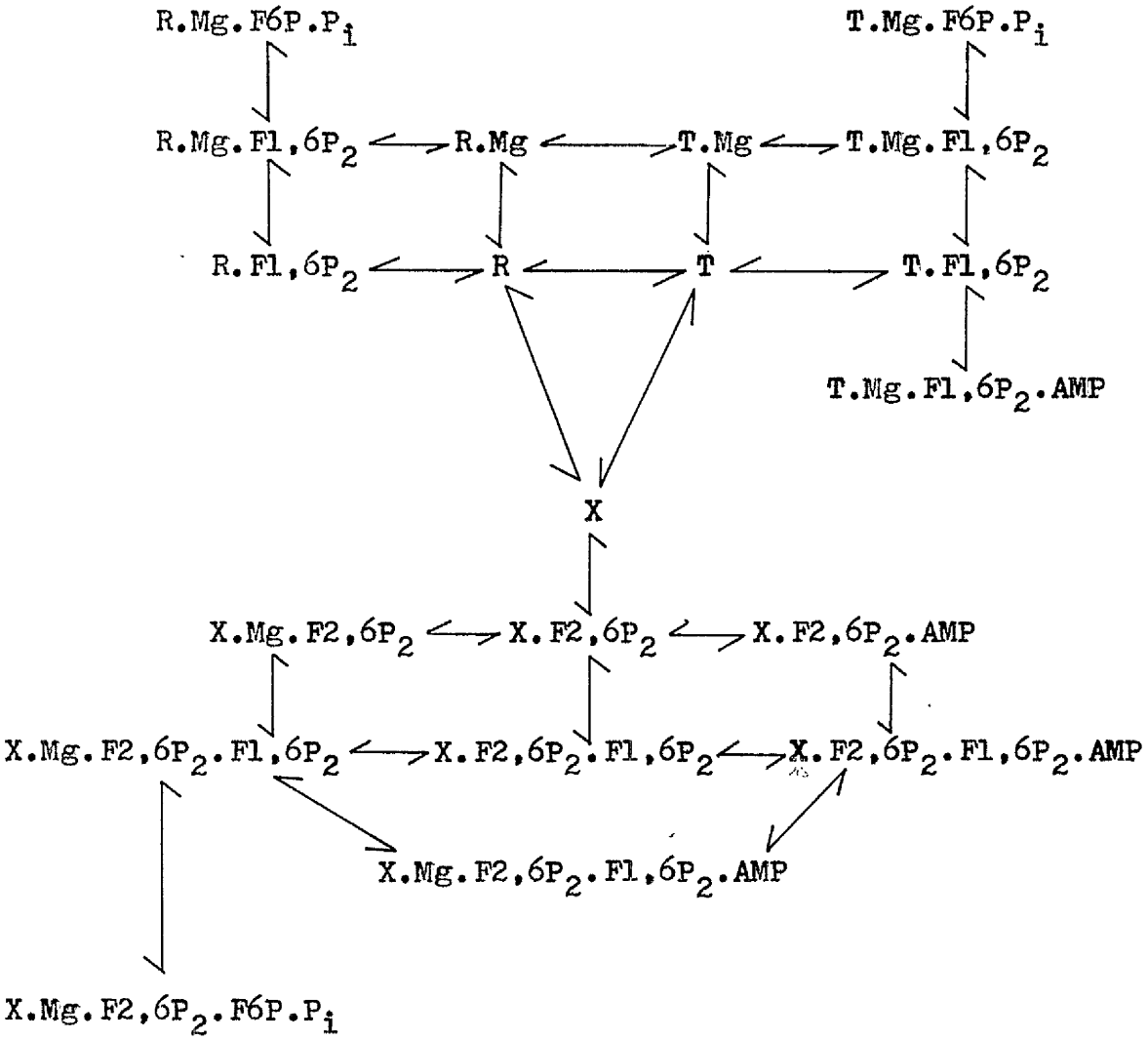


figure 5.7) rather than non-competitive (Nimmo and Tipton, 1975b) but this can also be accommodated if it is assumed that F1,6P_2 must bind to the T state before AMP can bind.

However, the discovery of F2,6P_2 and its effects on the cooperative interactions of Mg^{2+} ions and AMP demonstrate that the allosteric transitions of the enzyme are more complex than the previous model predicts. The most obvious difficulty is that in the presence of saturating concentrations of F2,6P_2, the enzyme responds hyperbolically to both AMP and Mg^{2+} ions, but responds sigmoidally to F1,6P_2. This is not possible on any variation of the basic two state model. Moreover, the evidence clearly suggests that F2,6P_2 itself can interact with the enzyme in two ways. Bearing this is mind, it is therefore necessary to postulate a 3 state model in which state X has an allosteric binding site for F2,6P_2. In this model, which is outlined in figure 7.2, state X can bind AMP with greater affinity than state T, Mg^{2+} ions with similar affinity to state R and F1,6P_2 with lower affinity than state R. In the absence of F2,6P_2, with only states R and T important, AMP is an uncompetitive inhibitor with respect to F1,6P_2. However, at saturating concentrations of F2,6P_2 the enzyme will be largely in state X. Since the presence of F2,6P_2 allows binding of AMP (see figure 5.29), AMP inhibition is non-competitive with respect to F1,6P_2. It is then possible to comprehend the following empirical observations within the framework of the new model (figure 7.2):

1. Saturating concentrations of F2,6P_2 reduce h for both AMP and Mg^{2+} ions to 1 since AMP and Mg^{2+} ions bind non-cooperatively to state X.

2. $F2,6P_2$ and AMP act synergistically since state X has an allosteric site for $F2,6P_2$ and has a higher affinity for AMP than state T.

3. By definition, $F2,6P_2$ binding is non-exclusive since it can bind to the catalytic site in states R and T. This explains why h for $F2,6P_2$ is fairly low and why it is not affected much by AMP or Mg^{2+} ions.

4. The kinetics for $F1,6P_2$ are sigmoid at high concentrations of $F2,6P_2$. Under these conditions the important states must be R and X, and the sigmoid kinetics would probably arise as a result of the transition of X to R at higher levels of $F1,6P_2$. In the presence of AMP the kinetics for $F1,6P_2$ at high concentrations of $F2,6P_2$ reverted to hyperbolic. However, considering states R and X, since $F1,6P_2$ binds non-exclusively, it would be expected that increasing concentrations of AMP would decrease h for $F1,6P_2$ to a value of 1.

5. The effect of NEM would be to abolish X as an available conformational state.

6. The model predicts that the dissocation constant for AMP would be lower with $F2,6P_2$ present than with $F1,6P_2$ present. The results presented in figure 5.29 show that the reverse was the case. However, this can be explained since the level of $F1,6P_2$ was high enough to give high substrate inhibition and this might well be greater at the low temperature at which the binding experiments were carried out.

7. The inhibition of purified rat liver $F1,6P_2$ase by AMP was found to be uncompetitive with respect to $F1,6P_2$ (this thesis; Tejwani et al, 1976) whereas in crude extracts of rat liver this inhibition was observed to be non-competitive (Taketa and Pogell, 1965). According

to the allosteric model, in the absence of $F2,6P_2$ the inhibition of $F1,6P_2$ase by AMP is uncompetitive with respect to $F1,6P_2$, but this inhibition becomes non-competitive in the presence of $F2,6P_2$. It is therefore possible that some of the $F2,6P_2$ which was present in the crude extract was bound to $F1,6P_2$ase; this would be gradually lost during purification, as is the case for PFK-1 (Furuya and Uyeda, 1980a). Moreover, owing to the very high affinity of $F1,6P_2$ase for $F2,6P_2$, it might be possible that highly purified $F1,6P_2$ases, such as the ox liver enzyme of Nimmo and Tipton (1975b), could retain sufficient $F2,6P_2$ that the inhibition of the enzyme by AMP would be non-competitive with respect to $F1,6P_2$.

The model can be used to predict the kinetic behaviour of the enzyme under certain conditions. For example, the conformational states R and T should still be available in the NEM-treated enzyme and one would therefore expect that AMP and Mg^{2+} ions should still give sigmoid kinetics. However, the available data do not allow calculation of parameters such as L', K_R and K_T for each ligand, so that the model could be tested by simulation. In order to calculate these parameters it would be necessary to obtain data on the binding of $F1,6P_2$, $F2,6P_2$ and, if possible, Mg^{2+} ions to the enzyme.

It should be emphasised that this model is the most simple explanation of the available data based on the ideas of Monod et al (1965)., and that other possibilities exist which could explain some of these kinetic results. For example, one can devise an alternative explanation for the sigmoid response to $F2,6P_2$ which does not involve cooperative binding of $F2,6P_2$. Since $F2,6P_2$ can interact with the enzyme in two different ways, the rate equation would be :

$$v = \frac{V_{max}\, S}{\left(1 + \frac{S}{K_i^{allo}}\right) S + K_m \left(1 + \frac{I}{K_i^{cat}} + \frac{I}{K_i^{allo}} + \frac{I^2}{K_i^{cat} \cdot K_i^{allo}}\right)}$$

where K_i^{cat} and K_i^{allo} refer to the catalytic and allosteric sites respectively; this equation contains the term I^2 and would therefore give upward-curving Dixon plots. By a similar argument, assuming that the enzyme obeys steady-state kinetics, and because the substrate can bind to more than one form of the enzyme (namely E and E.F2,6P_2), a formidable rate equation would be generated which would contain terms in S^2 as well as S. This could explain the sigmoid response to F1,6P_2.

The ~~the~~ most recent report of the interaction of F2,6P_2 with F1,6P_2ase, Gottschalk et al (1982) claimed that the effects of F2,6P_2 on swine kidney F1,6P_2ase were not mediated through an allosteric site, but were solely the result of F2,6P_2 binding at the catalytic site. This conclusion, however, was not fully supported by the evidence which they presented, although their findings did indicate that F2,6P_2 did not directly interact at the allosteric site for AMP. Their results were based on two rather doubtful assumptions, namely that the high substrate inhibition given by F1,6P_2 was not mediated through an allosteric site and that abolishing the inhibition by AMP, whether by limited proteolysis or by chemical modification, would also abolish any effects of the interaction of F2,6P_2 at an allosteric site; although AMP and F2,6P_2 interact synergistically the allosteric sites for these ligands need not overlap and therefore there is no guarantee that eliminating one site should necessarily affect interaction at the other site. None of the results presented in this paper (Gottschalk et al, 1982) are inconsistent with the allosteric model described in

Table 7.2

The criteria necessary to establish that an effect mediated by cyclic AMP occurs through phosphorylation of a protein *in vivo*

1 A protein substrate for cAMP-PrK should exist which bears a functional relationship to the process mediated by cyclic AMP. The rate of phosphorylation of that protein, in its native state, should be adequate to account for the speed at which the process occurs *in vivo* in response to cyclic AMP.

2 The function of the protein should be shown to undergo a reversible alteration *in vitro* by phosphorylation and dephosphorylation, catalysed by cAMP-PrK and by a protein phosphatase.

3 A reversible change in the function of the protein should occur *in vivo* in response to cyclic AMP.

4 Phosphorylation of the protein should occur *in vivo* in response to a hormone at the same site(s) phosphorylated by cAMP-PrK *in vitro*.

Taken from Nimmo, H.G. and Cohen, P. (1977)
Advances in Cyclic Nucleotide Research 8 145-266

this chapter.

7.2 The Hormonal Control of Rat Liver Fructose 1,6-Bisphosphatase

The evidence discussed in chapter 1 and the results presented and discussed throughout the thesis have indicated that the activity of Fl,6P_2ase can be regulated by glucagon, mediated through cyclic AMP-dependent phosphorylation. However, there are 4 criteria (summarised in table 7.2) which must be satisfied in order to establish that Fl,6P_2ase serves as a physiological substrate for cAMP-PrK *in vivo* (Nimmo and Cohen, 1977). The ability of rat liver Fl,6P_2ase to meet these criteria is discussed below.

1. Rat liver Fl,6P_2ase, in its native conformation, can be phosphorylated by cAMP-PrK *in vitro*. Using physiological concentrations of Fl,6P_2ase and the catalytic subunit of cAMP-PrK, the maximal incorporation of 1 mole of phosphate per mole of Fl,6P_2ase subunits was completed with 60 minutes (figures 4.1 and 4.3). However, the maximal effect of glucagon on Fl,6P_2ase *in vivo* is much quicker and appears to take place within 5-20 minutes (Taunton et al, 1974; Morikofer-Zwez et al, 1981). This difference could be explained if Fl,6P_2ase was fully activated before the maximal incorporation of phosphate into the enzyme was achieved. This possibility should be compared with the phosphorylation of rat liver PFK-1, where only 1 subunit per tetramer need be phosphorylated in order to obtain a fully active enzyme (Brand and Soling, 1975).

Alternatively, the glucagon effect may be entirely the result of a cyclic AMP-dependent reduction in the intracellular level of F2,6P_2. This is quite possible since glucagon can reduce the F2,6P_2 concentration in hepatocytes to basal levels in 5 minutes(Van Schaftingen et al, 1980b). The immediate effect of a fall in the F2,6P_2

concentration would be to arrest glycolysis and stimulate gluconeogenesis while the glucose output would be provided for the most part by the degradation of glycogen following the rapid activation of the enzymes involved. When the glycogen level had fallen the glucose output would be maintained by gluconeogenesis. Thus it could be argued that the phosphorylation of Fl,6P_2ase and the resulting activation of gluconeogenesis need not be as rapid as the phosphorylation of the key enzymes of glycogen metabolism; it would not be essential for gluconeogenesis to be fully active immediately. As discussed in Chapter 1, Pilkis et al (1980) have suggested that at physiological concentrations of Fl,6P_2ase and pyruvate kinase, these two enzymes are phosphorylated at similar rates even although pyruvate kinase is a much better substrate for cAMP-PrK. It is therefore possible that the phosphorylation of the key enzymes of gluconeogenesis and glycolysis could provide a delayed boost to gluconeogenesis as the level of glycogen decreases.

2. The results presented in Chapter 5 clearly demonstrated that the phosphorylation of rat liver Fl,6P_2ase by cAMP-PrK <u>in vitro</u> produced a significant increase in the affinity of the enzyme for its substrate Fl,6P_2. Although it was shown in Chapter 4 that the phosphorylation of the enzyme is reversible through dephosphorylation by phosphoprotein phosphatase 2A, a reversal of the phosphorylation-dependent decrease in K_m for Fl,6P_2 was not investigated. However, the finding of Ekman and Dalqvist-Edberg (1981) that a partially purified phosphoprotein phosphatase from rat liver reverses the phosphorylation-dependent decrease in the K_m for Fl,6P_2 would appear to satisfy the second criterion in table 7.2 .

3. Taunton et al (1974a) demonstrated a glucagon and cyclic AMP-dependent activation of rat liver F1,6P_2ase *in vivo* which could be reversed upon administration of insulin. However, this finding may not be rigorous enough to meet the third criterion in table 7.2, since the effect could clearly be the result of a cyclic AMP-dependent change in the level of F2,6P_2. In order to implicate phosphorylation, it would be necessary to purify the enzyme from glucagon-treated and control cells to show that these were phosphorylated and non-phosphorylated respectively and then to demonstrate a difference in K_m between the two forms of the enzyme similar to that observed upon phosphorylation of F1,6P_2ase *in vitro*.

4. The phosphorylation of rat liver F1,6P_2ase is known to occur *in vivo*, but only a small increase in the phosphate content of the enzyme was observed upon glucagon treatment (this thesis; Claus et al, 1981b). Although the site which is phosphorylated by cAMP-PrK *in vitro* has been sequenced (Humble et al, 1980; Pilkis et al, 1980), it has still to be demonstrated that phosphorylation occurs at this site in response to glucagon *in vivo*. It will therefore be necessary to isolate and sequence the phosphate-containing peptide(s) from F1,6P_2ase which has been phosphorylated in response to glucagon *in vivo*.

In conclusion, the evidence suggests that the cyclic AMP-dependent activation of the enzyme occurs through a fall in the level of F2,6P_2 in the cell and a phosphorylation-dependent increase in the affinity for substrate. The phosphorylation effect appears to be reversible *in vitro*, but whether a reversible phosphorylation-dependent change occurs *in vivo* remains to be determined. The site of phosphorylation in response to glucagon also merits some examination.

7.3 The Hormonal Regulation of Hepatic Gluconeogenesis

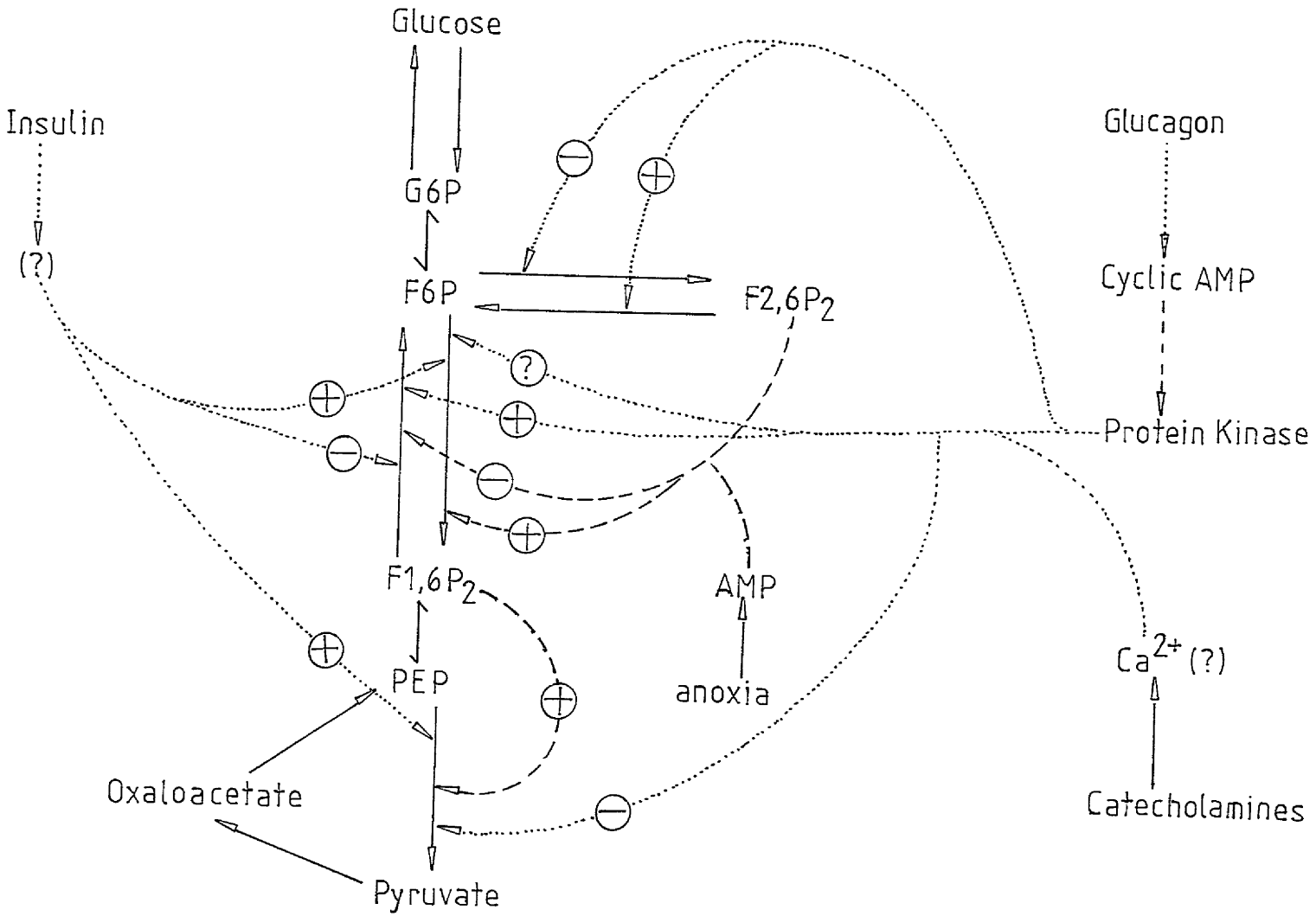
In a recent review of the hormonal control of hepatic gluconeogenesis, Pilkis et al (1978) argued that the major effect of glucagon, catecholamines and insulin on gluconeogenesis was to alter the flux of carbon through the phosphoenolpyruvate-pyruvate substrate cycle by affecting the activity of pyruvate kinase. In this hypothesis regulation of the flux through the F6P-F1,6P_2 substrate cycle played a secondary role in modulating the intracellular level of F1,6P_2 which is the major allosteric effector of pyruvate kinase. However, at the time when this model of regulation was postulated, the study of the hormonal regulation of pyruvate kinase had received considerable attention whereas the phosphorylation of F1,6P_2ase and PFK-1 had only just been recognised. Since recent studies have expanded previous knowledge concerning the hormonal regulation of gluconeogenesis and glycolysis, especially regarding control at the F6P-F1,6P_2 substrate cycle, it is necessary to update the previous model for the control of these pathways. Moreover, the need for reappraisal of the regulatory model is clearly emphasised by the very recent discovery of F2,6P_2 and its profound effects on F1,6P_2ase and PFK-1.

An outline of the major factors involved in the hormonal regulation of gluconeogenesis and glycolysis is given in figure 7.3. The overall direction and, for the most part, the rate of flux through these pathways appears to be governed through the actions of glucagon, insulin and, to a certain extent, catecholamines. This higher tier of control directs liver metabolism towards meeting the glucose requirements of other tissues in the body. The rate of flux is also modulated depending on the availability of substrates and the local concentrations of allosteric effectors; this may be regarded as a lower tier of "fine"

Figure 7.3

The hormonal regulation of gluconeogenesis and glycolysis

The information has been taken, for the most part, from reviews by Pilkis et al., (1978) in Vitamins and Hormones 36 383-460 and Hers and Van Schaftingen (1982) in Biochem. J. 206 1-12.


Glucose
Insulin
Glucagon
G6P
(?)
Cyclic AMP
F6P
F2,6P2
Protein Kinase
F1,6P2
AMP
Ca2+ (?)
PEP
anoxia
Oxaloacetate
Catecholamines
Pyruvate

control. However, it is obviously difficult to completely differentiate between hormonal and allosteric effects since $F2,6P_2$, which is a major factor in the hormonal control of gluconeogenesis, is an allosteric effector.

Hers and Van Shaftingen (1982) have pointed out that $F2,6P_2$ has the property, in common with cyclic AMP, that it is not an intermediate in any major metabolic pathway, but at micromolar concentrations has profound effects upon the regulation of at least two such pathways. They have also proposed that since the level of $F2,6P_2$ in hepatocytes is greatly increased upon incubation with glucose, it acts as a signal of glucose abundance to the metabolic machinery of the cell; this contrasts with the concept of cyclic AMP as a "hunger" signal. $F2,6P_2$ stimulates glycolysis and inhibits gluconeogenesis by the simultaneous activation of PFK-1 and inhibition of $F1,6P_2$ase. These effects are augmented by AMP which may act as a fine control <u>in vivo</u>. Although $F2,6P_2$ does not appear to have any direct influence on the activity of pyruvate kinase in crude extracts (Hers and Van Shcaftingen, 1982), it must have an indirect effect by regulating the F6P-$F1,6P_2$ substrate cycle. Thus, an $F2,6P_2$-dependent increase in the level of $F1,6P_2$ would produce a stimulation of pyruvate kinase activity and this is consistent with $F2,6P_2$ favouring glycolysis. In view of the dramatic effects of $F2,6P_2$ on the activities of $F1,6P_2$ase and PFK-1, hormonal regulation of these enzymes must be at least as important as that of pyruvate kinase.

The effects of glucagon, which are mediated through phosphorylation of the key enzymes by cAMP-PrK, favour gluconeogenesis and are therefore antagonistic to the effects of $F2,6P_2$. The phosphorylation-

dependent inhibition of PFK-2 and activation of $F2,6P_2$ase results in a fall in the intracellular concentration of $F2,6P_2$ and this, in turn, presumably relieves the restraint on $F1,6P_2$ase activity and diminishes the stimulation of PFK-1 activity. The resulting decrease in the level of $F1,6P_2$ would be expected to produce a decrease in the activity of pyruvate kinase. Over and above these allosteric mechanisms, the activation of $F1,6P_2$ase and the inactivation of pyruvate kinase are brought about through the phosphorylation of these enzymes by cAMP-PrK. Phosphorylation does not appear to alter the catalytic activity or allosteric properties of PFK-1 (Claus et al, 1980; Hers and Van Schaftingen, 1982) but it may reduce the affinity of the enzyme for $F2,6P_2$ (Furuya and Uyeda, 1980b).

As was discussed in Chapter 1, it is known that insulin opposes the effects of glucagon on hepatic gluconeogenesis and glycolysis but the mechanism of action of this hormone is at present unclear. One interesting possibility is that insulin affects the intracellular level of $F2,6P_2$. However, although it is known that glucose increases the intracellular concentration of $F2,6P_2$, there have been no reports concerning the effects of insulin on the level of this compound. This possibility remains to be examined.

The effects of catecholamines on hepatic gluconeogenesis and glycolysis are similar to, but less pronounced than the effects of glucagon. The action appears to involve the X receptors and is cyclic AMP-dependent. Catecholamines have also been shown to increase the level of $F2,6P_2$ in hepatocytes by a Ca^{2+} dependent mechanism (Hue et al, 1981). However, this effect is brought about indirectly through a stimulation of glycogenolysis which in turn increases the amount of F6P available to PFK-2.

Hormones do not appear to affect the activity of glucokinase, glucose 6-phosphatase, pyruvate carboxylase or phosphoenolpyruvate carboxykinase, and of these 4 enzymes it is known that $F2,6P_2$ has no positive or negative effect on the activity of glucokinase, glucose 6-phosphatase or phosphoenolpyruvate carboxykinase. However, glucagon can activate cytochrome c_1 and this increases proton efflux which in turn increases anion transport; this mechanism may serve to regulate gluconeogenesis by controlling the supply of oxaloacetate (Pilkis et al, 1978; Hers and Van Schaftingen, 1982). Thus there are a number of different mechanisms by which hormones can control gluconeogenesis. The relative importance of these in different conditions remains to be resolved.

7.4 Epilogue

Recently, considerable progress has been made towards understanding the mechanism and physiological significance of the action of hormones on $F1,6P_2$ase. However, several major questions, which have been pointed out in this thesis, still remain to be answered and should form the basis of further study. Immediate attention should be directed towards obtaining more data, through binding studies, in order to test the model for the allosteric transitions of the enzyme. Attention should also be focussed on an examination of possible phosphorylation-dependent changes in the properties of $F1,6P_2$ase *in vivo* and this will require a comparison of $F1,6P_2$ases isolated from glucagon-treated and control cells; isolation of the enzyme from glucagon-treated cells should also provide the opportunity to examine the phosphorylation site. Moreover it might be worthwhile to re-examine,using an assay system at

physiological ionic strength and phosphate concentration, possible changes in the inhibition of the enzyme by AMP and $F2,6P_2$ resulting from phosphorylation *in vitro*. A brief examination should also be carried out in order to determine whether there are any cyclic AMP- independent protein kinases in rat liver which phosphorylate $F1,6P_2$ase *in vitro*: a positive result would provide a further aspect of regulation for future study. The elucidation of the mechanisms of action of insulin and catecholamines is a problem which is general to many areas of metabolism and will undoubtedly receive considerable attention. The results from these and other studies should prove to be very interesting and should make a positive contribution towards our knowledge of the hormonal regulation of gluconeogenesis.

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